Characterisation of Human PETA-3:

A Member of the Transmembrane 4 Superfamily

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SUMMARY

The transmembrane 4 superfamily (TM4SF) of proteins, or tetraspans, consists of over 20 members, characterised by four conserved putative transmembrane domains. These molecules also possess a large divergent extracellular loop between the third and fourth membrane spanning domains. Although the exact biochemical function of the TM4SF remains unclear, several studies have shown these proteins to be components of multi-protein complexes, involved in cellular signalling and/or adhesive events.

Previously, a novel member of the TM4SF, PETA-3 (Platelet Endothelial Tetraspan Antigen-3), which preliminary studies had shown to be expressed by platelets and endothelium was cloned in this laboratory. Subsequently, PETA-3 was assigned CD151 at the VIth International Leucocyte Typing Workshop. Therefore the primary aims of this study were to i) characterise the tissue distribution of this molecule, ii) determine the subcellular localisation in platelets and endothelial cells, iii) investigate possible protein-protein interactions, and iv) examine the effect of anti-PETA-3 antibodies on cellular function.

Several members of the tetraspan superfamily, including CD9 and CD63, associate with each other and with β1 integrins. Since earlier studies showed that PETA-3 associated with these proteins, the localisation of these molecules was investigated. PETA-3 showed a broad distribution and was expressed by endothelium, epithelium, Schwann cells, dendritic cells and skeletal, smooth and cardiac muscle. Expression in skin and was mostly restricted to the basal cells of the epidermis and was downregulated upon differentiation, consistent with a possible role in anchorage to the basement membrane. Similarly, in the small intestine PETA-3 was expressed by crypt and villous enterocytes with a mostly basolateral distribution, but was not detectable on the brush border. CD9, CD63 and β1 integrin were also expressed broadly, and by many cell types positive for PETA-3. Although co-expression of these molecules was observed in several cell types, others failed to do so. The overall findings of

these experiments show colocalisation of PETA-3 with CD9, CD63 and β1 in particular tissues. Thus tetraspan/integrin complexes may occur in some cell types *in vivo*, however the different cellular and subcellular localisation of these antigens in other tissues also indicates distinct roles for these molecules. Additionally, the *in vivo* distribution of PETA-3, especially in polarised epithelium, was consistent with a possible role in cell-cell and/or cell-ECM interactions.

In cultured human umbilical vein endothelial cells, PETA-3 was present on the plasma membrane and predominantly localised to regions of cell-cell contact. Additionally, this protein was abundant within an intracellular compartment which accounted for up to 66% of the total PETA-3 expressed. Intracellular PETA-3 showed colocalisation with transferrin receptor and CD63 suggesting an endosomal/lysosomal localisation, which was supported by immunoelectronmicroscopy studies. Characterisation of the distribution of PETA-3 in platelets demonstrated expression on the plasma membrane, and to a lesser extent and intra-platelet membranes/vesicles and platelet granules.

Co-immunoprecipitation experiments investigating possible interactions of PETA-3 with other molecules demonstrated associations with several integrin chains including β1, β3, β4, α2, α3, α5, α6 and provide the first report of Transmembrane 4 Superfamily association with the α6β4 integrin. Using 2-colour confocal microscopy, the colocalisation of PETA-3 with integrin chains within cytoplasmic vesicles and regions of cell-cell contact was demonstrated.

In order to assess the functional implications of PETA-3/integrin associations, the effect of anti-PETA-3 antibodies on platelet and endothelial function was examined. Previous studies had shown that an IgG1 anti-PETA-3 antibody acted as a platelet agonist via crosslinking of the FcγRII receptor. However an IgG2a anti-PETA-3 antibody also acted as a weak platelet agonist, perhaps by a mechanism independent of FcγRII occupation. Anti-PETA-3 antibodies inhibited endothelial cell migration and modulated *in vitro* capillary

formation. However these same antibodies had no detectable effect on neutrophil trans-endothelial migration. The broad range of integrin associations and the intracellular colocalisation of PETA-3 with integrins, suggest a primary role for PETA-3 in regulating integrin trafficking and/or function.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text. I give consent for this thesis to be made available for loan and photocopying.

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Sincock, P.M., Fitter, S., Parton, R.G., Berndt, M.C., Gamble, J.R., and Ashman, L.K. PETA-3/CD151, a member of the transmembrane 4 superfamily, is localised to an endosomal/lysosomal compartment and the plasma membrane of endothelial cells, associates with multiple integrins and modulates cell function. (submitted to J. Cell Sci.)

Fitter, S., **Sincock, P.M.**, Jolliffe, C.N., and Ashman, L.K. The transmembrane 4 superfamily (TM4SF) protein CD151 (PETA-3) associates with β1 and αIIbβ3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. (submitted to Biochem. J.)

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Abbreviations

ADP	Adenosine Diphosphate
AJ	Adherens Junctions
ANOVA	Analysis of Variance
APAAP	Alkaline Phosphatase Anti-Alkaline Phosphatase
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
Az	Sodium Azide
BFA	Brefeldin A
bFGF	Basic Fibroblast Growth Factor
BMMNC	Bone Marrow Mononuclear Cells
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetra-acetic acid
FAK	Focal Adhesion Kinase
FBS	Feotal Bovine Serum
FcγRII	Low Affinity Receptor for IgG
FDC-P1	Factor Dependent Cell Line-Passage 1
FITC	Fluorescein-isothiocyanate
FL	Flourescence Channel
FSC	Forward Scatter
gp	Glycoprotein
HB-EGF	Heparin Binding Epidermal Growth Factor
HBSS	Hank's Buffered Saline Solution
HEPES	N-2-Hydroxyethylpiperazine N'2-ethane Sulfonic Acid
HEV	High Endothelial Venule
hMas20p	Human Mitochondrial Assembly Protein

HUVEC	Human Umbilical Vein Endothelial Cell
IAP	Integrin Associated Protein
ICAM	Intercellular Adhesion Molecule
IEL	Intra-Epithelial Lymphocyte
Ig	Immunoglobulin
IL	Interleukin
ILK	Integrin Linked Kinase
INF-γ	Interferon-γ
IP_3	Inositoltrisphosphate
kD	kiloDalton
LT_{50}	Lag Time to Induce 50% Maximal Response (minutes)
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MQ	Milli-Q
NP 40	Nonidet P 40
NRS	Normal Rabbit Serum
PBA	Phosphate Buffered Saline/Bovine Serum Albumin/Sodium Azide
PBS	Phosphate Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PETA-3	Platelet Endothelial Tetraspan Antigen-3
PFP	Platelet Free Plasma
PGE_1	Prostaglandin E_1
PI3-K	Phosphatidylinositol 3-Kinase
PI4-K	Phosphatidylinositol 4-Kinase
PIP_2	Phpsphatidylinositoldiphosphate
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PRP	Platelet Rich Plasma
PTK	Protein Tyrosine Kinase
S.D.	Standard Deviation

S.E.M.	Standard Error of the Mean
SH	Src Homology Domain
SSC	Side Scatter
TBS	Tris Buffered Saline
TC-PBS	Tissue Culture Phosphate Buffered Saline
TJ	Tight Junctions
TM4SF	Transmembrane 4 Superfamily
TNF-α	Tumour Necrosis Factor-α
TxA_2	Thromboxane
VCAM	Vascular Cell Adhesion Molecule
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor
WGA	Wheat Germ Agglutinin

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CHAPTER 1: INTRODUCTION

1.1 Identification of a Novel Surface Antigen

The use of monoclonal antibodies (mAb) in the identification and functional studies of novel surface antigens has provided much information towards the characterisation of these proteins. One such murine mAb, 14A2.H1, raised against human acute myeloid leukaemic cells, was shown to identify a 27kD glycoprotein (gp27) (Ashman *et al.*, 1991). Preliminary studies found gp27 to be predominantly expressed by platelets, megakaryocytes and endothelial cells (EC) (Ashman *et al.*, 1991). Furthermore 14A2.H1 was demonstrated to cause aggregation of human platelets in an FcγRII dependent manner (Ashman *et al.*, 1991; Roberts *et al.*, 1995). After submission to the Fourth and Fifth International Leucocyte Typing workshops, the 14A2.H1 mAb was found to be unique, suggesting that it identified a novel surface antigen (Cole *et al.*, 1989; von dem Borne, 1989; Ferro *et al.*, 1995).

In 1995, a cDNA clone encoding gp27 was isolated from a plasmid library derived from the megakaryoblastic cell line M-07e (Fitter *et al.*, 1995). The clone encoded an open reading frame of 253 amino acids and sequence comparison revealed 25-30% amino acid identity with several members of the recently described tetraspan, or transmembrane 4 superfamily (TM4SF). Since the expression of gp27 was mostly limited to platelets and endothelium and two of the most homologous tetraspans, CD9 and CD63, are also expressed by these cell types, the name PETA-3 (Platelet-Endothelial Tetraspan Antigen-3) was coined for this molecule. Investigation of the tissue distribution by Northern analysis demonstrated the presence of PETA-3 mRNA in all normal adult human tissues examined, with the exception of brain. Subsequently, PETA-3 was cloned from the adult T cell leukaemia cell line, SF-HT, and termed SFA-1 (SF-HT-activated gene 1) (Hasegawa *et al.*, 1996).

Three additional anti-PETA-3 mAbs, 11B1.G4, 14B5 and 11B5 were raised in this laboratory by immunisation of mice with M-07e cells and screening on transfectants. After submission of mAbs 14A2.H1 and 11B1.G4 to the VIth International Leucocyte Differentiation Antigen workshop, PETA-3 was assigned CD151 (Ashman *et al.*, 1997).

1.2 The Transmembrane 4 Superfamily

The TM4SF, also termed the tetraspans or tetraspanin family, was first described in 1990 when the sequences of two newly cloned molecules, a membrane protein from *Schistosoma mansoni* Sm23 (Wright *et al.*, 1990) and the target of an anti-proliferative antibody in human lymphoid cells CD81 (TAPA-1) (Oren *et al.*, 1990), showed homology with the previously described tumour associated antigen CD63 (ME491) (Hotta *et al.*, 1988) and the lymphoid antigen CD37 (Classon *et al.*, 1989). To date, over 20 members of the TM4SF have been described, and these are summarised in Table 1.1.

Although most TM4SF proteins have been described in humans and mammals, members have also been found in organisms such as *Schistosoma* (Wright *et al.*, 1990; Lee *et al.*, 1995), *Drosophila* (Kopczynski *et al.*, 1996) and *Caenorhabditis* (Tomlinson and Wright, 1996b; Wilson *et al.*, 1994). Furthermore, conservation of the genomic structure of the genes encoding these molecules across such a large evolutionary distance suggests they play a fundamental role in biology (reviewed in Wright *et al.*, 1993; Tomlinson and Wright, 1996a; Tomlinson and Wright, 1996b). Typically, these molecules are encoded by 7-8 exons and the positions of coding region introns are highly conserved. Interestingly, the genomic structure of mouse PETA-3 (Fitter *et al.*, 1998) is closest to those of *Schistosoma mansoni* (Sm23) (Wright *et al.*, 1990) and *Caenorhabditis elegans* (YKK8) (Tomlinson and Wright, 1996b), suggesting that the gene structure of mouse PETA-3 is more primordial than those of other mammalian TM4SF members.

Table 1.1 The Transmembrane 4 Superfamily

All members of the TM4SF described to date, their general distribution and associated molecules are listed. Table adapted from (Maecker *et al.*, 1997).

* Where possible only first author listed.

TM4SF Member	Other Names	General Distribution	Associated Molecules	Reference*
CD9	MRP-1 DRAP27	Platelets, eosinophils, basophils, pre B and activated T cells, neural cells	α3β1, α4β1, α5β1, α6β1, αIIbβ3, calnexin, G-proteins, diphtheria toxin receptor, proHB-EGF receptor, CD63, CD81	(Masellis-Smith *et al.*, 1994; Berditchevski *et al.*, 1996; Rubinstein *et al.*, 1994; Seehafer *et al.*, 1991; Mitamura *et al.*, 1992; Nakamura *et al.*, 1995; Rubinstein *et al.*, 1997, Radford *et al.*, 1996)
CD37	MB-1	Mature B Cells	MHC class II	(Angelisova *et al.*, 1994)
CD53	OX-44	Lymphocytes, monocytes, granulocytes	α4β1, MHC class II, unknown phosphatase, CD2	(Mannion *et al.*, 1996; Berditchevski *et al.*, 1996; Carmo *et al.*, 1995; Angelisova *et al.*, 1994; Bell *et al.*, 1992)
CD63	ME491, AD1, MLA1, NGA, PTLGP40, Granulo-physin	Platelets, monocytes, nonlymphoid cells	α3β1, α4β1, α6β1, CD9, CD81, αLβ2, unknown phosphatase	(Berditchevski *et al.*, 1996; Radford *et al.*, 1996; Berditchevski *et al.*, 1995; Skubitz *et al.*, 1996; Carmo *et al.*, 1995)
CD81	M38 TAPA-1	Most cells; high expression on germinal centre B cells	CD19/CD21/Leu-13 complex, MHC class II, CD4, CD8, α3β1, α4β1, α6β1, α4β7, CD9, CD63, CD82	(Matsumoto *et al.*, 1993; Takahashi *et al.*, 1990; Schick *et al.*, 1993; Angelisova *et al.*, 1994; Imai *et al.*, 1993; Imai *et al.*, 1995; Mannion *et al.*, 1996; Berditchevski *et al.*, 1996, Radford *et al.*, 1996)
CD82	C33, IA4, R2, 4F9, KAI1	Most haemopoietic and non-haemopoietic cells	MHC class II, CD4, CD8, α3β1, α4β1 α6β1, CD81	(Imai *et al.*, 1992; Imai *et al.*, 1993; Imai *et al.*, 1995; Mannion *et al.*, 1996; Hemler *et al.*, 1996; Angelisova *et al.*, 1994)
PETA-3	CD151, SFA-1	Platelets, T cells, endothelium	β1 integrins, CD9, CD63	(Ashman *et al.*, 1991; Ashman *et al.*, 1997; Hasegawa *et al.*, 1997)
NAG-2		Most cells; not in lymphoid cells, platelets or brain	α6β1, CD81, CD63, CD9	(Tachibana *et al.*, 1997)
CO-029	D6.1A	Colon carcinoma	α6β1	(Szala *et al.*, 1990; Claas *et al.*, 1998)
A15	TALLA-1, MXS1	Brain, spleen, skeletal muscle		(Takagi *et al.*, 1995)
SAS		Sarcomas		(Jankowski *et al.*, 1994; Jankowski *et al.*, 1995)
lbl		*Drosophila*		(Kopczynski *et al.*, 1996)
sm23		*Schistosoma mansoni*		(Wright *et al.*, 1990; Lee *et al.*, 1995)
sj25/TM4		*Schistosoma japonicum*		(Fan *et al.*, 1997)
YKK8		*Caenerhabditis elegans*		(Tomlinson *et al.*, 1996b)
		Divergent Members		
UPIa		Bladder epithelium	UPIb, UPII, UPIII	(Wu *et al.*, 1995)
UPIb	TI-1	Bladder epithelium	UPIa, UPII, UPIII	(Wu *et al.*, 1995)
Rom-1		Eye	Peripherin	(Bascom *et al.*, 1993)
Peripherin	RDS, ROCA-1	Nervous system, eye	Rom-1	(Moritz *et al.*, 1996)
L6		Carcinomas, epithelium		(Marken *et al.*, 1992)
TM4SF5		Pancreatic carcinoma		(Muller-Pillasch *et al.*, 1998)
il-TMP		Enterocytes, hepatocytes		(Wice *et al.*, 1995)

1.2.1 Structure of the TM4SF

Members of this superfamily are characterised by four highly conserved hydrophobic sequences which are believed to be membrane spanning (Figure 1.1). Within these transmembrane domains are hydrophilic residues which are conserved between TM4SF members. These include a single Asn in the first domain and several Glu or Gln residues within domains three and four (Wright and Tomlinson, 1994; Maecker *et al.*, 1997). Whether these residues form stable interactions with adjacent helices of the protein or are involved in intermolecular interactions is unclear. Interestingly, sequences with hydrophilic residues lying within membrane spanning regions are also found in ligand-gated ion channels. Polar residues within ion channels are a common feature and functional and mutational studies have demonstrated that ion selectivity is regulated by such residues (reviewed in Unwin, 1993). Thus the polar residues within the membrane spanning domains of the tetraspan superfamily may line the pore of an ion channel. However to date there is no definitive evidence to demonstrate that the TM4SF does indeed constitute a family of ion channels.

Based on gylcosylation patterns (Boucheix *et al.*, 1991; Wright and Tomlinson, 1994) and epitope mapping studies (Reynolds *et al.*, 1992; Tomlinson *et al.*, 1995; Tomlinson *et al.*, 1993; Levy *et al.*, 1991), the membrane topology of these molecules places the short amino and carboxyl terminal hydrophilic sequences on the intracellular side of the plasma membrane. For PETA-3, the cytoplasmic tails show nearly 100% conservation between human and mouse (Fitter *et al.*, 1995; Fitter *et al.*, 1998) (Figure 1.1), suggesting an important function for these domains. However between TM4SF members, the cytoplasmic tails vary greatly both in length and sequence, perhaps indicating specific roles. For example, the carboxyl tail of CD63 contains a putative lysosomal targeting motif, GY (Fukuda, 1991), which localises it to lysosomes (Metzelaar *et al.*, 1991). Other TM4SF members also show intracellular localisation with CD63 and CD9 in platelet granules (Nishibori *et al.*, 1993;

Figure 1.1 Proposed Membrane Topology of Human PETA-3

The proposed membrane topology of PETA-3 is illustrated. Black residues represent non-conserved amino acids between mouse and human PETA-3; the corresponding residue in mouse PETA-3 is shown. Red residues represent highly conserved cysteine motifs within the large extracellular loop. Green represents conserved polar residues within transmembrane domains.


Plasma Membrane

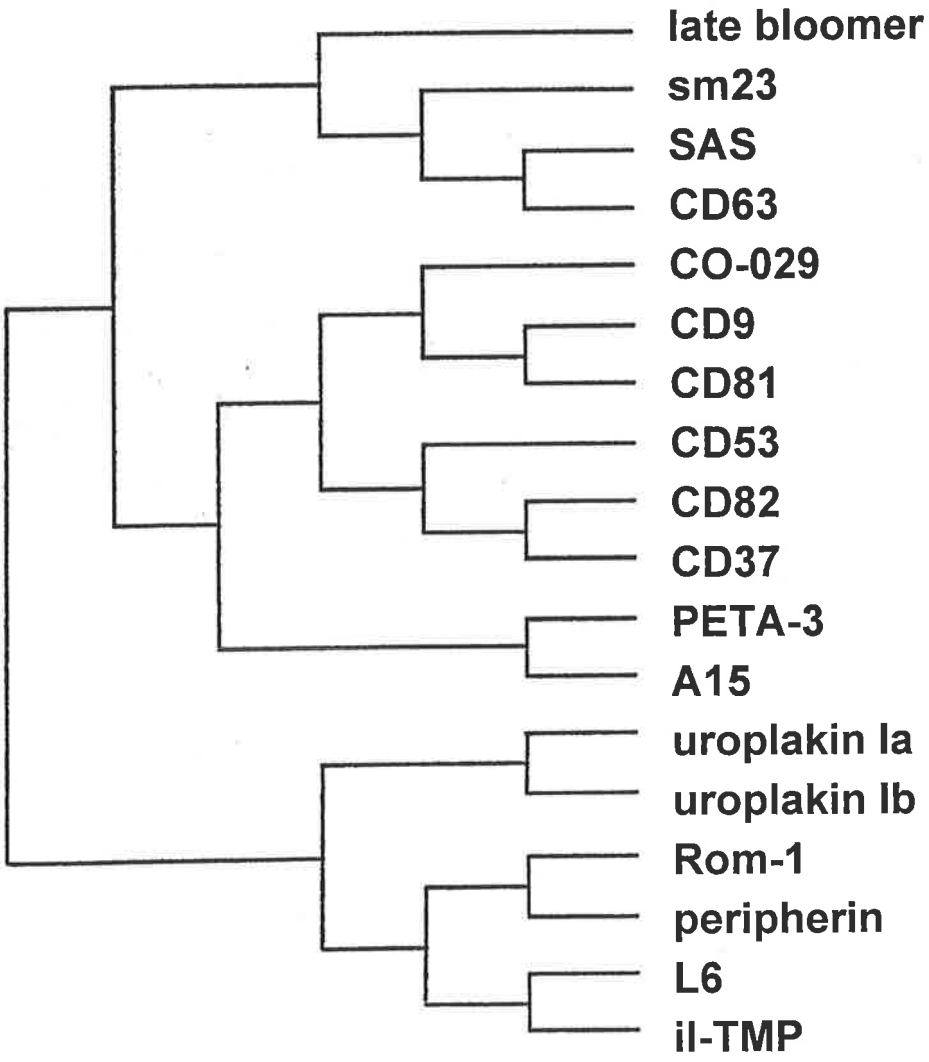
Cramer *et al.*, 1994), CD63 in Weibel-Palade bodies of EC (Vischer and Wagner, 1993), CD37 in intracellular vesicles of B cells (Schwartz-Albiez *et al.*, 1988), and Rom-1 and peripherin within intracellular membranes of photoreceptor cells in the retina (Moritz and Molday, 1996). However, apart from CD63, no obvious targeting motifs or conserved domains are apparent within their cytoplasmic tails.

Also characteristic of TM4SF proteins is a large divergent loop between the third and fourth membrane spanning domains (reviewed in Wright and Tomlinson, 1994; Maecker *et al.*, 1997). Within the large extracellular loop are two conserved cysteine containing motifs (CCG and PXSC) and a third conserved cysteine about 11 amino acids from the fourth transmembrane domain. Epitopes recognised by many antibodies against TM4SF members are sensitive to reduction, suggesting that at least two of these cysteine residues are critical for the correct folding of the molecule (Tomlinson *et al.*, 1993; Levy *et al.*, 1991; Oligino *et al.*, 1988). Apart from the conserved cysteine residues, the extracellular loop shows great variety in sequence, length and level of post-translational modification. This divergence within the extracellular loop suggests a specific role for each member of the TM4SF, although whether the loop is involved in ligand binding or interactions with other membrane components has not been established.

Within the TM4SF there are several members which, based on their amino acid homology, appear to be more distant relatives (Table 1.1). The dendrogram in Figure 1.2 shows the primary amino acid sequence homology of 18 TM4SF members and illustrates the divergence within the family. Those divergent members include peripherin, Rom-1, iL-TMP, L6 and the uroplakins UPIa and UPIb. Both peripherin (Moritz and Molday, 1996) and Rom-1 (Bascom *et al.*, 1993) have long carboxyl cytoplasmic tails not found in the other members. IL-TMP (Wice and Gordon, 1995) and L6 (Marken *et al.*, 1992) lack the highly conserved CCG motif in their large extracellular loops while the uroplakins UPIa and UPIb

Figure 1.2 Evolutionary Relationship Between TM4SF Members

The evolutionary relationship between TM4SF members is illustrated as a dendrogram with constant branch lengths as determined using the "pileup" multisequence analysis and the linked "grow trees" program in the GCG Wisconsin Sequence Analysis software package, version 8.0 (taken from Maecker *et al.*, 1997). The dendrogram does not include the recently identified members, NAG-2, YKK8, sj25/TM4 or TM4SF5.


late bloomer
sm23
SAS
CD63
CO-029
CD9
CD81
CD53
CD82
CD37
PETA-3
A15
uroplakin Ia
uroplakin Ib
Rom-1
peripherin
L6
il-TMP

(Wu *et al.*, 1995) are more closely related to each other and segregate further from the remainder of the TM4SF. Although not shown on the dendrogram, the newly described member, TM4SF5 (Muller-Pillasch *et al.*, 1998), also appears to be a more distant relative, as this molecule shows highest homology with L6 and lacks the CCG motif in its large extracellular loop.

1.2.2 Expression of the TM4SF

Whilst expression of some TM4SF members is restricted, such as CD37 (B cells) and CD53 (lymphoid and myeloid lineages), others, such as CD9, CD63, CD81, CD82, show widespread expression. TM4SF proteins are potentially involved in development as demonstrated by their differential expression in embryos versus adult tissues. For example CD9 is transiently expressed throughout the embryonic nervous system (Tole and Patterson, 1993), and remains in the nervous system and brain parenchyma of adults (Rossler *et al.*, 1992). Expression of mouse CD81 was observed during early development in oocytes and preimplantation embryos (Andria *et al.*, 1992) and has been found to be regulated during post-natal development of the brain (Sullivan and Geisert, 1998). Likewise, PETA-3 may play a role in neural development as expression in murine neural precursor cells has been detected by Northern analysis during embryonic days 5-15 (S. Fitter, unpublished data). Differential expression of TM4SF proteins has also been observed during lymphocyte differentiation as CD9, which is expressed by pre-B cells, is absent on mature resting B cells (Boucheix *et al.*, 1991), whereas the converse is the case with CD37 which is present on mature B cells, yet absent on pre-B cells and plasma cells (Schwartz-Albiez *et al.*, 1988). A similar pattern is found with CD53 expression on mature T cells (Tomlinson *et al.*, 1995).

Apart form being developmentally regulated, TM4SF expression may be modulated by cellular activation. Several TM4SF members, such as CD9 in platelets (Boucheix *et al.*,

1991), CD63 in platelets and EC (Nieuwenhuis *et al.*, 1987; Sohma *et al.*, 1994; Vischer and Wagner, 1993; Nishibori *et al.*, 1993), CD82 and PETA-3/SFA-1 in lymphoid cells (Gaugitsch *et al.*, 1991; Hasegawa *et al.*, 1997), are upregulated in response to cellular activation. In contrast, other members, CD53 in neutrophils (Mollinedo *et al.*, 1998) and TI-1 and il-TMP in epithelial cells (Kallin *et al.*, 1991; Wice and Gordon, 1995) are downregulated.

Differential expression of tetraspan antigens has also been described in malignant cells. Loss of CD63 expression is a marker of tumour progression in melanoma (Radford *et al.*, 1995), similarly, CD9 positivity in melanoma correlates with decreased metastatic potential (Atkinson *et al.*, 1984; Si and Hersey, 1993). Consistent with its association with decreased metastatic potential, CD9 was also cloned as a motility related protein-1 (MRP-1) (Miyake *et al.*, 1991). MAbs against CD9 have been shown to inhibit cell motility and metastatic potential of various transformed cell lines (Miyake *et al.*, 1991). Furthermore, ectopic expression of CD9 in tumour cells results in decreased motility (Ikeyama *et al.*, 1993). Expression of CD9 has also been found to be inversely correlated with metastatic potential and/or increased survival in non-small cell lung carcinoma and breast cancer (Higashiyama *et al.*, 1995; Adachi *et al.*, 1998; Miyake *et al.*, 1995). Like CD9, CD82 has a potential role as a tumour suppressor gene, as deletions in 11p11.2, the region encoding CD82, correlated with poor prognosis in prostate cancer (Dong *et al.*, 1995). Since this region encodes a variety of genes, the exact role of CD82 in tumour progression requires further investigation. However, other studies have also found decreased CD82 expression in a broad range of cancers (White *et al.*, 1998; Adachi *et al.*, 1998), thus further implicating this molecule as a possible tumour suppressor.

In contrast, other TM4SF members, CO-029, TM4SF5 and SAS (Sarcoma Amplified Sequence), which were originally described as tumour associated antigens, are upregulated by

transformed cells. CO-029 expression has been reported on pancreatic, colon, rectal and gastric carcinomas, while most normal tissues were negative (Szala *et al.*, 1990). Furthermore increased expression of CO-029 in transformed tissues was due to gene amplification (Jankowski *et al.*, 1994). Like SAS, TM4SF5 was observed to be highly expressed on pancreatic carcinomas (Muller-Pillasch *et al.*, 1998). Thus expression of some TM4SF members correlates with tumour progression (CO-029, TM4SF5 and SAS) while other members (CD9, possibly CD63 and CD82) appear to play a negative regulatory role. However, despite the reported differential expression of TM4SF members during development and cellular activation, the exact role these molecules play has not been elucidated. Upregulated expression of TM4SF proteins is also observed in haemopoietic malignancies. Although not expressed on normal myeloid progenitor cells, CD9 is a marker for 90% of non-T acute lymphoblastic leukaemias and 50% of acute myeloid and chronic lymphoid leukaemias (Ashman *et al.*, 1987; Boucheix *et al.*, 1991). Also, PETA-3 was originally identified as platelet antigen expressed by a subset of acute myeloid leukaemias (Ashman *et al.*, 1991) and the anti-PETA-3 mAb 14A2.H1 failed to bind normal myeloid progenitor cells. Additionally, PETA-3 was subsequently re-cloned as a molecule upregulated in phytohaemagglutinin stimulated or Human T cell Leukaemia virus-1 transformed T cells (Hasegawa *et al.*, 1996).

The expression, or lack of expression of several TM4SF members has also been associated with several disease states. Expression of the rat homologue of CO-029 (D6.1A) was found to interfere with coagulation, giving rise to massive bleeding near tumours derived from cell lines transfected this molecule (Claas *et al.*, 1998). Furthermore, administration an of anti-D6.1A mAb was able to partly reverse the bleeding disorder, suggesting a direct role for D6.1A in this phenomenon. In humans, loss of CD53 expression has been associated with recurrent bacterial, viral and fungal infections, and this molecule appears crucial for normal

neutrophil function (Mollinedo, 1997). Finally the generation of CD81 null mice has demonstrated a role for this molecule in B cell responses (Maecker and Levy, 1997). Interestingly, T cell development in these mice was normal, despite the proposed role of CD81 in thymocyte development (Boismenu *et al.*, 1996; see Section 1.2.4). B cells derived from CD81 null mice show decreased levels of CD19, suggesting that CD81 expression is required for normal levels of expression of CD19 with which it complexes and for optimal antibody production by B cells (see Section 1.2.4).

1.2.3 The TM4SF are Components of Multi-Protein Complexes

Although the exact biochemical function of the TM4SF is unknown, many studies have shown tetraspans to mediate a broad range of biological responses (reviewed in Wright and Tomlinson, 1994; Maecker *et al.*, 1997). These functional studies have mostly been performed by perturbation of TM4SF proteins using mAbs, as no ligands for these molecules have been described to date. Functional responses invoked in this manner include the regulation of cellular differentiation, proliferation, activation and motility. Furthermore, the responses elicited by mAb binding appear to be associated with multi-protein complexes of which the tetraspan is a component. From these studies there have emerged two broad categories of TM4SF molecules; those involved in immune signalling complexes (see Section 1.2.4) and those associated with integrins (Section 1.2.5). MAbs against tetraspans associated with immune signalling complexes generally modulate cellular differentiation, proliferation and activation events, while those against integrin associated members tend to regulate cellular motility and adhesive behaviour. To add further complexity, many TM4SF proteins may associate with other tetraspans and several, including CD9, CD53, CD63, CD81 and CD82, are associated with both immune receptors and integrins (reviewed in Wright and Tomlinson, 1994; Maecker *et al.*, 1997). For example, CD81 and CD82 are both components

of lymphocyte co-receptors (Imai and Yoshie, 1993, Imai and Yoshie, 1995). Additionally, CD81 is present in integrin complexes along with CD9 and CD63 (Berditchevski *et al.*, 1995; Rubinstein *et al.*, 1996).

Apart from being components of immune and integrin complexes, TM4SF/TM4SF associations have also been reported. For example, the association of uroplakins Ia with Ib in the asymmetric unit membrane of urothelial cells (Wu *et al.*, 1995) and peripherin with Rom-1 in photoreceptors (Moritz and Molday, 1996) have been described. The uroplakins Ia and Ib have been demonstrated to form hetero-oligomers which are thought to primarily play a structural role in the bladder urothelium (Wu *et al.*, 1995), whilst the function of peripherin/Rom-1 complexes in photoreceptors is less clear.

1.2.4 Association of TM4SF Proteins with Immune Signalling Complexes

Perhaps better characterised are those TM4SF members involved in immune complex signalling. Both CD81 and CD82 associate with each other and the T lymphocyte co-receptor molecules CD4 and CD8 (Imai and Yoshie, 1993; Imai *et al.*, 1995). Furthermore, association of CD81 and CD82 with CD4 competed the binding of lck, a Src family kinase, to CD4 (Imai and Yoshie, 1993). CD81 is also a component of the CD19/CD21/CD81/Leu^{13} complex on B cells (Matsumoto *et al.*, 1993). This complex amplifies signal transduction events initiated by membrane bound immunoglobulin (Ig), the B cell receptor, to promote B cell activation (Bradbury *et al.*, 1992; Tedder *et al.*, 1997). Therefore CD81 may promote the stable formation of the CD19/CD21/CD81/Leu^{13} complex or may initiate inter-complex associations, perhaps with integrins. CD81 also appears to be involved in T cell immune responses and T cell development, as anti-CD81 mAbs preferentially promote T cell production of interleukin (IL)-4, and synergise with anti-CD3 mAb to promote thymocyte proliferation (Secrist *et al.*, 1996; Todd *et al.*, 1996). Additionally CD81 has been reported be

a component of complexes containing the other TM4SF members CD37, CD53 and CD82, as well as major histocompatibility complex (MHC) class II, CD19 and CD21 in B cells (Angelisova *et al.*, 1994), although the significance of this finding has yet to be defined.

Functionally, ligation of CD81 or CD82 by mAb usually results in similar effects as these two TM4SF proteins are often associated. Stimulation with anti-CD81 mAb suppressed proliferation of lymphoid cells (Oren *et al.*, 1990), altered cellular morphology (Geisert *et al.*, 1996; Lin *et al.*, 1992) and blocked syncytium formation (Imai and Yoshie, 1993; Imai *et al.*, 1992). Anti-CD82 mAb also show effects on syncytium formation (Imai and Yoshie, 1993; Imai *et al.*, 1992), cellular morphology, provide costimulatory signals promoting T cell activation (Nojima *et al.*, 1993; Lebel-Binay *et al.*, 1995a) and stimulate tyrosine phosphorylation and increased intracellular calcium in monocytic cells (Gil *et al.*, 1992; Lebel-Binay *et al.*, 1995b). Induction of homotypic adhesion and other adhesive behaviour has been observed with anti-CD81 mAb; this may be due to associated integrins, and will be addressed in Section 1.2.5. MAb against CD37 also induce homotypic adhesion of B cells, however no integrin associations have been described for this molecule (Barrett *et al.*, 1991).

Association of CD53 with CD2 in a rat natural killer cell line and T cells has been reported, and anti-CD53 mAb initiate phosphatidylinositol signalling pathways in these cells (Bell *et al.*, 1992). Additionally, stimulation with anti-CD53 mAb led to increased cytosolic calcium, tyrosine phosphorylation, promoted cell proliferation and synergised with ligation of the T cell receptor by an anti-T cell receptor mAb. Anti-CD53 mAb also induced phosphatidylinositol signalling and increased intracellular calcium in macrophages, monocytes and weakly in B cells (Bosca and Lazo, 1994; Olweus *et al.*, 1993; Rasmussen *et al.*, 1994). Based on studies using inhibitors of protein kinase C (PKC) and G-proteins (Olweus *et al.*, 1993), signalling via CD53 ligation appears to be predominantly mediated though tyrosine kinases, and may be regulated by an as yet unidentified phosphatase

associated with CD53 (Carmo and Wright, 1995). Similarly, several studies have demonstrated that ligation of CD9 provides a co-stimulatory signal for T cell activation (Tai *et al.*, 1996; Toyo-oka *et al.*, 1997; Park *et al.*, 1998; Li and Tait, 1998). In rat basophilic leukaemia cells, CD63 has been shown to associate with the high affinity receptor for IgE (FcεRI) (Kitani *et al.*, 1991). Although treatment of rat basophilic cells with anti-CD63 mAb abrogated IgE-mediated histamine release, the exact role of CD63 in this complex is not understood.

1.2.5 Association of TM4SF Members with Integrins

The association of TM4SF proteins with integrins is a relatively new finding, with studies reporting these observations emerging in the last three years. However, during this short time a myriad of data investigating the integrins involved and the possible role of TM4SF proteins in these complexes has been produced (reviewed in Hemler *et al.*, 1996; Maecker *et al.*, 1997).

The TM4SF members, notably CD9, CD63, CD81 and CD82, have been demonstrated to associate with integrins, a family of cell surface receptors comprised of an α and β heterodimer (see Section 1.3). Reported integrin associations are predominantly with those of the β1 subfamily, including α3β1 (Berditchevski *et al.*, 1995; Nakamura *et al.*, 1995; Berditchevski *et al.*, 1996; Hadjiargyrou *et al.*, 1996), α4β1 (Rubinstein *et al.*, 1994; Mannion *et al.*, 1996) and α6β1 (Berditchevski *et al.*, 1995; Berditchevski *et al.*, 1996; Hadjiargyrou *et al.*, 1996) (reviewed in Hemler *et al.*, 1996; Maecker *et al.*, 1997). Immunoprecipitation studies have shown CD9 to be associated with both α4β1 and α5β1 in the pre-B cell line NALM-6, while in the erythroleukaemic derived cell line HEL, CD9 was only associated with α4β1 (Rubinstein *et al.*, 1994). Similarly, CD81, CD82, CD53 and CD63 were shown to associate with α4β1 (Mannion *et al.*, 1996). CD63 has also been demonstrated to associate

with α3β1 and α6β1 integrins in HT1080 fibrosarcoma cells and in transfected K562 cells expressing either the α3 or α6 chain (Berditchevski *et al.*, 1995). Furthermore CD9, CD63 and CD81 were shown to associate with each other and α3β1 to form tetraspan/integrin and tetraspan/tetraspan/integrin complexes (Berditchevski *et al.*, 1996; Rubinstein *et al.*, 1996). To add further complexity, TM4SF proteins have been reported to associate with other integrins subfamilies, namely CD9 with αIIbβ3 in platelets (Slupsky *et al.*, 1989; Indig *et al.*, 1997), CD81 with α4β7 in B cells (Mannion *et al.*, 1996) and CD63 with αLβ2 (Skubitz *et al.*, 1996) in neutrophils. Additionally, the association of CD9 with heparin-binding epidermal growth factor (HB-EGF), the receptor for diphtheria toxin, has been demonstrated (Mitamura *et al.*, 1992; Iwamoto *et al.*, 1994) in monkey cells. Although not alone sufficient for diphtheria toxin binding, co-expression of CD9 with HB-EGF enhanced toxin binding 10-fold. Therefore CD9 may to contribute to diphtheria toxin binding through a combinatorial epitope of CD9/HB-EGF or by conformational alteration of HB-EGF induced by the association of CD9. Additionally this complex has been shown to contain the integrin α3β1 (Nakamura *et al.*, 1995). Finally, the reports of CD63 associations with phosphatidylinositol 4-kinase (PI4-K) (Berditchevski *et al.*, 1997a), an unknown phosphatase (Carmo and Wright, 1995) and of CD9 with small GTP binding proteins (Seehafer and Shaw, 1991), suggest TM4SF proteins may link integrin complexes with signal transduction pathways.

The association of TM4SF proteins with integrins appears to have functional consequences, as anti-TM4SF mAbs may modulate cellular motility and adhesion. Perturbation of CD9 with mAbs resulted in homotypic adhesion and enhanced adhesion of pre-B cells to bone marrow fibroblasts via activation of α4β1 and α5β1 (Masellis-Smith and Shaw, 1994), augmented adherence of neutrophils to EC (Forsyth, 1991) and induced homotypic adhesion of pre-B cells (Masellis-Smith *et al.*, 1990). Similarly, anti-CD81 mAb

induced B-cell adhesion to tonsil interfollicular stroma (Behr and Schriever, 1995) and αLβ2 dependent homotypic adhesion of thymocytes (Todd *et al.*, 1996). CD63 also plays a role in modulation of adhesion as mAb block neutrophil binding to immobilised serum components and EC (Toothill *et al.*, 1990; Smith *et al.*, 1995; Skubitz *et al.*, 1996). It is important to note, that anti-integrin mAbs inhibit some of the responses induced by anti-TM4SF mAbs (Masellis-Smith and Shaw, 1994; Shaw *et al.*, 1995; Behr and Schriever, 1995). Therefore the modulation of adhesive behaviour by anti-TM4SF mAbs, in some cases at least, appears to be indirectly mediated. Additionally the interaction of anti-TM4SF mAbs with Fc receptors may contribute to some of the stimulatory effects observed.

Since cellular adhesion is inversely correlated with migration (Palecek *et al.*, 1997), it comes as no surprise that TM4SF members are also capable of modulating cell motility. This phenomenon is especially apparent for CD9, which has been demonstrated to both promote and inhibit cell motility. Transfection of CD9 into a poorly motile B cell line (Raji) resulted in an α4β1, α6β1 dependent increase in cell motility on their cognate ligands (Shaw *et al.*, 1995). Conversely, expression of CD9 in several highly motile cell lines abrogated their motility (Ikeyama *et al.*, 1993). CD9 has also been demonstrated to play a role in Schwann cell migration and adhesion *in vitro* (Anton *et al.*, 1995). Expression of CD63 and its association with β1 integrins has been reported to decrease motility in melanoma cells (Radford *et al.*, 1996).

Several studies have investigated the regions of the TM4SF protein and integrin chains involved in the interaction of these two molecules. Based on experiments with chimeric α4 integrin chains, the cytoplasmic domain of the α4 integrin chain did not appear necessary for its association with CD81 or the β1 chain whilst the transmembrane and extracellular juxtamembrane domains were required (Mannion *et al.*, 1996). Interestingly, the presence of divalent cations was not required for CD81/α4β1 association, however CD81 failed to

associate with an α4 mutant with disrupted calcium binding domains. Similar studies using CD9/CD81 chimeras demonstrated that the large extracellular loop between transmembrane domains three and four and/or the fourth transmembrane domain of CD9 was required for the specific association of CD9 with the precursor of the β1 integrin chain (Rubinstein *et al.*, 1997).

The mechanism by which these molecules modulate integrin function remains unclear. It has been postulated that TM4SF proteins may act as ion channels associated with integrins or that their association/dissociation may alter the conformation of integrin complexes, thus regulating function. Alternatively, the association of TM4SF members with each other may form a network across the plasma membrane, the so-called "tetraspan web" (Rubinstein *et al.*, 1996), in which they may act as molecular facilitators providing co-ordination of integrin complexes (Maecker *et al.*, 1997). Finally these molecules may be components of outside-in signalling cascades, linking receptors such as integrins with signalling molecules such as phosphatases (Carmo and Wright, 1995), kinases (Skubitz *et al.*, 1996; Berditchevski *et al.*, 1997a) and small G-proteins (Seehafer and Shaw, 1991). To date there is little evidence to either support or refute these proposals and further investigation will be required to examine the biochemical significance of TM4SF/integrin associations.

1.3 The Integrin Superfamily of Adhesion Molecules

Members of the integrin superfamily of receptors (Hynes, 1987) are comprised of α and β chain heterodimers which are anchored to the cytoskeleton. To date 16 α chains and 8 β chains have been described which are all members of the Ig superfamily. The α and β chains give rise to 22 combinations of heterodimers (Table 1.2). Additionally, splice variants within the extracellular or intracellular domains of several integrin chains have been reported (See Table 1.2). It is the association of the α and β chain which defines ligand specificity,

Table 1.2 Integrin Heterodimers and their Ligands

Integrin heterodimers are listed by β chains. Other names refer to CD classifications for specific chains or nomenclature for specific αβ heterodimers (in parenthesis). Isoforms of integrin chains are indicated (e) for extracellular variants or (c) for cytoplasmic variants.

* Where possible only first author listed.

Abbreviations used; COL: Collagen, FN: Fibronectin, vWF: von Willebrand Factor, VN: Vitronectin, FG: Fibrinogen, TSP-1: Thrombospondin, OP: Osteopontin, LN: Laminin, ICAM: Inter-Cellular Adhesion Molecule, VCAM: Vascular Cell Adhesion Molecule, MadCAM: Mucosal Addressin Cell Adhesion Molecule, VLA: Very Late Antigen, LFA: Leukocyte Functional Antigen.

Hetero-dimer	Other Names	Major Ligand/s	Reference*
β1[c]	CD29		(Argraves *et al.*, 1987)
α1	CD49a (VLA-1)	COL, LN	(Takada and Hemler, 1989)
α2	CD49b (VLA-2, GPIaIIa)	COL, LN, FN	(Takada *et al.*, 1991)
α3[i]	CD49c (VLA-3)	FN, LN, COL, Epiligrin	(Takada *et al.*, 1989)
α4	CD49d (VLA-4)	FN, VCAM-1	(Argraves *et al.*, 1987; Fitzgerald *et al.*, 1987a)
α5	CD49e (VLA-5, GPIcIIa)	FN	(Tamura *et al.*, 1990)
α6[e,c]	CD49f (GPIcIIa)	LN	(Kramer *et al.*, 1991)
α7[e,c]		LN	(Bossey *et al.*, 1991)
α8		FN	(Palmer *et al.*, 1993)
α9		LN, OP, Tenascin	(Vogel *et al.*, 1990)
αv	CD51	VN, FN, OP	
β2	CD18		(Kishimoto *et al.*, 1987; Law *et al.*, 1987)
αL	CD11a (LFA-1)	FG, Factor X, ICAM-1, iC3b	(Larson *et al.*, 1989)
αM	CD11b (Mac-1, Mo-1, CR3)	ICAM-1, ICAM-2, ICAM-3, Factor X, iC3b	(Corbi *et al.*, 1988; Arnaout *et al.*, 1988; Hickstein *et al.*, 1989)
αX	CD11c (p150,95)	FG, iC3b, C4, ICAM-1, ICAM-3	(Corbi *et al.*, 1987)
αD		ICAM-1, ICAM-3	(Van der Vieren *et al.*, 1995)
β3[c]	CD61		(Fitzgerald *et al.*, 1987b; Rosa *et al.*, 1988; Zimrin *et al.*, 1988)
αIIB[e]	CD41 (GPIIb-IIIa)	FB, FN, vWF, VN, TSP-1	(Poncz *et al.*, 1987; Uzan *et al.*, 1988)
αv	CD51	VN, FB, vWF, TSP-1, FN, OP, COL, LN, PECAM-1?	(Suzuki *et al.*, 1987)
β4[c]			(Tamura *et al.*, 1990; Hojervorst *et al.*, 1990; Suzuki *et al.*, 1990)
α6	CD49f	LN	(Tamura *et al.*, 1990)
β5			(Ramaswamy *et al.*, 1990; Suzuki *et al.*, 1990; McLean *et al.*, 1990)
αv		VN, FN, OP	(Suzuki *et al.*, 1987)
β6			(Sheppard *et al.*, 1990)
αv		FN, Tenascin	(Suzuki *et al.*, 1987; Busk 1992)
β7			(Yuan *et al.*, 1990; Erle *et al.*, 1991)
α4		FN, VCAM-1, VCAM-2, ICAM-3, TSP-1, MadCAM-1	(Cerf-Bensussan *et al.*, 1992)
αE		E-cadherin	(Cerf-Bensussan *et al.*, 1992)
β8			(Moyle *et al.*, 1991)
αv		VN, FN, LN, COL	(Suzuki *et al.*, 1987)

although there is a great deal of promiscuity with ligand binding, such that one αβ heterodimer may recognise a broad range of ligands (reviewed in Hynes, 1987; Hynes, 1992; Loftus and Liddington, 1997). For example the αvβ3 heterodimer recognises at least 8 different ligands. To add further complexity, several integrin heterodimers may bind the same ligand as α1β1, α2β1, α3β1, α6β1, α7β1,α9β1, αvβ3, α6β4 and αvβ8 are all receptors for laminin (Table 1.2). Initially the ligands reported for integrin receptors were extracellular matrix (ECM) proteins (collagens, laminins, fibronectin and vitronectin). However further studies have shown that immobilised soluble proteins, including von Willebrand Factor (vWF) and osteopontin, soluble proteins (fibrinogen) or cell surface molecules (VCAM-1 and ICAM-1) may also be substrates (Table 1.2). Through their binding of ECM components (and other ligands) members of the integrin superfamily of adhesion receptors link the cytoskeleton to the extracellular environment. Cells respond to their immediate microenvironment through integrin ligation and regulate interactions with neighbouring cells and the ECM through modulation of both integrin expression and function.

Integrins are widely expressed throughout many tissues and cell types, although restriction of individual chains is apparent. For example, the β1 integrin chain is expressed broadly whilst β2 integrins are expressed solely by leukocytes. β1 integrins are mostly involved in binding to ECM components, whilst β2 integrins are required for leukocyte extravasation across the endothelium and other cell mediated immune functions, for example superoxide production (reviewed in Gahmberg *et al.*, 1998; Carlos and Harlan, 1994). Therefore, the *in vivo* distribution of different integrin heterodimers appears related to their individual functions.

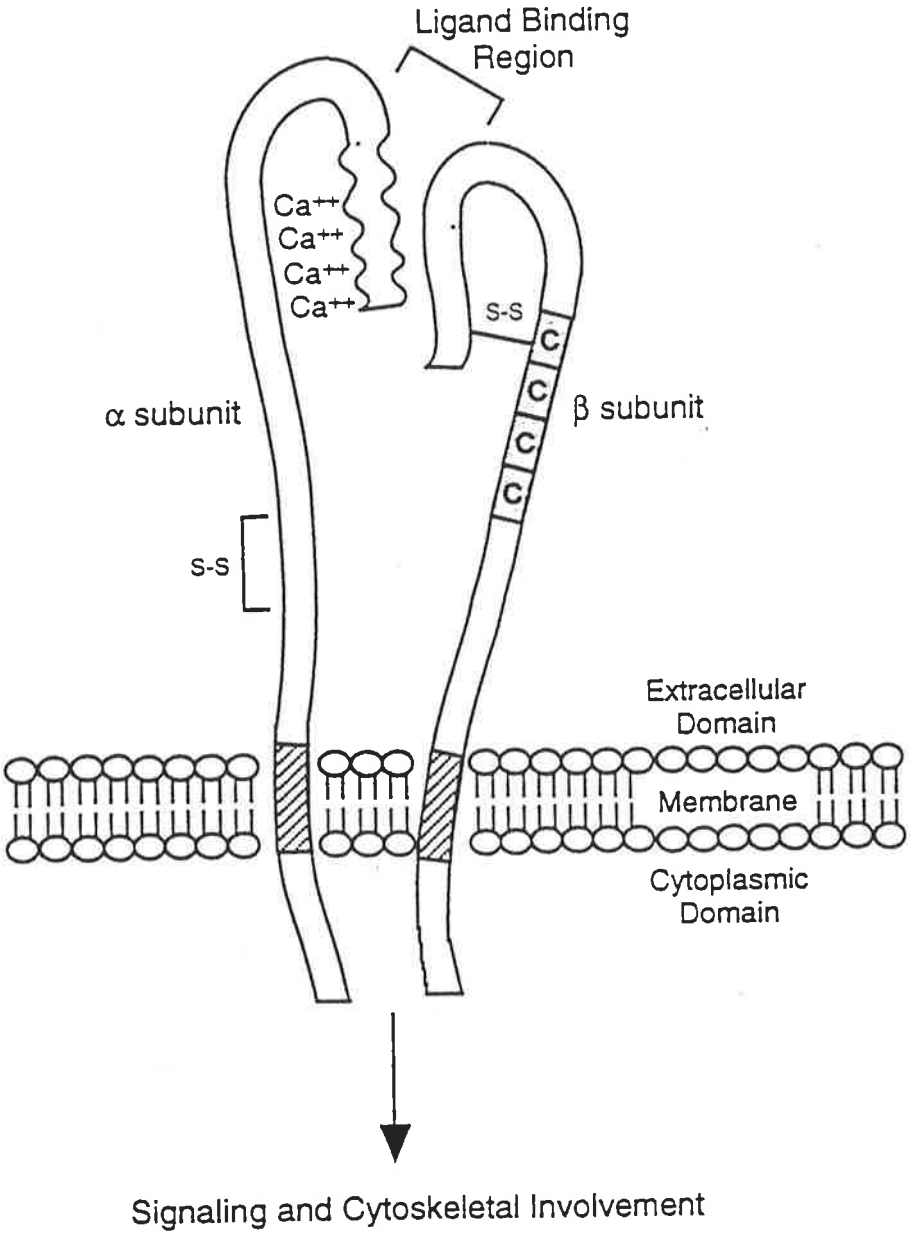
1.3.1 Integrin Structure

The integrin heterodimer consists of a large extracellular domain, comprised of both the α and β chain, approximately 1000 and 750 amino acids each respectively, which are non-covalently associated (Figure 1.3) (Hynes, 1992). High affinity ligand binding to the extracellular region requires both α and β subunits, as has been demonstrated for the platelet αIIb/β3 complex (Kunicki *et al.*, 1981; Brass *et al.*, 1985; Fitzgerald and Phillips, 1985; Shattil *et al.*, 1985). Divalent cations, Ca^{++}, Mg^{++} and Mn^{++}, are also required for ligand binding and highly conserved cation binding motifs are present within several α and β chains (Ginsberg *et al.*, 1986; Michishita *et al.*, 1993; Elices *et al.*, 1991; Gailit and Ruoslahti, 1988; Kirchhofer *et al.*, 1990; Kirchhofer *et al.*, 1991; Loftus and Liddington, 1997). In general, α chains contain multiple motifs (three-four) whilst β chains usually contain a single site. Furthermore, these conserved motifs flank, or in some cases lie within, the proposed ligand binding domain. Occupation of these sites by divalent cations potentiates ligand binding, as seen by binding of fibrinogen to αIIb which is of highest affinity when all four divalent cation binding sites are occupied by Ca^{++} (Gulino *et al.*, 1992). Integrin affinity may also be modulated by the particular cation present (Bazzoni *et al.*, 1998), for example α3β1 affinity for laminin and fibronectin, which is highest in the presence of Mn^{++}, moderate in Mg^{++} and lowest in Ca^{++} (Elices *et al.*, 1991). Interestingly, the affinity of α3β1 for collagen remains unaltered in the presence of either of these divalent cations.

The intracellular cytoplasmic carboxyl termini of both α and β integrin chains are relatively short, around 20-70 amino acids, with the exception of the intracellular domain of β4 which is over 1000 residues (Suzuki and Naitoh, 1990; Hojervorst *et al.*, 1990). Despite its short length, the intracellular region of the integrin heterodimer is responsible for anchoring to the cytoskeleton and transduction of signalling events.

Figure 1.3 Schematic Representation of a Typical Integrin Heterodimer

Integrins are composed of non-covalently associated α and β chains. Both chains contain a transmembrane domain and short cytoplasmic tails, 20-70 amino acids (except for β4 which is ~1000 amino acids). Intracellular domains are capable of association with the cytoskeleton and signalling molecules. The extracellular domain of the α chain contains several calcium binding motifs (Ca^{++}) and the β chain contains four cysteine-rich repeats (C). The large extracellular amino terminus of the β chain is stabolised by intrachain disulfide bonding (S-S). Both α and β subunits comprise the extracellular ligand binding site.


Ligand Binding Region
Ca++
Ca++
Ca++
Ca++
S-S
C
C
C
C
α subunit
β subunit
S-S
Extracellular Domain
Membrane
Cytoplasmic Domain
Signaling and Cytoskeletal Involvement

1.3.2 Association of Integrins with Cytoskeletal Components

When cells are plated onto ECM, they adhere then undergo several morphological changes resulting in flattening and spreading onto the adhesive surface. Additionally, accumulation of particular proteins, including several integrins and cytoskeletal components, occurs at sites where cells are in direct contact with the sub-stratum. These sites, termed focal contacts or focal adhesions (Abercrombie *et al.*, 1971; reviewed in Burridge *et al.*, 1997; Jockusch *et al.*, 1995), are required for the long term adhesion of cells to their sub-stratum and provide anchoring points for the cytoskeleton, thus giving rise to cellular morphology. Integrins, especially those of the β1 subfamily, play a central role in the formation of focal contacts through their binding of extracellular ligand and association with cytoskeletal components via their cytoplasmic tails. However, the particular αβ heterodimers recruited to focal contacts depend on the cell type examined and the substrate involved. The association of integrins with the cytoskeleton is also required for the transduction of signals initiated upon integrin ligation. To date, a plethora of molecules, including cytoskeletal components, kinases and adaptor molecules have been shown to directly associate with the intracellular domains of integrin chains; Table 1.3. and Figure 1.4.A.

Integrins of the β1 subfamily are anchored to the actin cytoskeleton via direct interaction of the β1 chain with the carboxyl terminal domain of talin (Pfaff *et al.*, 1998; Knezevic *et al.*, 1996; Horwitz *et al.*, 1986) and α-actinin (Otey *et al.*, 1990; Otey *et al.*, 1993) (Figure 1.4.A). Both talin and actin are anti-parallel homodimers of 270kD and 100kD subunits respectively (Rees *et al.*, 1990; Blanchard *et al.*, 1989). Apart from binding the cytoplasmic region of the β1 chain, talin also interacts with phospholipid membranes (Niggli *et al.*, 1994), vinculin (a major component of focal contacts) (Burridge and Mangeat, 1984; Gilmore *et al.*, 1993), and actin (Mugumuma *et al.*, 1990; Kaufmann *et al.*, 1991). Similarly, α-actinin associates with vinculin (McGregor *et al.*, 1994) and actin (Kuhlmann *et al.*, 1992),

Table 1.3 Molecules Associated with Integrin Cytoplasmic Domains

(Adapted from Hughes and Pfaff 1998).

* Where possible only first authors listed.

** Other Integrin Associated Molecules refers to protein interactions which have not been directly demonstrated to be via the integrin cytoplasmic domains.

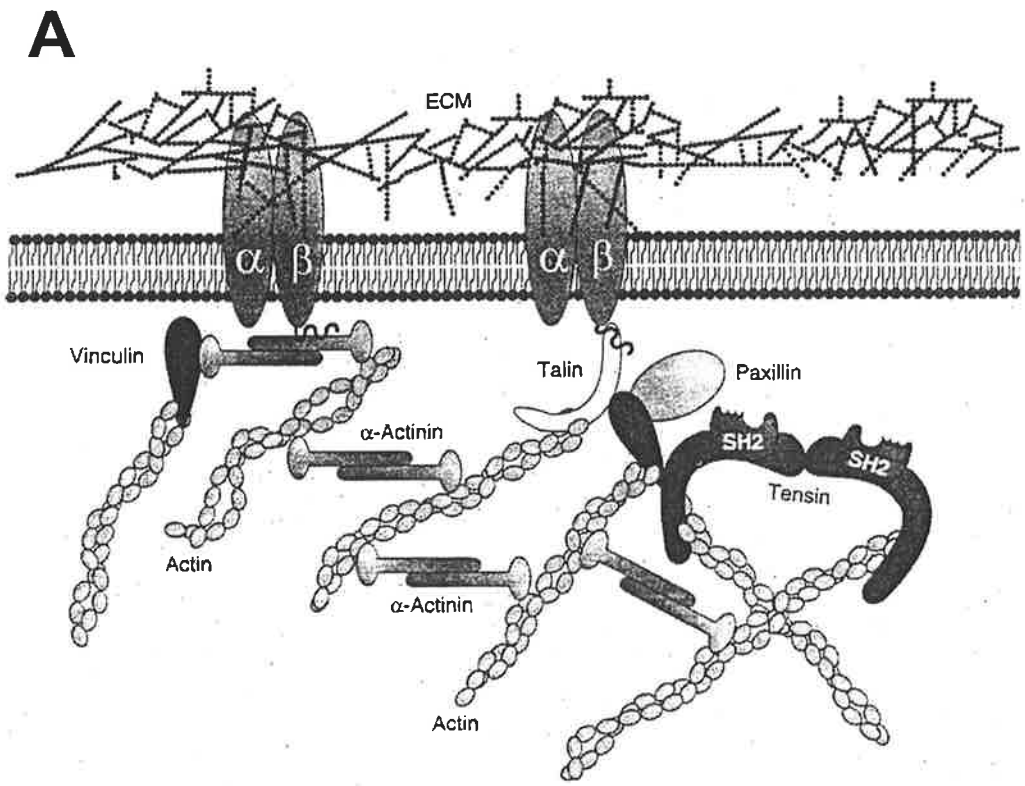
Abbreviations used; CIB: Calcium and integrin binding protein, PTK: Protein Tyrosine Kinase, ICAP-1: Integrin Cytoplasmic domain-Associated Protein-1, Rack-1: Receptor for activated PKC, IAP: Integrin Associated Protein, TM4SF: Transmembrane 4 Superfamily

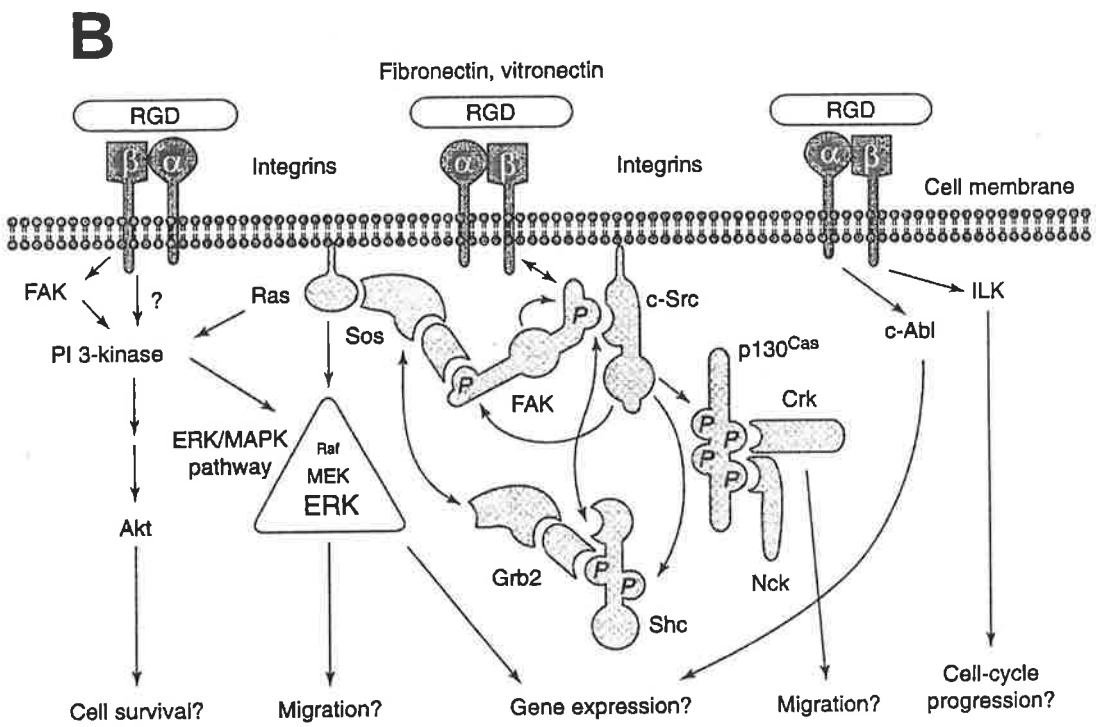
Molecule	Integrin Tail	Features	Reference*
Calreticulin	α	Expression correlates with integrin-mediated cell adhesion	(Coppolino *et al.*, 1995; Opas *et al.*, 1996; Zhu *et al.*, 1997; Coppolino *et al.*, 1997; Rojiani *et al.*, 1991; Dedhar, 1994)
F-Actin	α2	Cytoskeletal Protein	(Kieffer *et al.*, 1995)
CIB	αIIb	Calcium binding protein	(Naik *et al.*, 1997)
Talin	αIIb and β	Cytoskeletal Protein	(Horwitz *et al.*, 1986; Knezevic *et al.*, 1996; Pfaff *et al.*, 1998)
α-Actinin	β	Cytoskeletal Protein	(Otey *et al.*, 1990)
Skelemin	β	Myosin and intermediate filament associated protein	(Reddy *et al.*, 1996)
FAK	β	PTK localised to focal adhesions	(Schaller *et al.*, 1995)
ILK	β	Contains ankyrin repeats and threonine kinase domain	(Hannigan *et al.*, 1996)
Paxillin	β	Adapter with SH2 and SH3 binding motifs and LIM domains	(Tanaka *et al.*, 1996)
ICAP-1	β1 only	Ligation of β1 modulates phosphorylation state	(Chang *et al.*, 1997)
Filamin	β1A, β2, β7	Cytoskeletal Protein	(Pfaff *et al.*, 1998; Sharma *et al.*, 1995)
Cytoadhesin-1	β2 only	Guanine nucleotide exchange activity for ADP-ribosylation factor; overexpression increases αLβ2 mediated adhesion	(Kolanus *et al.*, 1996; Meacci *et al.*, 1997)
β_3-endonexin	β3 only	Overexpression increases αIIbβ3 affinity and function	(Kashiwagi *et al.*, 1997; Shattil *et al.*, 1995)
Rack1	β1, β2, β5	Interaction with integrins PMA dependent	(Liliental *et al.*, 1998)
Other Integrin Associated Molecules** **:**			
IAP (CD47)	αvβ3	Anti-IAP mAbs modulate αvβ3 affinity; receptor for C-terminal domain of thrombospondin	(Brown *et al.*, 1990; Gao *et al.*, 1996)
TM4SF Proteins	Many	Anti-TM4SF mAbs modulate cell adhesion/migration	(reviewed in Hemler *et al.*, 1996; Maecker *et al.*, 1997) Also see table 1.1
Emmprin	β1	Extracellular matrix metalloproteinase inducer	(Berditchevski *et al.*, 1997b)
CD98	Activated Integrins	Early T cell activation marker; modulates integrin affinity	(Fenczik *et al.*, 1997)

Figure 1.4 Integrin Cytoskeletal Interactions and Signalling Pathways

Panel (A) shows the arrangement of cytoskeletal proteins and their interaction with integrin cytoplasmic domains. Integrin αβ heterodimers bind the ECM and are anchored to actin filaments indirectly, via the associations of talin and α-actinin with integrin cytoplasmic domains (taken from Clark and Brugge, 1995).

Panel (B) shows the proposed signalling pathways utilised by integrins. Integrin engagement leads to the activation of FAK, allowing the association of and subsequent activation of Src. The recruitment and activation of Src leads to enhanced phosphorylation of FAK, enabling Grb2 binding of FAK. Activation of FAK, Src (and other Src-family PTKs) and Grb2, may promote the association Grb2 with Shc, facilitating the activity of Ras and the Ras-MAPK pathway (via Sos). Ras and FAK can also activate PI3-K, which apart from facilitating MAPK activation, might promote cellular survival via downstream targets such as Akt. The association of FAK with Src promotes the activation of $p130^{Cas}$ and its association with Src. Activated $p130^{Cas}$ may lead to migration events through the stimulation of the JNK MAPK pathway (via the adaptors Crk and Nck). The activation of ILK and c-Abl by integrin ligation may also modulate cell cycle progression and gene expression respectively (taken from Schlaepfer and Hunter, 1998).


A
ECM
α
β
α
β
Vinculin
Talin
Paxillin
α-Actinin
SH2
SH2
Tensin
Actin
α-Actinin
Actin


B
Fibronectin, vitronectin
RGD
RGD
RGD
Integrins
Integrins
Cell membrane
FAK
?
Ras
Sos
c-Src
ILK
c-Abl
PI 3-kinase
p130Cas
FAK
Crk
ERK/MAPK
pathway
Raf
MEK
ERK
Akt
Grb2
Shc
Nck
Cell survival?
Migration?
Gene expression?
Migration?
Cell-cycle
progression?

as well as zyxin, a 82kD phosphoprotein. Zyxin contains several cysteine-rich zinc binding domains which are believed to mediate protein-protein interactions. This molecule has also been found associated with focal contacts (Crawford *et al.*, 1992).

More recently, other components of the cytoskeleton have been demonstrated to associate with the β1 chain. These include paxillin, an adaptor protein containing Src homology (SH) domains 2 and 3 (Tanaka *et al.*, 1996), and skelemin, an intermediate filament associated protein (Reddy *et al.*, 1996). Interactions with the α chain tail have also been reported for calreticulin (Coppolino *et al.*, 1995) and F-actin (Kieffer *et al.*, 1995). In addition, protein tyrosine kinases (PTK) including focal adhesion kinase (FAK) (Schaller and Parsons, 1994) and integrin linked kinase (ILK) (Hannigan *et al.*, 1996) may interact directly with the β chain cytoplasmic tail. Finally, the receptor for activated PKC (Rack1) was demonstrated to associate with β1, β2 and β5 integrin cytoplasmic domains in kidney and lymphoblastoid cell lines (Liliental and Chang, 1998). However the association of Rack1 was dependent on pretreatment with phorbol esters, which activate PKC, suggesting that Rack1 may link activated PKC directly to integrins.

Two selective integrin binding proteins are β_3-endonexin and cytoadhesin-1, which specifically bind the β3 and β2 cytoplasmic domains respectively. Interestingly, overexpression of these molecules results in increased affinity of either αIIbβ3 (Kashiwagi *et al.*, 1997) or αLβ2 (Kolanus *et al.*, 1996) for their ligands, suggesting these molecules play a role in modulation of integrin affinity (see Section 1.3.3). The cytoskeletal structural protein Filamin, an actin cross-linking protein, has also been demonstrated to specifically associate with the cytoplasmic domain of the β2 integrin chain (Sharma *et al.*, 1995).

As mentioned previously, the cytoplasmic domain of the β4 integrin is very large in comparison to other integrin chains, over 1000 amino acids. The β4 chain has been identified as a major component of hemidesmosomes (Stepp *et al.*, 1990; Sonnenberg *et al.*, 1991; Jones

et al., 1991), which are adhesion structures involved in anchoring intermediate filaments of the cytoskeleton to the basement membrane. Similar to hemidesmosomes, desmosomes are structures within lateral cell contacts responsible for anchoring the intermediate filaments of neighbouring cells to plaques within the cell junction (reviewed in Garrod, 1993). However, unlike desmosomes, in which cell-cell interactions are mediated by cadherins, the cell-basement membrane binding of hemidesmosomes is mediated by the α6β4 integrin. The α6 chain is the only α chain known to heterodimerise with the β4 chain and is predominantly localised to the basal surface of epidermal basal cells (Sonnenberg *et al.*, 1991). Within the cytoplasmic domain of β4 lie several fibronectin type III repeats (Hojervorst *et al.*, 1990; Tamura *et al.*, 1990; Suzuki and Naitoh, 1990) analogous to the structure of desmoglein, the cadherin-like component of desmosomes. These repeats are proposed to be involved in interactions with the dense plaque, lying directly below the plasma membrane, and possibly with intermediate filaments. Thus it is the association of the integrin cytoplasmic domains with cytoskeletal components and other molecules which is responsible for the targeting of integrins to focal contacts or hemidesmosomes, providing structural integrity, modulating integrin affinity (see Section 1.3.3) and initiating subsequent signalling events (see Sections 1.3.4 and 1.3.5) (reviewed in Williams *et al.*, 1994).

1.3.3 Signalling via Integrins: Inside-Out

Some cell types are capable of rapidly modulating integrin function by altering integrin ligand binding affinity (Bennet and Vilaire, 1979) thus enabling rapid attachment to or detachment from substrate as required. This process of inside-out signalling, or affinity modulation, has been described for several integrins including members of the β1, β2, β3 and β7 subfamilies (Crowe *et al.*, 1994; Altieri and Edgington, 1988; Bennet and Vilaire, 1979; Shimizu *et al.*, 1990; Hughes and Pfaff, 1998). Although the mechanism of affinity

modulation remains unclear, many studies have examined this phenomenon using the platelet specific αIIbβ3 complex. Platelets, or thrombocytes, (Section 1.5) express an abundant amount of the αIIbβ3 integrin which serves as the receptor for fibrinogen (a soluble plasma protein required for platelet aggregation). Treatment of platelets with soluble agonists, such as adenosine diphosphate (ADP) or thrombin, leads to rapid upregulation of αIIbβ3 affinity for fibrinogen (Bennet and Vilaire, 1979). These agonists do not themselves act directly on the integrin but rather transduce signals through their own receptors activating PTK and G-proteins, and other downstream events, which modulate integrin affinity (Shattil and Brass, 1987). This proposal is supported by the finding that inhibition of these signalling pathways leads to the failure of integrin affinity modulation by soluble agonists (Shattil and Brass, 1987; Fujimoto *et al.*, 1982; Dustin and Springer, 1989).

Affinity modulation of the αIIbβ3 complex results in a low level of phosphorylation of the cytoplasmic domain of β3, probably mediated by PKC (Smyth *et al.*, 1992). However this does not appear to be solely sufficient to explain affinity modulation. Furthermore, affinity modulation of β2 integrins does not appear to involve phosphorylation of the β2 chain and mutation of the phosphorylation site does not perturb integrin function (Chatila *et al.*, 1989; Buyon *et al.*, 1990; Merrill *et al.*, 1990; Hibbs *et al.*, 1991).

Although phosphorylation of the cytoplasmic domains does not appear crucial for integrin affinity modulation, the cytoplasmic domains themselves are nonetheless critical for this process (reviewed in Ginsberg *et al.*, 1992; Schwartz, 1992; Sastry and Horwitz, 1993). Currently, the working model for integrin affinity modulation involves the cell type specific recruitment of "activating" molecules or complexes to the integrin cytoplasmic tails. Possible candidate molecules include the focal adhesion kinase FAK (Section 1.3.4), calreticulin (Rojiani *et al.*, 1991; Dedhar, 1994), α-actinin, or talin.

Binding of activating molecules to the integrin cytoplasmic tails has been proposed to alter the conformation of the integrin heterodimer. This altered conformation is believed to be transmitted across the plasma membrane via a membrane proximal sequence, a lysine followed by a short hydrophobic stretch then a charged region (Williams *et al.*, 1994). In α chains this sequence is XGFFKR and in β chains LLviXhDR, where X is a non-conserved amino acid and lower case letters are less conserved residues. The importance of the membrane proximal GFFKR motif has been highlighted by mutation of this region which leads to constitutive αIIbβ3 activation (O'Toole *et al.*, 1994). Transmission of the altered conformation to the extracellular domains, via the transmembrane proximal regions, is proposed to lead to increased ligand affinity. Other sequences in the integrin cytoplasmic domain involved in activation or protein-protein interactions include the highly conserved NPXY or NPXF motifs (O'Toole *et al.*, 1995). The exact mechanism and the molecules involved in the "activation" of the high affinity state remains an area of ongoing research (reviewed in Hughes and Pfaff, 1998; Williams *et al.*, 1994).

1.3.4 Signalling via Integrins: Outside-In

The majority of integrin ligands are immobilised ECM components, thus allowing clustering of ligand-occupied integrins. This promotes anchoring of the cytoskeleton, via focal adhesions and generates mechanical forces which give rise to stress fibres and shape the cytoskeleton. However, concurrent with the structural aspects of integrin ligation is an array of biochemical changes within the cytosol which are capable of modulation of cellular functions including survival, differentiation, state of activation and growth (reviewed in Hynes, 1992; Clark and Brugge, 1995; Schwartz *et al.*, 1995; Schlaepfer and Hunter, 1998). In a similar fashion to growth factor and cytokine receptors, integrins activate protein tyrosine kinases, increase inositol lipid turnover and raise intracellular Ca^{++} and pH in response to

ligand binding. Furthermore, several of the molecules involved in integrin mediated signalling pathways are shared by growth factor receptors (summarised in Table 1.4) allowing cross-talk between integrins and growth factor receptor pathways.

Protein phosphorylation is one of the earliest events detected after ligation of integrins and was first described in platelets (Ferrell and Martin, 1989; Golden *et al.*, 1990). Activation of some PTK may be mediated through the direct interaction with the cytoplasmic domain of the integrin β chains, as perturbation of this region results in loss of PTK phosphorylation upon integrin ligation (Guan *et al.*, 1991; Balzac *et al.*, 1994; Lukashev *et al.*, 1994; Akiyama *et al.*, 1994). The major PTK involved in integrin signalling and the pathways these molecules initiate are summarised in Table 1.4 and Figure 1.4.B. To date, two PTK have been shown to associate directly with the β chain, FAK (Schaller *et al.*, 1992) and ILK (Hannigan *et al.*, 1996). FAK is an abundant cytosolic protein which is found in most cell types, localises to focal adhesions and is phosphorylated upon integrin engagement (Schaller *et al.*, 1992). This protein contains a central catalytic domain flanked by a large carboxyl and amino terminal domains. Within the amino terminus lie integrin binding and Src binding domains (Cobb *et al.*, 1994), whilst the carboxyl terminus contains a focal adhesion targeting sequence (Hildebrand *et al.*, 1993), binding sites for cytoskeletal components (paxillin) (Turner and Miller, 1994) and binding sites for the adaptor molecules, Grb2 (Schlaepfer *et al.*, 1994) and $p130^{Cas}$ (Polte and Hanks, 1995). The PTK Csk and the adaptor molecule Crk may associate with FAK via their association with paxillin (Sabe *et al.*, 1994; Birge *et al.*, 1993). Additionally, integrin ligation has been demonstrated to induce the association of FAK with the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) (Chen and Guan, 1994a; Chen and Guan, 1994b). Thus, FAK plays a central role in integrin signalling by acting as both a kinase and a scaffold protein for both signalling and cytoskeletal components (reviewed in Schlaepfer and Hunter, 1998). Studies investigating the phenotype of FAK null mice found

Table 1.4 Components of Integrin Signalling Pathways

(Adapted from Clark and 1995).

* Where possible only first author listed.

Molecule	Characteristics	Reference*
Protein Kinases		
FAK	Localised to focal contacts via FAT, activated by β1, β2 and β3 ligation, associates with Src, Csk, Grb2, PI3-K, paxillin and integrin β chain cytoplasmic domains	(Schaller *et al.*, 1992; Lipfert *et al.*, 1992; Shattil *et al.*, 1994; Scott *et al.*, 1995; Hildebrand *et al.*, 1993; Cobb *et al.*, 1994; Schaller *et al.*, 1995)
ILK	Serine Threonine kinase	(Hannigan *et al.*, 1996)
Src	Associates with cytoskeleton, FAK and paxillin in an integrin dependent manner; activity increased during cell spreading	(Cobb *et al.*, 1994; Kaplan *et al.*, 1995)
Fgr	Src family PTK, Activated by TNF-α in a β2 dependent manner	(Berton *et al.*, 1994)
Csk	Suppresses Src family kinase activity, associates with FAK and paxillin	(Bergman *et al.*, 1995)
Syk	Expressed by haemopoietic cells, activated by β1 and β3 integrin engagement	(Poole *et al.*, 1997; Clark *et al.*, 1994b)
Fyn	Src family kinase	(Cobb *et al.*, 1994)
PKC	Localised to focal contacts, activated by DAG and calcium	(Chun *et al.*, 1993; Vuori *et al.*, 1993)
MAPK	Activated by integrin cross-linking	(Wary *et al.*, 1996)
JNK	Activated by integrin engagement	(Mainiero *et al.*, 1997)
c-ABL	Proto-oncogene, PTK	(Lewis *et al.*, 1996)
SH2-SH3 Containing Proteins		
$p130^{cas}$	Possible substrate of Src?; integrin induced association with Crk and Nck,	(Nakamoto *et al.*, 1996)
Crk	Associates with paxillin, C3G, mSOS1; Integrin induced association with $p130^{cas}$	(Vuori *et al.*, 1996)
Nck	Possible link into JNK MAP kinase pathway	(Schlaepfer *et al.*, 1997)
Grb2	Adaptor linking to Ras activation; associates with FAK and mSOS1	(Schlaepfer *et al.*, 1994)
PI3-K	Integrin-dependent associtaion with FAK, Src, α-actinin	(Chen *et al.*, 1994a; Chen *et al.*, 1994b)
PLC	Activated by integrin engagement	(Kanner *et al.*, 1993)
Shc	Adaptor, associates with FAK, Grb2	(Wary *et al.*, 1996)
Small Molecular Weight GTPases		
Ras	Activated by integrin engagement in T cells	(Kapron-Bras *et al.*, 1993)
Rho	Essential for focal adhesion and stress fibre formation	(Chong *et al.*, 1994; Clark *et al.*, 1998)
mSOS1	Activates Ras, associates with Grb2, Crk	(Tanaka *et al.*, 1994)
C3G	Associates with Crk and Grb2; GDP-GTP exchange Protein	(Tanaka *et al.*, 1997)
Phospholipid Mediators		
PIP-5K	Lipid kinase, activated by Rho	(McNamee *et al.*, 1992; Chong *et al.*, 1994)
$cPLA_2$	Phospholipase, activated by MAPK, Produces arachidonic acid, the precursor of the cyclo-oxygenase pathway	(Chun *et al.*, 1992, Chun *et al.*, 1993)
5-Lipoxygenase	Oxygenates arachidonic acid into leukotrienes	(Chun *et al.*, 1992)

disruption of this gene to be lethal, resulting in defective mesoderm development (Furuta *et al.*, 1995). Cell lines derived from these mice displayed retarded motility, yet showed enhanced focal contact formation, suggesting a role for FAK in the turnover of focal contacts (Ilic *et al.*, 1995). The serine/threonine kinase ILK, was identified by a yeast two-hybrid screen using the β1 cytoplasmic domain and was demonstrated to bind and phosphorylate the β1 cytoplasmic domain *in vitro* (Hannigan *et al.*, 1996). ILK activity was found to be reduced upon fibronectin binding, and overexpression of this molecule, led to disrupted adhesion and morphology of epithelial cells and anchorage-independent growth (Hannigan *et al.*, 1996). Overexpression of ILK has also been demonstrated to upregulate expression of cyclin-dependent kinases (Radeva *et al.*, 1997), thus regulating cell cycle progression (Figure 1.4.B).

Recently, another PTK, Pyk2 (Proline-rich Tyrosine Kinase 2), was demonstrated to be phosphorylated upon integrin engagement (Astier, 1997) and to colocalise with vinculin to focal contacts (Li *et al.*, 1996). Pyk2 shares approximately 45% homology with FAK, however the mechanism by which integrin ligation leads to Pyk2 phosphorylation has not been established as there is no evidence to suggest that this PTK directly interacts with integrins. Like FAK, Pyk2 also associates with paxillin (Ostergaard *et al.*, 1998) and the adaptor $p130^{Cas}$ (Xiong *et al.*, 1998). Translocation and activation of the c-ABL proto-oncogene by integrin ligation has also been demonstrated in fibroblasts adhering to fibronectin (Lewis *et al.*, 1996). Although the mechanism of c-ABL activation by integrins has not been elucidated, it suggests that integrin signalling may directly influence gene expression.

1.3.5 Downstream Signalling Events

The association of Src and Grb2 with FAK suggests that integrin ligation may feed into the small GTPase linked ras-MAPK (mitogen activated protein kinase) pathway (Figure 1.4.B), as MAPK activation has been demonstrated in fibroblasts adhering to fibronectin

(Schlaepfer *et al.*, 1994). Apart from activation of transcription factors, MAPK may also activate cytoplasmic phospholipase A_2 (PLA_2) (Clark and Hynes, 1996), leading to hydrolysis of glycerophospholipids to produce arachidonic acid which is utilised by the cyclo-oxygenase pathway to produce eicosinoids and leukotrienes. Activity of PLA_2 has been shown to be necessary for cell spreading as inhibitors of PLA_2 block this response (Chun and Jacobson, 1992). The production of leukotrienes is also required for actin polymerisation (Peppelenbosch *et al.*, 1993). Therefore activation of PLA_2 appears to be required for normal cytoskeletal changes in response to integrin ligation. The activation of PLA_2 also plays a central role during platelet activation (Section 1.5.3). In addition to the Ras-MAPK pathway, Ras may also activate PI3-K (Figure 1.4.B). The role of PI3-K in integrin mediated signalling is not entirely clear, however evidence suggests possible involvement in cytoskeletal rearrangement (Shattil *et al.*, 1994; Vuori and Ruoslahti, 1993), the promotion of cell survival via activation of the serine threonine kinase Akt (Khwaja *et al.*, 1997) or the enhancement of MAPK activation (King *et al.*, 1997).

The activation of $p130^{Cas}$ by binding of Nck and Crk might lead to migratory events, as both Crk and Nck may lead into the JNK MAP kinase pathway (Tanaka *et al.*, 1997). The activation of phospholipase C (PLC) by ligation of integrins (Langholz *et al.*, 1997) may also promote cytoskeletal rearrangements and migratory events. PLC generates second messengers by hydrolysis of phosphatidyldiphosphate (PIP_2) to diacylglycerol (DAG), which subsequently activates PKC, and inositoltrisphosphate (IP_3). The activation of PKC is thought to enhance cell spreading and the formation of focal contacts, whilst IP_3 production promotes increased intracellular calcium (reviewed in Clark and Brugge, 1995).

Signalling pathways initiated by integrins are capable of cross-talk with growth factor or G-protein coupled receptor pathways. One of the key molecules providing co-ordination between these two pathways is the small molecular weight GTPase Rho (reviewed in Clark

and Brugge, 1995). Although not required for the initial phosphorylation of FAK and Src during focal contact formation, the subsequent phosphorylation of FAK and paxillin was found to be Rho dependent (Clark *et al.*, 1998). Additionally, the requirement of Rho activity during stress fibre formation and cell spreading has also been demonstrated.

1.3.6 Cell Biology of Integrins

As mentioned previously, binding of integrins to ECM leads to cytoskeletal changes and the initiation of signalling events. Integrin ligation may modulate many aspects of cellular function including proliferation, differentiation and survival (reviewed in Hynes, 1992; Clark and Brugge, 1995; Schwartz *et al.*, 1995; Schlaepfer and Hunter, 1998). For example, the anchorage of cultured endothelial or epithelial cells to immobilised sub-stratum is required for spreading and promotes cell-cycle progression (Ingber, 1990; Meredith *et al.*, 1993; Bottazzi and Assoian, 1997). Integrin ligation has also been shown to be inversely correlated with differentiation in haemopoietic (Utsumi *et al.*, 1991; Vuillet-Gaugler *et al.*, 1990) and epithelial cells (Adams and Watt, 1990).

Apart from their role in proliferation and differentiation, integrins also promote cell survival (reviewed in Meredith and Schwartz, 1997; Giancotti, 1997; Bates *et al.*, 1995). Endothelial (Meredith *et al.*, 1993), epithelial (Frisch and Francis, 1994) and muscle cells (Vachon *et al.*, 1996) have been demonstrated to undergo apoptosis when binding of integrins to ECM was perturbed. The term "anoikis", meaning homelessness, was established to describe the induction of cell death upon the loss of integrin ligation (Frisch and Francis, 1994). Anoikis ensures that cells remain viable within their correct micro-environment and provides a mechanism by which cells escaping tissues may be quickly eliminated. However the circumvention of anoikis, either by constitutive activation of integrin signalling pathways, FAK (Frisch *et al.*, 1996b), ras or src activation (Frisch and Francis, 1994), or through the loss

of apoptotic signalling pathways by Bcl-2 overexpression (Frisch and Francis, 1994) or inactiviation of caspases (Boudreau *et al.*, 1995; Frisch *et al.*, 1996a), may lead to metastasis, as transformed cells often display anchorage-independent growth (Stoker *et al.*, 1968). Therefore integrins play a key role in maintaining tissue architecture and ensuring survival within the context of a specific micro-environment.

1.3.7 Cell Migration: The role of Integrins

Cell migration plays a pivotal role in embryogenesis, inflammation, angiogenesis, wound healing and tumour metastasis. Most of the studies examining the process by which cells migrate have been performed using 2-dimensional *in vitro* assays. However, the molecules involved and the mechanism of cell migration in culture, are believed to be directly relevant to the 3-dimensional *in vivo* microenvironment (reviewed in Sheetz *et al.*, 1998; Lauffenberger and Horwitz, 1996; Tooney *et al.*, 1993).

One of the earliest responses observed when an immobile adherent cell initiates migratory behaviour is the establishment of polarity. Redistribution of the actin cytoskeleton to a particular region of the plasma membrane has been observed in neutrophils stimulated with chemoattractant (Coates *et al.*, 1992). Additionally several molecules, including integrins and their cytoskeletal complexes (Lawson and Maxfield, 1995; Schmidt *et al.*, 1993) redistribute to what will become the leading edge. After induction of polarity, actin polymerisation and membrane extension occurs (Condeelis, 1993; Stossel, 1993) in the direction the cell will ultimately take. Such extensions are rich in actin and other cytoskeletal components and may occur as either filamentous protrusions containing bundles of actin fibres, filapodia, or large sheet-like structures containing an actin meshwork, lamellipodia. As the membrane extends out, firm attachment to the sub-stratum occurs along the leading edge. This is the site of focal adhesion formation and accumulation of β1 integrins (Izzard and

Lochner, 1980; Regen and Horwitz, 1992). These aggregates of adhesion complexes remain in place and increase in size as the cell body moves over them. As membrane extension continues forward, these complexes are effectively removed from the leading edge allowing the subsequent formation of new ones. Therefore there must be some mechanism by which adhesion complexes are replenished and targeted to the leading edge of the migrating cell. Integrins, cytoskeletal components and other surface molecules appear to be actively recruited to the cell periphery, especially the leading edge, during migration. Furthermore, recycling and vesicular trafficking of integrins (and other molecules) through the endocytic pathway may also provide replenishment (Bretscher, 1992; Lawson and Maxfield, 1995). Internalisation and recycling has been reported for several integrins including, $\alpha 5\beta 1$, $\alpha 6\beta 4$, $\alpha v\beta 3$ (Bretscher, 1992; Sczekan and Juliano, 1990; Panetti and McKeown-Longo, 1993; Bretscher, 1989; Raub and Kuentzel, 1989). Furthermore several integrin chains contain NPXY coated pit-mediated internalisation motifs in their cytoplasmic tails (Chen *et al.*, 1990). However, these domains do not appear to be directly involved in integrin internalisation, yet are still required for migration (Vignoud *et al.*, 1994; Filardo *et al.*, 1995). Therefore the mechanism by which integrins are internalised, sorted and recycled to the leading edge of migrating cells remains unclear.

As the body of the cell moves forward, detachment from the sub-stratum occurs at the rear of the cell, allowing continued movement forward. The rate at which detachment of the trailing edge from the substratum occurs appears to be the limiting factor of migration (Chen, 1981). Rapidly migrating cells may detach their trailing edge by "ripping" of integrin complexes (Regen and Horwitz, 1992). This process leaves most of the $\beta 1$ integrins attached to the substratum and an accumulation of integrin-associated cytoskeletal components within the rear of the cell. However, the process of "ripping" has only been observed in fibroblasts and has not been reported for other cell types. Alternatively, integrin complexes may detach

from the sub-stratum and remain in the membrane of the trailing edge. The mechanism by which integrin function at the trailing edge is modulated to induce detachment remains unknown, however the biomechanical force of myosin dependent contraction, and the downregulated anchorage of integrins to the cytoskeleton in this region appear important (Jay *et al.*, 1995; Wilson *et al.*, 1991; Crowley and Horwitz, 1995; Schmidt *et al.*, 1993; Palecek *et al.*, 1998). For integrin aggregates which remain on the cell membrane several fates may ensue. A proportion of aggregates appear to be transported along the cell periphery back to the leading edge to form new adhesions. The remainder may be dispersed and endocytosed into cytoplasmic vesicles (Palecek *et al.*, 1996; Regen and Horwitz, 1992). Some of the integrin containing vesicles have been proposed to recycle to the leading edge (Bretscher, 1992; Tooney *et al.*, 1993) thus replenishing the supply of integrin. This process of integrin recycling back to the leading edge has been demonstrated for $\alpha v\beta 3$ in migrating neutrophils (Lawson and Maxfield, 1995). The ability of other integrins to recycle back to the leading edge and whether this process occurs in other cell types has not been addressed. Interestingly, a subset of internalised integrin containing vesicles has been observed to accumulate in the perinuclear region of cells (Lauffenberger and Horwitz, 1996). Whether these complexes are destined for degradation in lysosomes or reside within perinuclear recycling vesicles for subsequent recycling back to the plasma membrane has not been investigated.

1.4 Endothelial Cells

The vascular endothelium provides the primary barrier between the blood constituents and extravascular tissue. EC interact directly with luminal contents, the basement membrane upon which they reside, as well as each other to provide a selectively permeable, non-thrombogenic barrier. Furthermore, the vascular endothelium is a dynamic structure, capable of extensive remodelling, and is responsible for regulating many biological functions

including angiogenesis, wound healing, recruitment of leukocytes to sites of inflammation and regulation of thrombosis (reviewed in Shattil and Ginsberg, 1997; Shattil, 1995; Luscinskas and Lawler, 1994; Smyth *et al.*, 1993).

Many aspects of EC function are mediated by cell surface receptors. Four major classes of adhesion receptors have been described on EC, namely cadherins, Ig superfamily members, selectins and integrins (reviewed in Humphries and Newham, 1998; Carlos and Harlan, 1994). Of the cadherin family, VE-cadherin (Vascular Endothelial-cadherin) is predominantly expressed and plays a role in EC-cell interactions (see Section 1.4.3). Similarly, platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) an Ig superfamily member plays a major role in cell-cell contact (see Section 1.4.3). Unlike the constitutive expression of VE-cadherin and PECAM-1, members of the selectin family, E-selectin and P-selectin, are expressed upon EC activation (reviewed in Carlos and Harlan, 1994). These molecules predominantly mediate the binding and extravasation of leukocytes. Upregulated E-selectin expression results from transcriptional activation, whilst P-selectin resides within Weibel-Palade bodies and is rapidly exposed upon degranulation. As mentioned above, these structures also contain the TM4SF member CD63.

Although the integrin heterodimers expressed by EC may vary depending on the vascular bed from which they arise and state of cellular activation, most integrins of the β1 subfamily, αvβ3, α6β4 and αvβ5 have been detected (reviewed in Luscinskas and Lawler, 1994; Smyth *et al.*, 1993; Shattil, 1995). These molecules are predominantly localised to the basal aspect, however recent studies have also detected expression on the apical/luminal surface (Gawaz *et al.*, 1998; Conforti *et al.*, 1992).

1.4.1 Endothelial Cell Function and the Role of Integrins

Many aspects of EC function are mediated by integrins (reviewed in Luscinskas and Lawler, 1994; Smyth *et al.*, 1993; Shattil and Ginsberg, 1997) and several of these have been discussed previously (Sections 1.3.6 and 1.3.7). The association of TM4SF members with this family of adhesion receptors, and the expression of CD9, CD63 and PETA-3 by EC, also indicates important roles for these molecules in EC biology.

The central role integrins play in vascular development, or vasculogenesis, has been highlighted by the phenotypes of several integrin or integrin ligand null mice (reviewed in Hynes and Bader, 1997). Deletion of the genes encoding α5 (α5β1) (Yang *et al.*, 1993) and αv (αvβ3 and αvβ5) (Hynes and Bader, 1997) results in perturbed vascular development. Loss of α5 results in lethality at embryonic days 10-11, while mice lacking αv mostly die *in utero* at embryonic days 10.5-12.5. Those that survive this period succumb to cerebral haemorrhage within a day of birth. Similarly, disruption of the gene encoding the integrin ligand fibronectin, results in vascular collapse and lethality at embryonic days 9-10 (George *et al.*, 1993).

In addition to gene knockout studies, several *in vivo* models of angiogenesis have been established to investigate the role of integrins in the remodelling of existing EC to form new blood vessels. The process of angiogenesis, or neovascularisation, involves the sprouting of new blood vessels from existing ones and requires EC invasion through the basement membrane, migration and proliferation (Bischoff, 1997). Using the chick chorioallantoic membrane assay, Cheresh and colleagues have demonstrated inhibition of angiogenesis by mAb against αvβ3 (Brooks *et al.*, 1994). Interestingly the inhibition by αvβ3 mAb was specific, as anti-β1 mAbs had no detectable effect. Subsequently, αvβ5 was also shown to be involved in the angiogenic process in a similar manner as αvβ3 (Friedlander *et al.*, 1995).

The role of integrins in angiogenesis has also been investigated using *in vitro* techniques. EC utilised for *in vitro* experiments have been derived from a variety of sources, including human umbilical vein EC (HUVEC), bovine aortic EC (BAEC) and human microvessel EC (HMEC) (reviewed in Luscinskas and Lawler, 1994). Although there exists some variation in the molecules expressed and cellular behaviour of these EC from various sources, they have nonetheless provided a useful model for dissecting the mechanism of EC function under controlled conditions.

Plating of EC onto collagen type I, fibrin (Montesano *et al.*, 1985; Gamble *et al.*, 1993) or the complex basement membrane Matrigel (Kubota *et al.*, 1988), results in adhesion, alignment and subsequent formation of a network consisting of capillary-like tubes (Meyer *et al.*, 1997). The formation of tubes on collagen type I and fibrin gels was enhanced by addition of PMA, whilst the process appeared to be constitutive on Matrigel. MAbs which block ligand binding to $\alpha 2\beta 1$ or $\alpha v\beta 3$ have been observed to modulate the ability of EC to form capillaries on collagen type I and fibrin gels respectively (Gamble *et al.*, 1993). The modulation of tube formation observed in these studies was associated with increased tube numbers and an increase in the diameter of tubes formed. Increased tube diameter was found to correlate with multiple cells lining the lumen of such vessels, whilst tubes formed by untreated EC were within single cells. Thus in this model, anti integrin mAbs appear to promote tube formation, perhaps by shifting the equilibrium away from cell-matrix binding and towards cell-cell interactions. In contrast, tube formation on Matrigel, which required RNA and protein synthesis, was abrogated by mAbs which block ligand binding of the $\alpha 6$ or $\beta 1$ integrin chains (Davis and Camarillo, 1995). It is important to note that the concentration of blocking mAb used in this study was insufficient to perturb cellular migration and/or adhesion. Therefore ligation of $\alpha 6\beta 1$ by mAb appears to stimulate an anti-angiogenic signal.

In addition to vasculogenesis and angiogenesis, the role of integrins in leukocyte recruitment to activated endothelium, the extravasation of leukocytes across the EC barrier and the activation of leukocytes has been investigated extensively (reviewed in Gahmberg *et al.*, 1998; Dejana *et al.*, 1995, Luscinskas and Lawler, 1994; Carlos and Harlan, 1994). Upon activation by pro-inflammatory stimuli, including TNF-α, IL-1 or LPS, EC upregulate surface expression of a variety of adhesion molecules. Under flow conditions, the current model of leukocyte binding and diapedesis proposes that leukocytes make initial contact with activated EC, roll, become arrested as firm adhesion occurs, then undergo diapedesis. The initial interaction of leukocytes with EC appears to be mediated by the selectins, E and P-selectin, which are expressed by EC and bind the fucosylated tetrasaccharide sialyl Lewis X on leukocytes and activated lymphocytes (reviewed in Tedder *et al.*, 1995). Additionally L-selectin expressed by leukocytes, which is also a ligand for sialyl Lewis X, may be involved in extravasation across lymphoid associated EC. For the promotion of stronger adhesion, leukocytes require stimulation by a variety of activating agents including IL-8 or platelet activating factor. Activation of leukocytes leads to increased expression and affinity modulation of β2 integrins (predominantly αLβ2) which, through the binding of ICAMs and E-selectin, mediates stronger cell-cell contact (reviewed in Carlos and Harlan 1990). Additionally α4β1 binding to EC expressed VCAM-1 (vascular cell adhesion molecule-1) plays a primary role by promoting leukocyte arrest (Elices *et al.*, 1990). Once arrested, αLβ2 binding to ICAMs mediates cell spreading and migration to EC intercellular margins. Homotypic adhesion of PECAM-1, expressed by leukocytes and EC, and binding of β2 integrins to ICAMs are involved in mediating diapedesis (reviewed in Gahmberg *et al.*, 1998; Carlos and Harlan, 1994).

1.4.2 Integrin Associated Protein

TM4SF molecules associate with integrins within the plane of the plasma membrane and are capable of influencing cellular function. Similarly, another transmembrane protein, integrin associated protein (IAP, CD47, OA-3), has also been demonstrated to associate with integrins and to modulate integrin function. IAP is a 50kD glycoprotein which was found to co-purify with αvβ3 from placenta and to co-immunoprecipitate with β3 from platelet lysates (Brown *et al.*, 1990; Lindberg *et al.*, 1993). Unlike the promiscuous association of TM4SF members with many integrin subfamilies, the association of IAP with integrins is restricted to αvβ3 and the β3-like adhesion molecule in leukocytes. Cloning of the cDNA encoding this molecule showed it to be a member of the Ig superfamily (Lindberg *et al.*, 1993). Interestingly, the amino acid sequence of IAP showed 100% homology to the previously cloned ovarian carcinoma antigen OA3 (Campbell *et al.*, 1992). However the significance of IAP expression in ovarian carcinoma remains unclear.

Hydrophobicity analysis suggests IAP is a transmembrane protein with five membrane spanning domains, a short intracellular carboxyl tail (16 amino acids) and a large extracellular IgV domain toward the amino terminus. Anti-IAP mAbs have been shown to inhibit neutrophil transendothelial migration (Cooper *et al.*, 1995), the activation of neutrophils, monocytes and EC by ECM components, and chemotaxis of EC and neutrophils (Brown *et al.*, 1990; Schwartz *et al.*, 1993). Anti-IAP mAbs also block the binding of vitronectin coated beads to αvβ3 and binding of neutrophils to ECM components (Lindberg *et al.*, 1993). Therefore IAP appears to directly regulate both ligand binding and functionality of αvβ3, although the exact mechanism has not been defined. This mechanism appears to differ from that of the TM4SF which appear to modulate cellular behaviour, as opposed to integrin affinity *per se* (Section 1.2.5). More recently, IAP was demonstrated to bind the carboxyl terminal RFYVVM motif of thrombospondin, thus acting as a receptor for this protein (Gao *et*

al., 1996) and an anti-IAP mAb, which blocked ligand binding, was demonstrated to inhibit chemotaxis of EC to thrombospondin. Additionally a peptide corresponding to the carboxyl terminus of thrombospondin was able to induce platelet aggregation, suggesting that IAP acts as a thrombospondin receptor on platelets (Dorahy *et al.*, 1997).

1.4.3 Endothelial Cell Junctions

The regulation of EC proliferation, motility, permeability of the monolayer and leukocyte extravasation are mediated through cell-cell interactions. Since many of these functions are also mediated by integrins, the components of these structures and the molecules involved are discussed below. To date, three major junctions have been described in EC, adherence junctions (AJ), tight junctions (TJ) and gap junctions (reviewed in Dejana *et al.*, 1995).

AJ, or *zona adherentes* (adherent junctions), are mediated by the cation-dependent adhesion of cadherins between apposing plasma membranes. Cadherins are single membrane spanning proteins that homotypically bind other cadherins, via their N-terminal extracellular portion. Their intracellular cytoplasmic tail interacts with the actin cytoskeleton, via the binding of catenins. Although VE-cadherin is restricted to EC, cadherins found in other cell types are also expressed by EC (Lampugnani *et al.*, 1992).

TJ, or *zona occludins* (occluding junctions), were originally described as a region of fusion of plasma membrane outer leaflet. These structures mediate the establishment of cellular polarity by "partitioning" of the plasma membrane (reviewed in Gumbiner, 1993). A major component of these structures is occludin, a 65kD protein with four predicted transmembrane domains, which associates with a variety of cytoplasmic molecules (Furuse *et al.*, 1993). Interestingly, occludin null mice display morphologically and functionally normal TJ, suggesting the presence of alternative molecules (Saitou *et al.*, 1998). Indeed two other

components of TJ were recently identified, claudin-1 and claudin-2, which although smaller than occludin, also have four predicted transmembrane domains (Furuse *et al.*, 1998).

Gap junctions consist of channels formed by a hexamer of proteins, termed connexons which, when bound to a like channel on the opposing membrane, link the cytoplasm of neighbouring cells (reviewed in Goodenough *et al.*, 1996). These channels, some of which are regulated, allow the passage of solutes and small metabolites and may assist in coordinating signals between cells. Connexons belong to the connexin superfamily which, like the major components of TJ, also have four predicted transmembrane domains. EC have been shown to express several connexons, Co43, Co40, and Co37, which form inter-cellular channels both between EC and between EC and underlying smooth muscle cells.

Apart from the organised junctional structures mentioned above, several other molecules are localised to sites of EC cell-cell contact. Perhaps the best studied of these is PECAM-1 (Newman *et al.*, 1990; DeLisser *et al.*, 1994; reviewed in Newman, 1998). A member of the Ig superfamily, PECAM-1 is a 130kD glycoprotein expressed by platelets, monocytes, neutrophils, a subset of T cells, and is strongly localised to EC margins. The extracellular domain contains six Ig loops, each encoded by a separate exon, and alternate splicing leads to several PECAM-1 isoforms which contain or have lost palmitylation, phosphorylation or cytoplasmic protein interaction sites (Kirschbaum *et al.*, 1994). The major ligand of PECAM-1 is itself (Albelda *et al.*, 1991; Sun *et al.*, 1996), and homophilic binding mediates EC cell-cell and leucocyte-EC interactions. In addition, other ligands for PECAM-1 have been described and include $\alpha v\beta 3$ integrin (Piali *et al.*, 1995; Buckley *et al.*, 1996) and glycosaminoglycans (DeLisser *et al.*, 1993), however there remains some doubt as to the physiological relevance of these proposed ligands. PECAM-1 has been demonstrated to play a major role in leukocyte extravasation as treatment of leukocytes or EC with blocking mAb abrogated this process both *in vitro* (Muller *et al.*, 1993) and *in vivo* (Vaporciyan *et al.*, 1993;

Bogen *et al.*, 1994; Murohara *et al.*, 1996). Although the molecule lacks a catalytic kinase domain in its 118 amino acid cytoplasmic tail, phosphorylation of PECAM-1 has been reported in EC (Lu *et al.*, 1996) and platelets (Modderman *et al.*, 1994). Furthermore, phosphorylation of Tyr_{663} creates a docking site for the protein tyrosine phosphatase SHP-2 (Jackson *et al.*, 1996). Although the downstream signalling events initiated by SHP-2 binding to phosphorylated PECAM-1 are unclear, SHP-2 has been implicated in the activation of Src family kinases and the small GTP binding protein ras, via its association with the Grb2/Sos complex (reviewed in Streuli, 1996). Indeed the potential of signalling initiated by PECAM-1 engagement has been recently investigated using mAb. These studies have demonstrated that crosslinking of PECAM-1 leads to activation of β1, β2 and β3 integrins, in a variety of haemopoietic cells and platelets. Conversely, integrin ligation has been shown to modulate the level of PECAM-1 phosphorylation suggesting cross-talk between these two receptors (reviewed in Newman, 1998).

In addition to PECAM-1, two integrins have been described in EC cell-cell contacts. Although integrins expressed by EC are predominantly localised to the basal surface, α2β1 and α5β1 are localised to cellular margins (Lampugnani *et al.*, 1991). Furthermore the localisation of these two integrins correlates with that of their ligands, laminin, collagen type IV and fibronectin, which are arranged in fibres along EC margins. Although the function of these molecules in regions of EC-EC contact is unclear, perturbation with blocking mAb or RGD peptide leads to disruption of the EC monolayer and in the case of α5β1 mAb, increased permeability (Lampugnani *et al.*, 1991). This response is in contrast to that observed with PECAM-1 blocking mAbs, which have no effect on established EC monolayers, but do abrogate the formation of the monolayer if added prior to the establishment of cell-cell contact (Muller *et al.*, 1993).

1.5 Platelets

Platelets, or thrombocytes, are small disc shaped particles derived from megakaryocytes in the bone marrow and are the smallest formed element in whole blood. The fusion of microvesicles with the plasma membrane and subsequent budding from megakaryocytes releases platelets into the circulation (Tavassoli, 1980). Once in the circulation, platelets play a central role in maintaining the circulatory system by responding to vascular injury through the formation of a haemostatic plug. In humans, platelets are present at 150,000-400,000 per microliter of blood and have a life span of 8-12 days, after which they are cleared from the circulation by the reticuloendothelial system within the spleen, bone marrow and liver (Kelton, 1987). Although platelets are cytoplasmic fragments and contain no nuclei they are metabolically active and capable of complex biological processes. Such processes include the recognition of normal vasculature, interaction with subendothelial matrix exposed by damaged endothelium and subsequent adhesion and aggregation events involved in the initiation and maintenance of thrombus formation (reviewed in Body, 1996; Blockmans *et al.*, 1995).

1.5.1 Platelet Ultrastructure

Platelets are discoid anuclear cells about one tenth the size of a red blood cell, approximately 3μm in diameter by 1μm thick (reviewed in White, 1979). The plasma membrane is a typical bilamellar phospholipid membrane with deep invaginations and channels which make up the open canalicular system which greatly increases the platelet surface area and assists with secretory mechanisms. Immediately below the plasma membrane lies the cytoskeleton, composed primarily of actin filaments. The cytoskeleton maintains platelet structure and is involved in the anchoring of several membrane

glycoproteins thus assisting in stabilising the plasma membrane. Upon activation, cytoskeletal rearrangements lead to the platelet shape change, contraction and pseudopod formation (reviewed in Fox, 1993).

Intracellular organelles include α and dense granules (Holmsen and Weiss, 1979), mitochondria, glycogen particles, lysosomes and peroxisomes (reviewed in Body, 1996; Blockmans *et al.*, 1995). Platelets also possess a dense tubule system which acts as the main store of intracellular calcium. Within the α and dense granules are a variety of platelet agonists and other mediators which are released upon degranulation (Gerrard *et al.*, 1977). The α granules which vary in size from about 300 to 500nm contain fibronectin, fibrinogen and several unique platelet proteins. These include platelet derived growth factor, platelet factor-4 (antiheparin), low activity antiheparin, vWF and β-thromboglobulin. Dense granules are composed of a crystailine matrix containing ADP, adenosine triphosphate (ATP), serotonin and calcium.

1.5.2 Characterisation of TM4SF Proteins in Platelets

Apart from PETA-3, platelets express at least two other members of the TM4SF, CD9 and CD63. High expression of CD63 has been detected in platelet α-granules and CD63 is rapidly translocated to the plasma membrane upon platelet activation (Nieuwenhuis *et al.*, 1987). Thus CD63 acts as a marker of platelet activation. Similarly CD9 has also been found in α-granules (Cramer *et al.*, 1996), however unlike CD63, CD9 is highly expressed (approximately 40,000 copies) on the platelet plasma membrane, has been demonstrated to associate with the αIIbβ3 integrin (Slupsky *et al.*, 1989; Indig *et al.*, 1997). Additionally, anti-CD9 mAbs act as platelet agonists (see Section 1.5.5). Investigation of PETA-3 expression by platelets has also demonstrated agonist activity of anti-PETA-3 mAbs (see

Section 1.5.5), however unlike CD9, PETA-3 appears to be present at a much lower copy number, approximately 10% the level of CD9 surface expression (Ashman *et al.*, 1991).

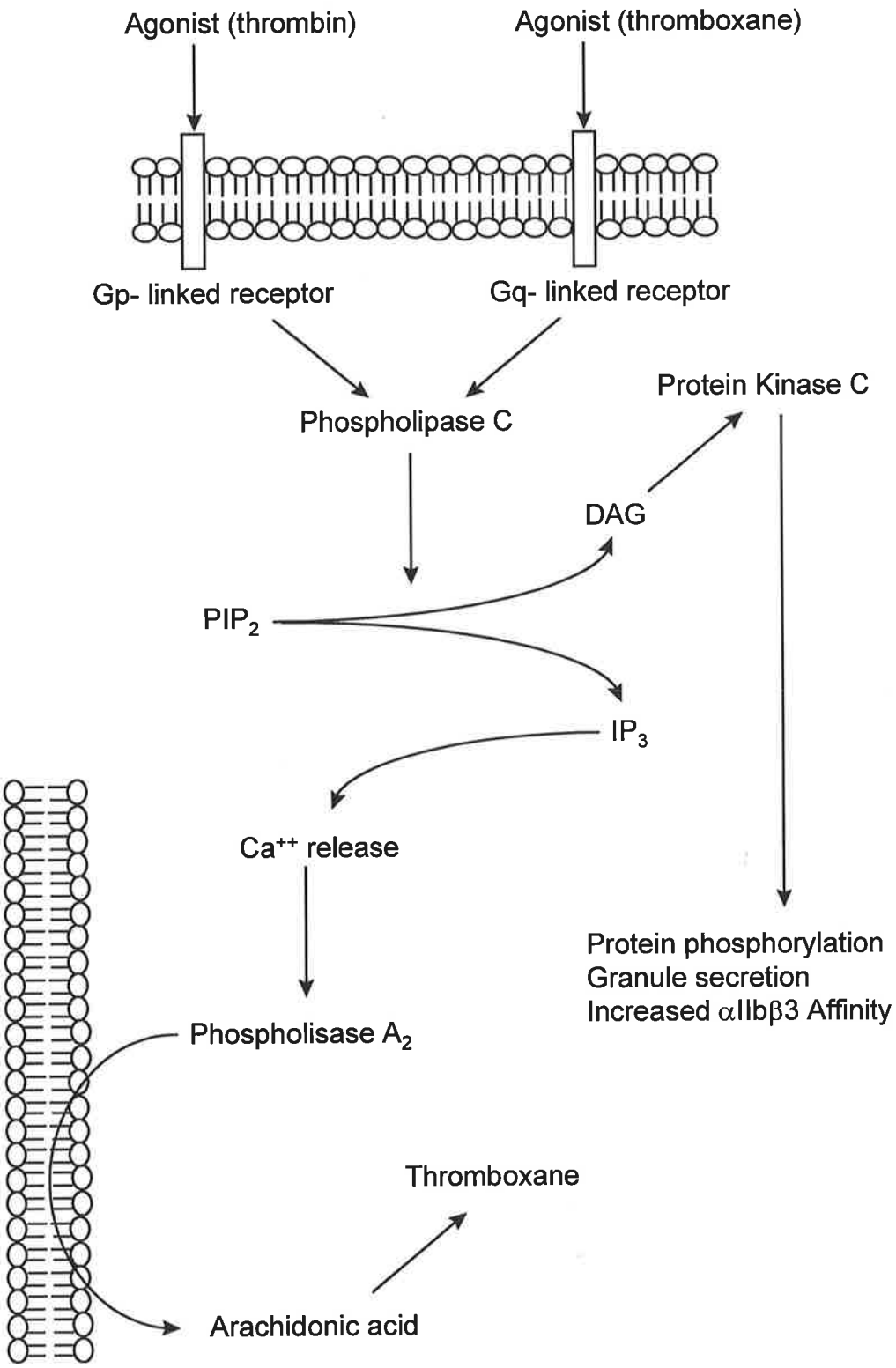
1.5.3 Mechanisms of Platelet Activation

Platelet activation occurs through signalling events initiated by membrane receptors for various platelet agonists (Figure 1.5) (reviewed in Blockmans *et al.*, 1995; Body, 1996, Fox, 1996). Such agonists include both soluble mediators, ADP, thrombin, fibrinogen, adrenaline, serotonin, platelet activating factor, thromboxane (TxA_2), prostaglandins and vasopressin, as well as immobilised substrates such as vWF and the subendothelial matrix proteins collagen, fibronectin, vitronectin and laminin. Platelets also respond to a variety of pharmacological agents such as calcium ionophores, poly-l-lysine and cyclic endoperoxide analogues. Platelet activation can be segregated into two components, an initial reversible activation response and irreversible activation leading to full platelet aggregation. Reversible activation, induced by weak agonists, including ADP, adrenaline or serotonin, is characterised by platelet shape change and formation of pseudopodia. If no further stimulation occurs, the platelets will revert back to a discoid shape and re-enter a resting state (Holmsen, 1987). Irreversible activation occurs when strong agonists, including collagen and thrombin, promote sufficient mediator release, through the secretion of granules, to provide further amplification. Continued stimulation leads to the release of dense granules and subsequent platelet aggregation (Holmsen, 1987).

Several platelet receptors, those for thrombin, serotonin, adrenaline, TxA_2, platelet activating factor and vasopressin, act through "classical" G-protein-linked mechanisms (Figure 1.5) (reviewed in reviewed in Blockmans *et al.*, 1995; Body, 1996, Fox, 1996). The phosphorylation of G-proteins activates PLC which subsequently hydrolyses PIP_2 to generate DAG and IP_3. DAG activates PKC while IP_3 promotes the release of calcium into the

Figure 1.5 Mechanisms of Platelet Activation

Several receptors for platelet agonists phosphorylate G-proteins upon ligand binding. Phosphorylation of G-proteins leads to activation of PLC which releases DAG and IP_3 from PIP_2. DAG activates PKC leading to further phosphorylation events and the release of other agonists, via the secretion of platelet granules. PKC activation also leads to the affinity modulation of αIIbβ3 (Section 1.3.3), promoting irreversible platelet activation. The production of IP_3 increases cytoplasmic calcium levels by mobilising intraplatelet stores from the dense tubular system. Increased calcium levels activate PLA_2 which releases arachidonic acid from membrane phospholipids. Arachidonic acid is utilised for the production of TxA_2, which upon release, promotes further platelet activation (taken from Body, 1996).


Agonist (thrombin)
Agonist (thromboxane)
Gp- linked receptor
Gq- linked receptor
Phospholipase C
Protein Kinase C
DAG
PIP2
IP3
Ca++ release
Protein phosphorylation
Granule secretion
Increased αIIbβ3 Affinity
Phospholisase A2
Thromboxane
Arachidonic acid

cytoplasm by binding its receptor on the dense tubular system (Majerus *et al.*, 1986). The increased intracellular calcium concentration leads to the activation of PLA_2 which in turn releases arachidonic acid from phospholipids (Billah *et al.*, 1980). Arachidonic acid is then utilised for the biosynthesis of eicosanoids, the most important of which are TxA_2 (Hamberg *et al.*, 1975) and prostaglandins G_2 and H_2 (Smith *et al.*, 1974), products of the aspirin inhibitable cyclo-oxygenase pathway (Roth *et al.*, 1975). TxA_2 released during platelet activation promotes further activation and secretion (Gryglewski, 1980). Release of ADP is also important for the promotion of irreversible activation, degranulation and aggregation (Holmsen, 1987). Although less well understood than the G-protein-linked platelet receptors, the ADP receptor on platelets is a purine type-2 receptor and ADP binding has been shown to inhibit adenylate cyclase activity, resulting in decreased c-AMP levels (Gordon, 1986; Cusack and Hourani, 1982).

Platelets also possess several surface glycoproteins which are members of the integrin superfamily. Most abundant of these is αIIbβ3 (gpIIb/IIIa), the fibrinogen receptor, which is present at 40,000-80,000 copies per platelet and is essential for the irreversible activation and aggregation of platelets (Marguerie *et al.*, 1979; reviewed in Ginsberg *et al.*, 1995). Other integrins expressed on the platelet surface are α2β1, α5β1, α6β1 (Hemler *et al.*, 1988) and αvβ3 (reviewed in Ginsberg *et al.*, 1995). Although not a member of the integrin superfamily, the receptor for vWF (gpIb/IX/V complex) is involved in platelet activation and aggregation by upregulating the affinity of the αIIbβ3 integrin upon binding of its ligand, immobilised vWF (Ikeda *et al.*, 1991). Another platelet receptor is gpVI which acts as a receptor for the potent platelet agonist collagen (Tsuji *et al.*, 1997).

Protein tyrosine kinases (PTK) and phosphatases have been demonstrated to play a central role in platelet signalling, as inhibitors of PTK abrogate platelet aggregation (reviewed in Clark *et al.*, 1994a). Upon stimulation by strong platelet agonists phosphorylation of

several cellular proteins occurs. The pattern of proteins phosphorylated takes place in "waves" corresponding with reversible and irreversible platelet activation. Reversible events are those detectable within a few seconds after stimulation by agonist and involve the rounding of the platelet into a spherical shape and the formation of pseudopodia. Platelets from patients with the bleeding disorder Glanzman's thrombasthenia, which lack functional αIIbβ3, or platelets treated with inhibitors of αIIbβ3 undergo reversible activation only (reviewed in Clark *et al.*, 1994a). During these early activation events rapid phosphorylation of the PTK Src (Section 1.3.4) and Syk (Clark *et al.*, 1994b; Poole *et al.*, 1997) occurs. The phosphorylation of Src is transient and is accompanied by its redistribution to the cytoskeleton. Syk phosphorylation is also transient with phosphotyrosine levels returning to baseline levels in approximately 1 minute (reviewed in Clark *et al.*, 1994a). Several other proteins which also become tyrosine phosphorylated during early activation include MAPK, Ras GTPase-activating protein (Section 1.3.5).

As platelet activation continues, affinity of αIIbβ3 is upregulated leading to the binding and subsequent inter-platelet crosslinking by fibrinogen (Marguerie *et al.*, 1979). At this point irreversible platelet aggregation occurs and two key events take place, the tyrosine phosphorylation of focal adhesion kinase FAK and the redistribution of Src, Syk and FAK to the cytoskeleton where they associate with integrins to form cytoskeletal complexes (Clark *et al.*, 1994b; reviewed in Clark *et al.*, 1994a; see Section 1.3.4). The activation of FAK and subsequent signalling pathways induced by ligation of integrins in nucleated cells has been previously discussed (Sections 1.3.4 and 1.3.5).

1.5.4 Platelet Activation by Monoclonal Antibodies

Apart from signalling through agonist receptors, platelets may also be activated by aggregated Ig (Humphrey and Jacques, 1955) or mAbs against platelet surface proteins (see

below). Several anti-platelet mAbs which cause aggregation have been characterised and bind surface molecules such as αIIbβ3 and gpIV (Horsewood *et al.*, 1991; Rubinstein *et al.*, 1991b), β_2-glycoprotein I (Arvieux *et al.*, 1993), β2 microglobulin (Rubinstein *et al.*, 1991a) and the TM4SF members CD9 (Worthington *et al.*, 1990) and PETA-3 (Ashman *et al.*, 1991; Roberts *et al.*, 1995). Platelet activation appears to occur through one of two possible mechanisms depending on the subtype of the mAb (De Reys *et al.*, 1993). The majority of anti-platelet mAbs are of the IgG1 subtype, and signalling is believed to be primarily due to Ig crosslinking of the specific antigen with the low affinity IgG receptor, FcγRII (CDw32) (Horsewood *et al.*, 1991; Worthington *et al.*, 1990; Mazurov *et al.*, 1992; Tomiyama *et al.*, 1992; Slupsky *et al.*, 1992). Furthermore, $F(ab')_2$ fragments of anti-platelet mAbs fail to bring about aggregation, demonstrating the dependence for crosslinking of the FcγRII. The FcγRII is a sialoglycoprotein with a molecular weight of 40kD and is present at about 1000 copies per platelet (for review of Fc receptors see Daeron, 1997). Monomeric IgG has a very low affinity for this receptor, however aggregated or crosslinked IgG bind with stronger affinity (Karas *et al.*, 1982). Pre-incubation with $F(ab')_2$ fragments of the anti-FcγRII mAb IV.3, which blocks Ig binding to the FcγRII, abrogates activation induced by anti-platelet mAbs and aggregated Ig, suggesting that FcγRII is the only functional IgG receptor on platelets (Looney *et al.*, 1986). A functional polymorphism has also been described for human FcγRII (Looney *et al.*, 1988). The polymorphism resides at position 131, encoding either a His or Arg residue in the second extracellular Ig domain (Clark *et al.*, 1989; Tate *et al.*, 1992). Arg131 has been demonstrated to be essential for high affinity binding of murine IgG1, whilst His131 greatly impairs murine IgG1 binding (Clark *et al.*, 1991).

Anti-platelet mAbs of the IgG2 subtype are thought to induce platelet aggregation through the activation of complement, since IgG of this subtype interacts poorly with the FcγRII (Jones *et al.*, 1985) and pre-incubation with IV.3 $F(ab')_2$ fragments does not inhibit

IgG2a responses (De Reys *et al.*, 1993). Binding of IgG2a mAb to the platelet surface may activate complement and as platelets express several complement receptors, the binding of activated complement components may lead to platelet activation. Lysis or activation of platelets may also occur through the generation of membrane attack complexes. Pretreatment of platelet rich plasma (PRP) with the protease inhibitor leupeptin abrogates platelet aggregation by mAbs of the IgG2a subtype, but not IgG1, further demonstrating the involvement of complement in IgG2a induced platelet activation (De Reys *et al.*, 1993).

1.5.5 Monoclonal Antibodies Against CD9 and PETA-3 Act as Platelet Agonists

Like most mAbs of the IgG1 subtype, anti-CD9 and anti-PETA-3 mAb are able to induce platelet activation and mediator release by crosslinking of FcγRII, the low affinity IgG receptor (Worthington *et al.*, 1990; Ashman *et al.*, 1991; Roberts *et al.*, 1995). However, apart from signalling via the FcγRII receptor, there have been several reports of intrinsic signalling by CD9 ligation. Studies have shown that although unable to induce full aggregation, ligation of CD9 induces signals similar to those of αIIbβ3 and CD42 ligation (Slupsky *et al.*, 1997) and can promote phosphorylation the PTK Syk (Ozaki *et al.*, 1995). Furthermore, full aggregation of platelets could be induced by immobilised CD9 $F(ab')_2$ fragments (Griffith *et al.*, 1991). Since CD9 has been shown to associate with β1 integrins and the platelet αIIbβ3 complex (Section 1.2.5), crosslinking of CD9/integrin complexes by mAb might mediate signalling events. However the strength of the signal generated may be dependent on the context of the stimulus. Clustering of integrin complexes by immobilised mAb may provide the necessary secondary signals leading to full platelet activation, whilst soluble $F(ab')_2$ fragments appear to mediate weaker sub-threshold signalling events.

The activation of platelets by the anti-PETA-3 mAb 14A2.H1 has also been demonstrated to be FcγRII dependent as pre-incubation with IV.3 or 14A2.H1 $F(ab')_2$

fragments abrogated aggregation (Ashman *et al.*, 1991; Roberts *et al.*, 1995). Activation by 14A2.H1 was also found to be partially inhibitable by aspirin and totally inhibitable by PGE_1 (Roberts *et al.*, 1995). The inhibition of platelet activation by PGE_1, which activates adenylate cyclase, demonstrated that aggregation was not simply due to passive inter-platelet crosslinking, or agglutination. Suboptimal concentrations of 14A2.H1 were also shown to synergise with other platelet agonists, ADP, collagen, adrenaline and serotonin suggesting that signalling occurred independently of these pathways. The synergy of 14A2.H1 with other platelet agonists was also blocked by pre-incubation with IV.3 or 14A2.H1 $F(ab')_2$ fragments. Interestingly platelet aggregation by 14A2.H1 showed varied responses between donors which was proposed to be due to the previously discussed polymorphism within the FcγRII (Roberts *et al.*, 1995).

1.6 Aims

TM4SF proteins have been demonstrated to be components of multi-protein complexes involved in a broad range of physiological and cellular processes. Preliminary studies using the anti-PETA-3 mAb 14A2.H1 had demonstrated expression on platelets and endothelium (Ashman *et al.*, 1991). Further studies of PETA-3 mRNA distribution by northern blotting analysis showed mRNA expression in all tissue homogenates examined with the exception of brain (Fitter *et al.*, 1995). Since PETA-3 was expressed by endothelium it was unclear as to whether the vascular component of the tissues examined accounted for their positivity by Northern analysis. Therefore the first aim of this project was to carry out a comprehensive analysis of PETA-3 expression in human tissues. Early experiments in this study revealed substantial intracellular pools of PETA-3 in EC and megakaryoblastic cell lines. Hence the second aim of this project was to characterise this intracellular pool by confocal microscopy and immunoelectron microscopy. Preliminary biochemical studies,

carried out by S. Fitter in this laboratory, had demonstrated the association of PETA-3 with CD9, CD63 and β1 integrins in haemopoietic cell lines (Fitter *et al.*, submitted). Therefore the third aim of this project was to investigate the association of PETA-3 with integrins and other TM4SF proteins in EC. Finally the role of PETA-3 in EC and platelet function *in vitro* was investigated by the use of mAbs 14A2.H1 and 11B1.G4.

CHAPTER 2: MATERIALS AND METHODS

2.1 Tissue Culture

2.1.1 Tissue Culture Medium

Dulbecco's Modified Eagle's Medium (DMEM) was prepared by mixing one sachet of DMEM powder (GIBCO BRL, Life Technologies, Inc., Grand Island, NY, US), Cat. No. 12800-017) and 3.7g of $NaHCO_3$ (BDH, Merck,Victoria, Aust. Cat. No. 10247) in 900ml of Milli-Q purified water (MQ-water) (tap-water deionised using a Milli-Q RO60 system and purified through two beds of ion exchange resins, a carbon filter and an organic filter of a Milli-Q system - Millipore, USA). To the dissolved solution, N-2-Hydroxyethylpiperazine N`-2-ethanesulfonic acid (HEPES) pH 7.2 (Boehringer-Mannheim, Australia, Cat. No. 737151) was added from sterile stock solutions to give a final concentration of 15mM. Likewise, penicillin (Sigma, St Louis, MO, USA, Cat. No. P3032) and streptomycin sulfate (Sigma, Cat. No. S9137) were added to final concentrations of 100IU/ml and 100µg/ml respectively. The pH was adjusted to 7.0 by adding 4ml of 1M HCl and the volume adjusted to 1L using MQ-water. The medium was filter sterilised using an Acrocap 0.22µm filter unit (Gelman Sciences, Michigan, USA, Cat. No. 4480) and a Millipore pump with a filling bell (Millipore, USA, Cat. No. SVGB1010) and stored at 4°C. RPMI 1640 medium was prepared by dissolving one sachet of RPMI 1640 powder (GIBCO BRL, Cat.No. 31800-02) and 2g of $NaHCO_3$ in 900ml of MQ-water. Sterile stock solutions of HEPES, penicillin and streptomycin sulfate were added and the medium was filter sterilised as indicated above. The pH was adjusted to 7.4 with 2.5ml of HCl and the volume to 1L. F-12 medium was prepared by dissolving one sachet of F-12 powder (GIBCO BRL, Cat.No. 21700-026) and 1.18g of $NaHCO_3$ (BDH, Cat. No. 10247) in 900ml of MQ-water. Sterile stock solutions of penicillin and streptomycin sulfate were added, the volume was then made up to 1L and the medium

was filter sterilised as indicated above. For the culture of cells, DMEM, RPMI 1640 and F-12 media were supplemented with 10% v/v foetal bovine serum (FBS) (GIBCO BRL, Cat. No. 200-6140PJ, Batch No. 660; heat inactivated at 56°C for 30 minutes) as well as glutamine (BDH, Cat. No. 37107) to a final concentration of 2mM and warmed to 37°C. Fresh glutamine (2mM final) was added to medium stored at 4°C for more than 7 days.

M199 medium (Cytosystems, Sydney, Australia) was supplemented with 20% FBS, 20mM HEPES, 2mM glutamine, 1mM sodium pyruvate (Multicel, Trace Biosciences Pty. Ltd, Australia, Cat. No. 21-154-0100v), 1% non-essential amino acids (Multicel, Cat. No. 21-145-0100v), 3% $NaHCO_3$ (BDH), 100IU/ml penicillin and gentamycin (Sigma), 25μg/ml EC growth factors (Collaborative Research, Bedford, MA, US) and 20μg/ml heparin (GIBCO BRL).

Analytical grade reagents were used in the preparation of all solutions. Solutions used to wash cells were Hank's balanced salt solution (HBSS) and Tissue Culture Phosphate Buffered Saline (TC-PBS). HBBS contained 0.14M NaCl, 5mM KCl, 0.3mM $Na_2HPO_4.12H_2O$, 0.4mM KH_2PO_4, 4.2mM $NaHCO_3$, 5.5mM glucose and 1% phenol red (M & B, England, Cat. No. P152/18/61) dissolved in 0.1M NaOH in MQ-water with a final pH of 7.4. TC-PBS contained 0.14M NaCl, 3mM KCl, 8mM $Na_2HPO_4.12H_2O$ and 1mM KH_2PO_4 in MQ-water with a final pH of 7.4. The solutions were sterilised by autoclaving at 130°C for 20 minutes and stored at 4°C until use.

2.1.2 Cell Lines

All cell lines were maintained in tissue culture flasks (Corning Costar Corporation, Cambridge, MA, USA, Cat.No. 25110-25) containing the indicated tissue culture medium and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were either subcultured or the tissue culture medium was replaced every 3 to 5 days depending on the growth rate. All

suspension cell cultures were maintained for a maximum of 3 months and adherent cultures for a maximum of 6 weeks, after which cultures were re-established from cryopreserved stocks. Cell densities were calculated using a haemocytometer and exclusion of trypan blue (0.8% w/v in saline) diluted 1/2 was used to determine cell viabilities. All cell lines used in this study were screened for the presence of mycoplasma, all of which were negative.

The human megakaryoblastic cell line M-07e (Avanzi *et al.*, 1988) was obtained from Dr P. Crozier (University of Auckland, Auckland, New Zealand) and maintained at log phase in DMEM/10% FBS supplemented with conditioned medium (5%) produced from Chinese hamster ovary cells transfected with either mouse GM-CSF or IL-3 cDNA (provided by Dr A. Lopez Hanson Centre for Cancer Research, Adelaide, Australia).

The murine factor dependent cell line, FDC-P1 (Dexter *et al.*, 1980), was obtained from Dr T. Gonda (Hanson Centre for Cancer Research, Adelaide, Australia). Transfected FDC-P1 cells were prepared by retroviral transfection of cDNA encoding human PETA-3, CD63 or the control vector puromycin (S. Fitter, unpublished), using methodology essentially as described in (Zannettino *et al.*, 1996). FDC-P1 transfectants were cultured in DMEM medium supplemented with 10% FBS and murine GM-CSF or murine IL-3 (Section 2.1.5).

The murine L-cell cell line transfected with genomic DNA encoding human CD9 (T8.9) has been described previously (Cole *et al.*, 1989). T8.9 cells were cultured in F-12 medium supplemented with 10% FBS and 2% HAT. Confluent monolayers of T8.9 cells were harvested by rinsing briefly in TC-PBS/0.01M Ethylenediaminetetra-acetic acid (EDTA) followed by 30 seconds trypsin treatment (a solution of 0.054% w/v trypsin (Difco, Detroit, MI, USA, Cat. No. 0152-13-1) and 0.54mM EDTA in HBBS, purified through a low protein binding 0.22μm filter (Millipore, USA, Cat. No. SLGV025LS) and stored at -20°C). Detached cells were resuspended in media supplemented with 2% FBS and centrifuged at 1200xg for 5 minutes before being seeded into fresh flasks.

Mouse hybridomas were obtained as described (Section 2.3.1). Hybridomas were maintained in RPMI 1640 supplemented with 10% FBS and 2% 50x HAT (13.6mg/L, hypoxanthine, 0.176mg/L aminopterin, 3.876mg/L thymidine) (Cytosystems, Cat. No. 21-110-0100v). The 12F12 hybridoma was obtained uncloned from Dr A. Zannettino (Hanson Centre for Cancer Research, Adelaide, Australia). This hybridoma was sub-cloned by limiting dilution and screened by flow cytometry (data not shown). For the collection of hybridoma culture supernatant, medium was collected from log phase cultures at a density of 6-8x10^5 cells/ml, centrifuged at 200xg to pellet any remaining cells. The culture supernatant was then supplemented with 0.1% sodium azide (Az) and stored at 4°C until use. For the generation of ascitic fluid, BALB/c mice were primed with 0.2ml mineral oil pristane 1-4 weeks prior to intraperitoneal injection with 1-2x10^6 hybridoma cells in TC-PBS in accordance with animal ethics approval from the Institute for Medical and Veterinary Science and University of Adelaide, Adelaide, Australia. After development of ascities, mice were sacrificed and ascitic fluid collected. After centrifugation at 200xg, ascitic fluid was aspirated, supplemented with 0.1% Az and stored at 4°C until use. All mAb culture supernatants and ascitic fluid were routinely screened on cell lines to verify activity.

2.1.3 Cryopreservation of Cell Lines

Cells in culture were harvested at log phase, washed in HBBS and resuspended at 2x10^7 cells/ml in medium containing 10% FBS. An equal volume of cryoprotectant (30% FBS, 20% dimethyl sulfoxide (BDH, Cat. No. 10323) and 50% RPMI) was then added dropwise over several minutes. Cells were placed into cryotubes (Lab Tek Nunc, Roskilde Denmark, Cat No. 3-66656) in 1ml aliquots and cooled to -100°C using a controlled rate freezer (Paton Industries, South Australia). The freezing rate was -5°C per minute down to

0°C, -1°C per minute to -25°C then -5°C per minute to -100°C. Cryotubes were then transferred liquid nitrogen for long term storage.

For thawing of cryopreserved cells, cryotubes from liquid nitrogen were rapidly warmed to 37°C in a water-bath. The cell suspension was transferred to a 10ml tube and an equal volume of appropriate medium/10% FBS (pre-warmed to 37°C) was added dropwise, mixed and allowed to stand for 5 minutes. Cells were then washed three times by centrifugation at 200xg for 5 minutes in medium supplemented with 2% FBS before being placed into flasks containing appropriate medium.

2.1.4 Human Umbilical Vein Endothelial Cells

EC, isolated from human umbilical cords by collagenase treatment (Wall *et al.*, 1978), were provided by Dr J. Gamble (Hanson Centre for Cancer Research, Adelaide, Australia). Isolated cells were cultured in 25cm^2 tissue culture flasks (Corning, Cat.No. 25110-25) precoated with gelatin and maintained in endotoxin free M199 supplemented as described (Section 2.1.1). Cells were maintained at 37°C with 5% CO_2 in air. After three days in culture the medium was aspirated and replaced with fresh medium supplemented with growth factors and heparin. On the fourth day, confluent monolayers were harvested by rinsing briefly in TC-PBS/0.01M EDTA followed by trypsin treatment (as described for T8.9 cells, Section 2.1.2). Detached cells were resuspended in M199 medium supplemented with 2% FBS and centrifuged at 200xg for 5 minutes before being seeded into fresh gelatin coated flasks. Cells were then cultured for three days before being sub-cultured as described. All experiments were performed using cells from passage 1 to 4.

2.1.5 Cytokines

Recombinant human tumour necrosis factor-α (TNF-α) (batch number 3056-55 at 0.5μg/ml, $2x10^7$ units/ml), interferon-γ (IFN-γ) (batch number N9327AX) and vascular endothelial growth factor (VEGF) (all from Genentech, San Francisco, CA, US), and recombinant human basic fibroblast growth factor (bFGF) (R&D Systems, UK) were kindly provided by Dr J. Gamble (Hanson Centre for Cancer Research, Adelaide, Australia). Recombinant human IL-1β, (IL-1) (batch number 4686-029 at 100μg/ml, $3.2x10^7$U/ml) and IL-4 (IL-4) (batch number 5479-046 at 5.27μg/ml) (both from Immunex Corporation Seattle, WA). Recombinant human IL-10 (IL-10) ($1-2x10^7$U/mg) was the gift of Dr R. de waal Malefyt (DNAX, Palo Alto, CA, US).

Recombinant mouse GM-CSF at $4x10^4$U/ml, synthesised using a yeast expression system, was a gift from Drs T. Wilson and N. Gough (Walter and Eliza Hall Institute, Melbourne, Australia). Recombinant mouse IL-3 (IL-3) at $8.3x10^5$U/ml, synthesised by insect cells infected with a recombinant baculovirus vector was a gift from Dr. A. Hapel (John Curtin School of Medical Research, Canberra, Australia).

2.2 Tissue Sections

2.2.1 Preparation of Human Tissues

Normal adult human tissue specimens were obtained with ethics approval from the Royal Adelaide Hospital Ethics Committee. Tonsil and gut were obtained through surgical procedures and embedded within 1 hour. Postmortem tissues including brain, skeletal muscle, cardiac muscle, skin, kidney, liver, lung, pancreas, spleen, thyroid and adrenal gland were obtained within 6 hours of death. Solid tissues were placed in Tissue Tek OCT compound (Miles Inc., Elkhart, IN, USA, Cat. No. 4583) and frozen by immersion into iso-pentane pre-cooled in liquid nitrogen. To maintain muscle morphology, skeletal and cardiac muscle

specimens were not embedded in OCT, but plunged directly into liquid nitrogen. After several minutes in liquid nitrogen, tissue blocks were placed on dry ice before storage at -70°C until use.

Peripheral blood was collected into heparinized tubes (Greiner Laborteknik, Frickenhausen, Germany, Cat. No. 455083) and blood smears prepared by drawing a drop of whole blood (50µl) along the length of an ethanol cleaned microscope slide using the edge of another slide. Bone marrow mononuclear cells (BMMNC) were obtained from normal volunteers and Ficoll purified by placing 5ml bone marrow aspirate into a 10ml tube and underlaying with 4ml Lymphoprep (Nycomed Pharma, Norway, Batch No. 502327). After centrifugation at 400xg for 25 minutes at room temperature, BMMNC at the interface of Lymphoprep and plasma were carefully removed. BMMNC were then washed three times in HBSS by centrifugation at 500xg for 5 minutes. Slides of BMMNC were prepared by resuspending $5x10^5$ cells/ml in 100% FBS and 100µl aliquots centrifuged at 28xg onto ethanol-cleaned microscope slides in a Cytospin 3 centrifuge (Shandon Scientific Ltd., England). Blood smears and cytocentrifuge slides were allowed to air dry and stored at -70°C before use.

2.2.2 Cryostat Sectioning of Human Tissues

Tissue blocks were removed from -70°C and placed at -20°C for 30 minutes before cryostat sectioning. 5µm sections were cut using a Cryostat (Bright Instrument Company Ltd., Huntingdon, Cambridgeshire, UK) onto ethanol cleaned glass microscope slides. After air drying for up to 2 hours, cryostat sections were stored at -20°C in the presence of self-indicating 2-4mm silica gel (Ajax Chemicals, Australia, Cat. no. 3681) overnight.

2.3 Antibodies

2.3.1 Primary Antibodies

All primary antibodies used in this study are summarised in Table 2.1. The anti-PETA-3 mAb 14A2.H9 was raised in this laboratory by fusion of splenocytes from Balb/c mice immunised with primary acute myeloid leukaemia blasts (purified from leukaemic patients) with the X-63.Ag8.653 myeloma cell line (Ashman *et al.*, 1991). The anti-PETA-3 mAbs 11B1.G4 and 14B5 were raised in a similar manner, with the exception that Balb/c mice were immunised with M-07e megakaryoblastic leukaemia cells. The resultant hybridoma supernatants were screened on FDC-P1 cells transfected with PETA-3 cDNA. The generation and specificity of these anti-PETA-3 mAbs has been previously described (Ashman *et al.*, 1991; Ashman *et al.*, 1997). The anti-PETA-3 rabbit polyclonal antibody (PETA-Rabbit) was raised against PETA-3 purified from platelets as described (Fitter *et al.*, 1995). The specificity of this reagent was verified as described (Section 2.3.5).

Anti-CD9 mAb, 1AA2.H9 and anti-CD63 mAb, 12F12 were raised in this laboratory as described (Cole *et al.*, 1989; Zannettino *et al.*, 1996). Anti-integrin mAbs were obtained as follows; the anti-β1 mAb, 61-2C4 (Levesque *et al.*, 1995) provided by Dr J. Gamble (Hanson Centre for Cancer Research, Adelaide, Australia), 8A2 (Kovach *et al.*, 1992) was kindly provided by Dr. N. Kovak (University of Washington, WA), anti-α2 16B4 (Serotec, Oxford, UK); anti-α3, P1B5 (GIBCO BRL); anti-α4, HP2/1 (GIBCO BRL); anti-α5, PHM2 (Becker *et al.*, 1981; Hancock *et al.*, 1983) provided courtesy of Dr R. Faull (Royal Adelaide Hospital, Adelaide, Australia), anti-α6, G_oH3 (PharMingen, San Diego, CA), anti-β3 AP-3 (American Type Culture Collection, (ATCC) Rockville, MD), anti-β4 439-9B (PharMingen) and anti-αIIbβ3 10E5 provided by P. Dyson (Hanson Centre for Cancer Research, Adelaide, Australia). The anti-PECAM-1 mAb B2B1 was raised in this laboratory and has been described previously (Cole *et al.*, 1989). Anti-PECAM-1 mAb 3D2, anti-vWF 49-5C3 and

Table 2.1 Antibodies used in this Study

Name	Specificity	Isotype	Reference	Source
10E5	αIIbβ3		(Coller, 1985)	P. Dyson
16B4	α2	IgG1		Serotec
P1B5	α3	IgG1		GIBCO BRL
HP2/1	α4	IgG1		GIBCO BRL
PHM2	α5	IgG1	(Becker *et al.*, 1981; Hancock *et al.*, 1983)	R. Faull
G_0H3	α6	Rat IgG2a		Pharmingen
61-2C4	β1	IgG1	(Sincock *et al.*, 1997)	J. Gamble
8A2	β1	IgG1	(Kovach *et al.*, 1992)	N. Kovach
AP-3	β3	IgG1		ATCC
439-9B	β4	Rat IgG2b		Pharmingen
100/3	γ-adaptin	IgG2b	(Robinson and Pearse, 1986)	Sigma
1AA2.H9	CD9	IgG1	(Ashman *et al.*, 1987; Cole *et al.*, 1989)	L. Ashman
561	CD34			G. Gaudinack
H9H11	CD44			P. Simmons
12F12	CD63	IgG1	(Zannettino *et al.*, 1996)	A. Zannetino
1B11	E-selectin			J. Gamble
68-2A1	E-selectin	IgG2a		J. Gamble
IV.3	FcγRII	IgG2b	(Looney *et al.*, 1986)	Medarex
1B5	*Giardia*	IgG1	(Sincock *et al.*, 1997)	G. Mayrhofer
P2A4-2	ICAM-1	IgG1		J. Gamble
anti-p180	p180	Rabbit Polyclonal	(Savitz and Meyer, 1990)	P. Meyer
B2B1	PECAM-1	IgG1	(Cole *et al.*, 1989)	L. Ashman
3D2	PECAM-1	IgG1		J. Gamble
11B1.G4	PETA-3	IgG2a	(Sincock *et al.*, 1997; Ashman *et al.*, 1997)	L. Ashman
14A2.H1	PETA-3	IgG1	(Ashman *et al.*, 1991; Ferro *et al.*, 1995; Ashman *et al.*, 1997)	L. Ashman
14B5	PETA-3	IgG1	(Ashman *et al.*, 1997)	L. Ashman
11G5	PETA-3	IgG1	(Ashman *et al.*, 1997)	Serotec
PETA-Rabbit	PETA-3	Rabbit Polyclonal		M. Berndt
1D4.5	*Salmonella*	IgG2a	(O'Connor and Ashman, 1982)	L. Ashman
3D3.3	*Salmonella*	IgG1	(O'Connor and Ashman, 1982)	L. Ashman
TOM-20	hMas20p	IgG1	(Hanson *et al.*, 1996)	B. Wattenberg
OKT-9	Transferrin Receptor	IgG1	(Goding and Burns, 1981)	ATCC
55-7H1	VE-Cadherin			L. Wayner
49-5C3	vWF	IgG1		J. Gamble

anti-E-selectin mAbs 1B11 and 68-2A1 were provided by Dr J. Gamble. The anti-transferrin receptor mAb OKT-9 (Goding and Burns, 1981) was obtained from the ATCC, anti-γ-adaptin mAb 100/3 (Robinson and Pearce, 1986) (Sigma), anti FcγRII IV.3 (Medarex Inc., Annadale, NJ, US), anti-p180 rabbit polyclonal antisera was the generous gift of Dr P. Meyer (University of California and Los Angeles, CA) (Savitz and Meyer, 1990) and the anti-hMas20p mAb TOM-20 (Hanson *et al.*, 1996) was provided by Dr B. Wattenberg (Hanson Centre for Cancer Research, Adelaide, Australia). Anti-CD44, mAb H9H11 (unpublished) provided by Dr P. Simmons (Hanson Centre for Cancer Research, Adelaide, Australia), anti-CD34 mAb, 561 (unpublished) courtesy of Dr G. Gaudinack (The National Hospital, Oslo, Norway) and anti-VE-cadherin mAb, 55-7H1 provided by Dr L. Wayner. Isotype matched non-binding mAbs 1B5 (IgG1), anti-*Giardia* (Sincock *et al.*, 1997), provided by Assoc Prof G. Mayrhofer (University of Adelaide, Adelaide, Australia), 3D3.3 (IgG1) and 1D4.5 (IgG2a), anti-*Salmonella* (O'Connor and Ashman, 1982), both raised in this laboratory, or normal rabbit serum (NRS) (Hunter Antisera, Australia) were included in all experiments as specificity controls.

2.3.2 Production of F(ab')$_2$ Fragments

F(ab')$_2$ fragments were produced by Ficin digestion of whole Ig (Mariani *et al.*, 1992) followed by Protein-A purification at 4°C. Pilot reactions were performed and monitored by SDS polyacrylamide gel electrophoresis and coomassie blue staining to ensure optimal digestion of IgG1 and IgG2a Ig (data not shown). All reagents were made fresh in digestion buffer (50mM Tris-HCl, pH 7.0/2mM EDTA) unless stated otherwise. To 1ml of mAb, at 1mg/ml in phosphate buffered saline (PBS) (0.14M NaCl, 3mM KCl, 8mM $Na_2HPO_4.12H_2O$ and 1mM KH_2PO_4 in MQ-water with a final pH of 7.4, sterilised by autoclaving at 130°C for 20 minutes and stored at 4°C until use) 100µl of Ficin (Sigma, Cat. No. F-6008, Lot No.

50H8145) at 1mg/ml was added before the addition of 110µl of 10mM L-cysteine (Sigma, Cat. No. C-7755) to activate the reaction. After thorough mixing, digestion reactions were rotated for 8 hours at 37°C. The reaction was terminated by the addition of 121µl of 100mM N,N-ethylmalemide (Sigma, Cat. No. E-3876, Lot No. 129F0185). Finally the pH of the digestion mixture was adjusted to 8.2 using 1M Tris-HCl, pH 8.6/0.1% Az for Protein-A purification.

2.3.3 Protein A Purification of Antibodies and F(ab')$_2$ Fragments

Protein-A purification of hybridoma culture supernatants or mouse ascites was performed using a column containing 5ml of Protein-A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden, Cat. No. 17-0780-01, Lot No. 252713) buffered with 0.1M phosphate, pH 8.2. The column was stripped of any Ig bound by washing with 15ml 0.5M proprionic acid, pH 2.0. The column was then neutralised by washing with 25ml of 0.1M phosphate buffer, pH 8.2. The pH of culture supernatant or mouse ascites was adjusted to 8.2 with 0.1M phosphate buffer pH 8.2. Antibody was allowed to enter the column and equilibrated for 30 minutes. The column was then washed with 25ml of 0.1M phosphate buffer pH 8.2. Antibody was eluted by the addition of 0.1M citrate pH 5.5 for IgG1 mAbs or 0.1M citrate, pH 4.5 for IgG2a mAb and 1ml fractions were collected into polythene tubes (Techno-Plas, Australia) containing 1ml of 1M Tris-HCl, pH 8.6 to neutralize the citrate. Protein content of the eluted fractions was determined by OD_{280} readings using a Cary Varian spectrophotometer (Varian Australia Pty. Ltd., Australia). Elution of the antibody peak was estimated (OD_{280}>0.1) and these fractions were pooled and dialysed against PBS using 6-8000 cutoff Cellu Sep T_2 dialysis tubing (Membrane Filtration Products Inc., San Antonio, TX, USA) through three changes of PBS, with stirring, over 24 hours. Dialysed antibodies were then diluted 1/2 in glycerol, aliquotted into 1ml eppendorf tubes and stored at -20°C until use.

For Protein-A purification of $F(ab')_2$ fragments, the Protein-A-Sepharose column was stripped and neutralised as described before loading of the Ficin digestion mixture. The digestion mixture was allowed to enter the column and equilibrated for 30 minutes, to allow binding of any residual Fc. $F(ab')_2$ fragments were then eluted with 0.1M phosphate buffer and 1ml fractions were collected into polythene tubes. Protein content of the eluted fractions was determined by OD_{280} readings using a Cary Varian spectrophotometer. Elution of the $F(ab')_2$ peak was estimated (OD_{280}>0.2) and these fractions were pooled then dialysed against Tyrodes buffer (prepared by adding 5ml stock solution (160g/L NaCl, 4g/L KCl, 20g/L $NaHCO_3$, 1.16g/L $Na_2HPO_4.H_2O$ in 1L MQ-water) to 80ml MQ-water, adjusting pH to 7.35 before making up to 100ml with MQ-water). Dialysed $F(ab')_2$ fragments were concentrated by centrifugation at 500xg in Centricon Concentrators (Amicon Inc., Beverly, MA, USA, Cat. No. 4205) and the concentration of $F(ab')_2$ fragments determined by OD_{280} readings. $F(ab')_2$ in Tyrodes buffer were supplemented with 0.01% bovine serum albumin (BSA) (Sigma, Cat. No. A-7906, Lot No. 76H0911), sterilised by centrifugation, 13,000xg for 30 seconds through Spin-X columns (Corning) and stored at 4°C until use.

2.3.4 Verification of $F(ab')_2$ Activity and Purity

The activity of $F(ab')_2$ fragments was verified by titration on the megakaryoblastic cell line M-07e, which has been previously demonstrated to express the antigens recognised by mAbs 11B1.G4, 14A2.H1 (PETA-3) and 1AA2.H9 (CD9) (Fitter, Sincock, Jolliffe and Ashman, submitted). M-07e cells were harvested from culture, stained by indirect immunofluorescence and analysed by flow cytometry (Figure 2.1.A) as described (Sections 2.5.1 and 2.5.3).

The purity of Protein-A purified $F(ab')_2$ fragments was assessed by SDS polyacrylamide gel electrophoresis and Western blotting for mouse Ig as described (Sections

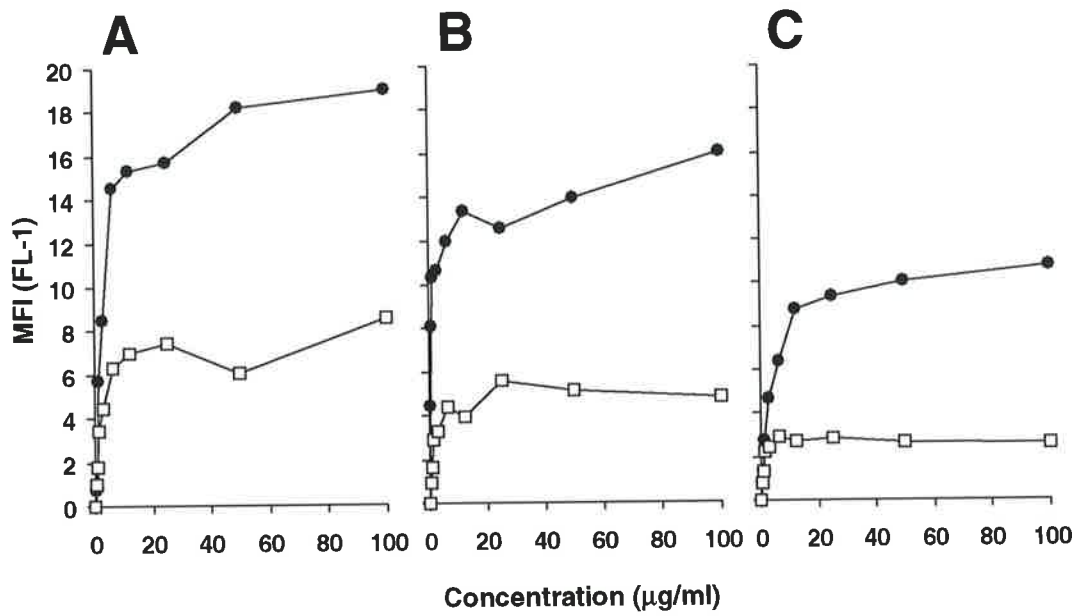
Figure 2.1 Verification of $F(ab')_2$ Activity and Purity

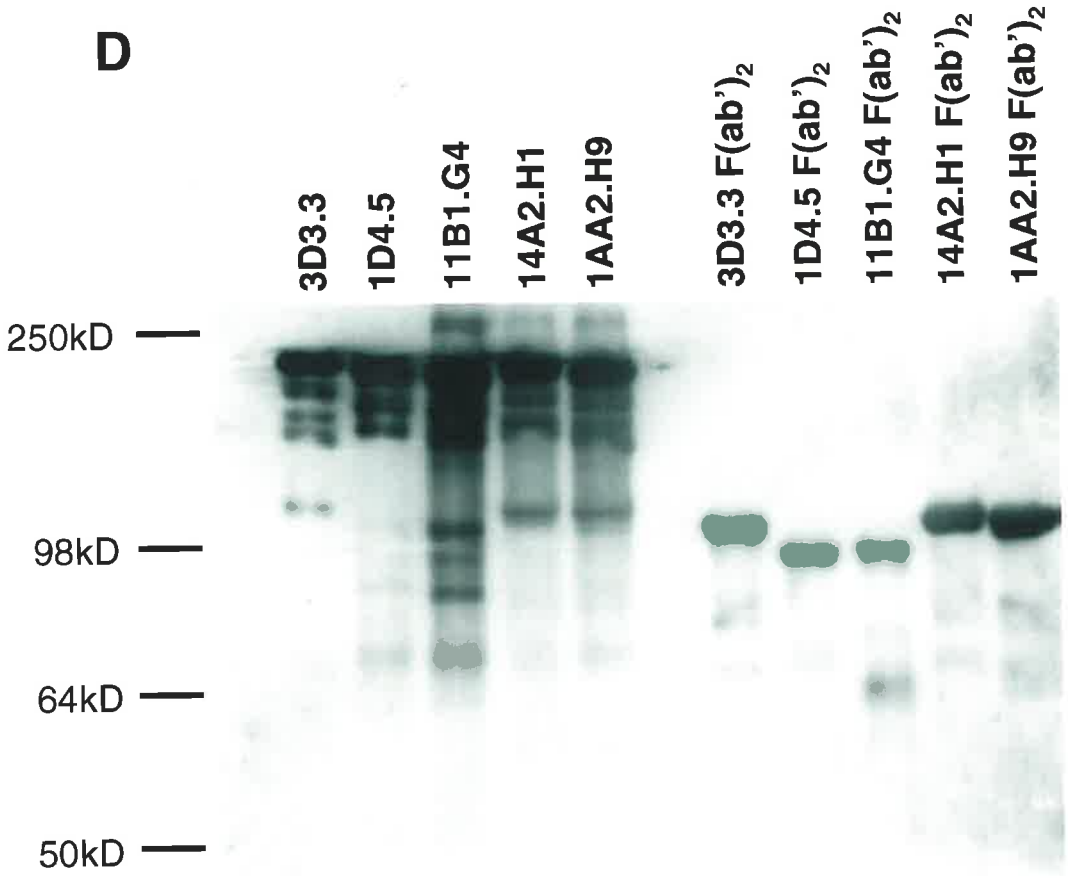
Purified $F(ab')_2$ fragments were screened by flow cytometry (Section 2.5) on the M-07e cell line. For comparison, uncut Ig was also titrated. Panels show binding of (A) 11B1.G4, (B) 14A2.H1 and (C) 1AA2.H9. $F(ab')_2$ fragments bound with approximately 40% efficiency of that of their respective whole Ig.

Closed Circles Whole Ig

Open Squares $F(ab')_2$ Fragments

Panel (D), whole Ig and $F(ab')_2$ fragments were seperated on 8% gels and Western blotting with sheep anti-mouse Ig alkaline phosphatase (Section 2.8.6).


A
B
C
MFI (FL-1)
0
2
4
6
8
10
12
14
16
18
20
0 20 40 60 80 100
Concentration (µg/ml)


D
3D3.3
1D4.5
11B1.G4
14A2.H1
1AA2.H9
3D3.3 F(ab')2
1D4.5 F(ab')2
11B1.G4 F(ab')2
14A2.H1 F(ab')2
1AA2.H9 F(ab')2
250kD
98kD
64kD
50kD

2.8.5 and 2.8.6). $F(ab')_2$ fragments were gel electrophoresed under non-reducing conditions through an 8% acrylamide gel and Western blotted as described (Figure 2.1.B).

2.3.5 Verification of PETA-Rabbit Specificity

Affinity purified PETA-Rabbit was produced and kindly provided by Dr M. Berndt (Baker Medical Research Institute, Melbourne, Australia). In order to verify the specificity of this reagent, immunofluorescent labelling (Section 2.5) of TM4SF transfected cell lines (Section 2.1.2) was performed as shown in Figure 2.2.A. Since the transmembrane domains of TM4SF members are most homologous (Section 1.2.1), the binding of PETA-Rabbit to permeabilised transfected cells was assessed. Cytocentrifuge slides of cells were fixed, labelled by indirect immunofluorescence and viewed by confocal microscopy as described (Section 2.6). MAbs specifically bound transfected cells expressing their respective TM4SF antigens (Figure 2.2.A). Additionally, PETA-Rabbit specifically bound FDC-P1 (PETA-3) cells only. Similar studies examining surface labelling were also performed using flow cytometry and gave similar results (data not shown). The specificity of PETA-3-Rabbit was also assessed by Western blotting of whole EC and platelet lysates as described (Sections 2.8.5 and 2.8.6). PETA-Rabbit specifically bound a protein of predicted size (~32kD) from both EC and platelet lysates, which corresponded with the band detected by the anti-PETA-3 mAb 11B1.G4 (Figure 2.2.B).

2.4 Immunohistochemical Techniques

2.4.1 Indirect Immunoperoxidase Staining

Fresh frozen sections were removed from -20°C and allowed to equilibrate to room temperature before staining. Slides were labelled and the specimens outlined using a Dakopen (Dakopatts, Denmark, Cat. no. S2002) to aid the application of solutions to the sections.

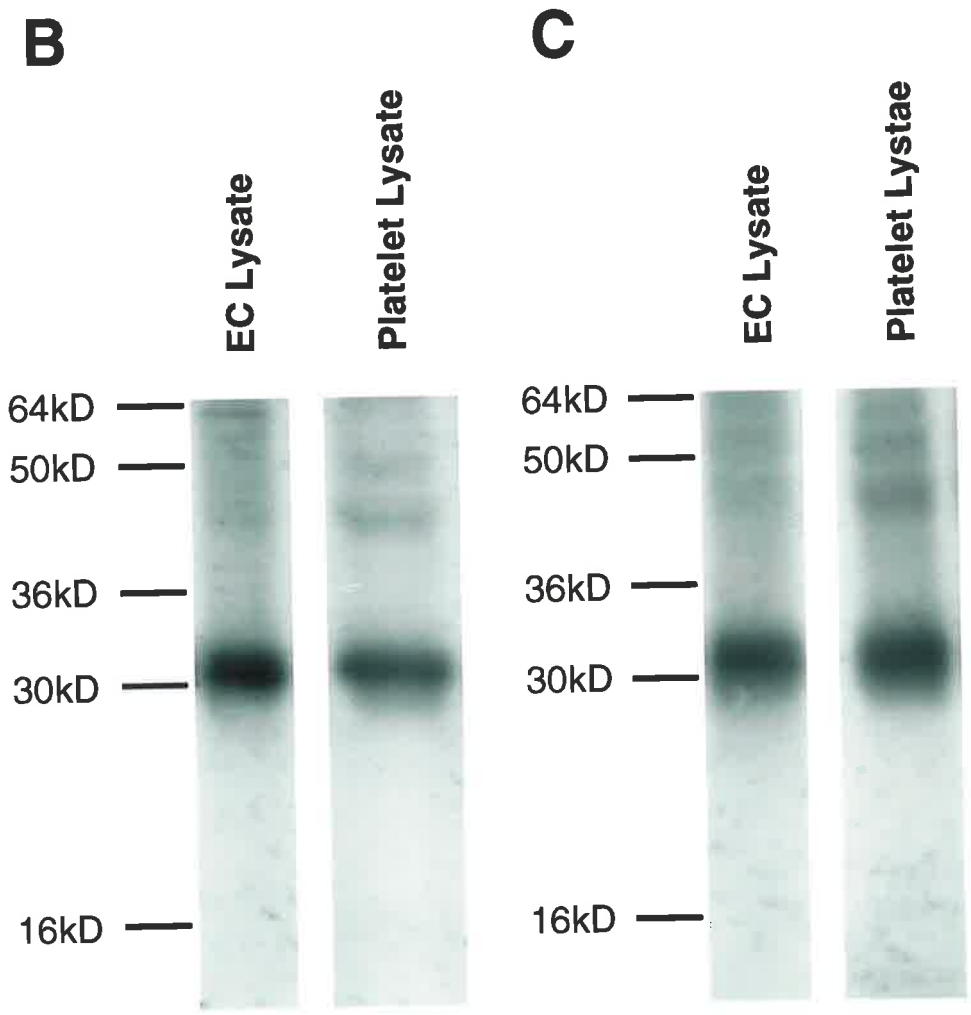
Figure 2.2 Verification of PETA-Rabbit Specificity

Panel (A) shows tabulated results of screening mAbs and PETA-Rabbit. Cells were permeabilised, stained by indirect immunofluorescence and viewed by confocal microscopy (Section 2.3.5). FDC-P1 (puro) refers to vector alone transfected cells. Staining was scored from weak (+) to very strong (+++).

Lower panels show Western blotting analysis of whole EC and platelet lysates for PETA-3 using (B) mAb 11B1.G4 or (C) PETA-Rabbit (Section 2.3.5). Both reagents specifically reacted with a single polypeptide of molecular weight ~32kD corresponding to PETA-3.

A

Antibody	Transfectant FDC-P1 (Puro)	FDC-P1 (PETA-3)	T8.9 (CD9)	FDC-P1 (CD63)
1B5/1D4.5	-	-	-	-
NRS	-	-	-	-
14A2.H1	-	+++	-	-
11B1.G4	-	+++	-	-
14B5	-	+++	-	-
PETA-Rabbit	-	++	-	-
1AA2.H9	-	-	+++	-
12F12	-	-	-	+++



Sections were then fixed in 95% ethanol for 10 minutes on ice and washed with three changes of ice cold PBS. MAb culture supernatant (30-50µl), supplemented with heat inactivated NRS, (10% final) was applied to sections and allowed to incubate for 1 hour at 4°C. After washing in three changes of cold PBS, sheep anti-mouse Ig conjugated horseradish peroxidase (Amersham Life Sciences, Burkinghamshire, UK, Cat. No. NA9310) diluted 1/20 in 10% NRS was applied and incubated for 1 hour at 4°C. Sections were then washed as before then brought to room temperature. Peroxidase detection was performed by immersion in 1.3mM 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Cat. No. D-5637) containing 0.07% hydrogen peroxide (Sigma, Cat. No. H-1009, Lot No. 22H3480) for up to 20 minutes at room temperature. Sections were then counterstained with haematoxylin (Section 2.4.3), washed twice in water to remove excess salt, then dehydrated through two changes of 100% ethanol followed by three changes of safsolvent (Ajax Chemicals, Cat. No. 2537). Slides were then mounted with Depex (BDH, 36125). Cells exhibiting mAb binding were stained brown. For black and white photomicrographs of immunoperoxidase stained sections, photos were taken using a #47 Kodak Wratten filter (Kodak, Rochester, NY, US) with maximum transmission at 440nm, to enhance DAB staining.

2.4.2 Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

The APAAP technique used for staining of cell smears was a derivation of that described by (Erber *et al.*, 1984). The slide box was allowed to warm to room temperature prior to removing slides. A circle was drawn around the cell smear with a Dakopen to enable localisation of applied solutions to the cells. Smears were then fixed in standard fixative (47.5% v/v acetone, 47.5% v/v methanol, 5% v/v formaldehyde) for 30 seconds on ice. Smears were rinsed in distilled water and then washed in three changes of Tris-buffered saline (TBS) (0.5M Tris-HCl, pH 7.6 and 1.5M NaCl in H_2O).

All subsequent steps were performed at room temperature. Primary mAb supernatant (30µl), supplemented with NRS (10% final) was applied to the smear and incubated for at least 30 minutes in a humidified chamber to ensure that the smears did not dry at any stage. Smears were then washed in three changes of TBS over 5 minutes and 20µl of diluted rabbit anti-mouse Ig (1/50 with 25% heat inactivated normal human serum in TBS) (Dakopatts, Denmark, Cat. no. Z259) was applied and incubated for 30 minutes, then washed as described above. A volume of 20µl of diluted APAAP mAb complex (1/100 in TBS) (Dakopatts, Denmark, Cat. no. D651) was then applied and incubated for a further 30 minutes prior to washing as above. Smears were then incubated with two further cycles of rabbit anti-mouse Ig/wash/APAAP mAb/wash, with 10 minute Ab incubations. After the final wash the slides were then incubated for 20 minutes in substrate. To prepare substrate 20mg napthol AS-MX phosphate free acid (Sigma, Cat. No. N-4875, Lot No. 93H5032) was dissolved in 2ml dimethylformamide (BDH, Cat. No. 10322) and made up to 100ml with 0.1M Tris-HCL, pH 8.2. To this was added 100µl levamisole (Sigma, Cat. No. L9756, Lot. No. 115H3412) to block endogenous phosphatases. Finally 10mg/ml Fast Red TR salt (Sigma, Cat. No. F1500, Lot. No. 91F-0433) was added immediately prior to use. After thorough mixing, substrate was filtered through 3M Whatman paper. After incubation in substrate, slides were counterstained with haematoxylin (Section 2.4.3). Once counterstained sufficiently, slides were rinsed in water and mounted in glycerol-glycine (1.4g glycine dissolved in 100ml H_2O and the pH adjusted to 8.6 with NaOH. To 30ml of this solution 70ml glycerol was added). Cells exhibiting antibody binding were stained red.

2.4.3 Haematoxylin Counterstaining

Indirect immunoperoxidase or APAAP stained slides were rinsed in water and counterstained with haematoxylin (a solution of 25% v/v Ethylene Glycol and 2% v/v glacial

acetic acid in MQ-water, containing 2g/L of anhydrous/monohydrate Haematoxylin, (0.2g/L sodium periodate ($NaIO_4$) and 17.6g/L aluminium sulfate ($Al_2(SO_4)_3.18H_2O$)) for 10 minutes. Slides were then quickly rinsed in water and immersed in 0.5% HCl in water for 5 seconds to remove excess haematoxylin. After rinsing in water, slides were then placed in Scott's gentle alkaline solution (3.5g/L $NaHCO_3$ and 20mg/L $MgSO_4.7H_2O$ in MQ-water) for 2 minutes to enhance haematoxylin staining. Sections were examined to determine the level of counterstaining, and if further staining was required, slides were returned to haematoxylin and processed as described.

2.5 Indirect Immunofluorescence Labelling For Flow Cytometry

2.5.1 Immunofluorescent Labelling of Cells

Expression of surface molecules was measured by indirect immunofluorescence and flow cytometry. Adherent cells were harvested as described (Section 2.1.2) and washed twice in cold PBA (PBS supplemented with 0.1% bovine serum albumin and 0.1% Az), whilst non-adherent cells were harvested directly from culture before washing. Cells were then resuspended at 10^7/ml in PBA containing 10% heat inactivated NRS, before incubation of $5x10^5$ cells with primary mAb for 1 hour on ice. For the detection of PETA-3, mAb 11B1.G4 was used, unless stated otherwise. After washing three times in cold PBA, bound mAb was detected by incubation for 1 hour on ice with r-phycoerythrin conjugated goat anti-mouse Ig (Southern Biotechnology Associates Inc., Birmingham, AL, USA, Cat. No. 1030-09, Lot. No. L327-Q487) diluted 1/50 in 10% NRS/PBA for 1 hour on ice. Alternatively, bound mAb or $F(ab')_2$ fragments were detected by fluorescein-isothiocyanate (FITC) conjugated sheep anti-mouse Ig (Silenus Laboratories, Australia, Cat. No. DDF) diluted 1/50 in 10% NRS/PBA. Cells were washed three times in cold PBA, before fixing in 0.5ml cold 1% paraformaldehyde.

Detection of intracellular antigens was performed using saponin permeabilisation (Openshaw *et al.*, 1995; Sander *et al.*, 1991). Cells were harvested and washed as above, fixed with 2% formaldehyde for 10 minutes at room temperature, washed twice in cold PBA and resuspended at 10^7/ml in cold PBA with 0.1% saponin (Sigma, Cat. No. S130-2). Following 10 minutes incubation on ice, primary mAb supplemented with 0.1% saponin/10% NRS/PBA was added to cells and incubated for 1 hour on ice. After washing three times in cold PBA with 0.1% saponin, bound mAb was detected by incubation with r-phycoerythrin conjugated goat anti-mouse Ig diluted 1/50 in 10% NRS/PBA with 0.1% saponin for 1 hour on ice. Cells were washed twice in cold PBA containing 0.1% saponin, followed by two washes in cold PBA without saponin, before fixing with 1% paraformaldehyde. For comparison with surface (unpermeabilised) staining, cells were fixed with 2% formaldehyde and stained as above with the omission of 0.1% saponin in all buffers. Percentage intracellular antigen was calculated by comparison of the mean fluorescence intensity of labelled formaldehyde fixed cells with formaldehyde fixed saponin permeabilised cells.

2.5.2 Immunofluorescent Labelling of Platelets

Immunofluorescent labelling of platelets was performed at room temperature using a modified protocol based on that of (Sedlmayr *et al.*, 1996). All washes were performed using PBS supplemented with 0.1% Az (PBS/Az). Whole blood was collected by venipuncture into EDTA (Greiner Laborteknik, Cat. No. 454036) and either immediately fixed with 0.1% paraformaldehyde, for resting platelets, or stimulated with bovine thrombin (0.12U/ml) (Behringwerke, Germany) for 5 minutes at room temperature before fixation, for activated platelets. Blood containing non-thrombin stimulated platelets was also supplemented with 10^{-6}M prostaglandin E_1 (PGE_1) (Pharmacia Upjohn Inc, Bridgewater, NJ, US). Fixed whole blood was then centrifuged at 1200xg and the plasma aspirated. Pellets were then

resuspended and 50µl aliquots were diluted 1/2 in PBS/Az before addition of 100µl primary mAb. After incubation for 1 hour, cells were washed in 1ml PBS/Az and the pellet resuspended in 30µl sheep anti-mouse Ig conjugated FITC (Silenus) diluted 1/10 in PBS/Az and incubated for 30 minutes in the dark. Cells were then washed once, as before, and centrifuged at 250xg for 30 seconds to pellet the majority of red blood cells. The platelet rich supernatant was then collected, transferred to polythene tubes (Techno-Plas) and stored on ice for up to 2 hours until analysis.

2.5.3 Flow Cytometry

For studies using cell lines and HUVEC, mAb binding was quantitated using an EPICS XL flow cytometer (Coulter, Hileah, FL, USA). PE-conjugated fluorochromes, were detected using fluorescence channel-2 (FL-2) on a logarithmic scale whilst FITC conjugates were analysed using fluorescence channel-1 (FL-1), again using a logarithmic scale. Indirect immunofluorescence labelling of platelets was analysed using a FacScan (Becton Dickinson, Franklin Lakes, NJ, US). Since platelets were labelled with FITC-conjugated secondary reagents, FL-1 was used for this analysis with a logarithmic scale. For all flow cytometric analysis, mean fluorescence intensity (MFI) was calculated from a minimum of 10,000 gated events for cells and 20,000 events for platelets.

2.6 Indirect Immunofluorescent Labelling for Confocal Microscopy

For confocal microscopy, HUVEC were plated at $5x10^4$ per well in chamber slides (Lab Tek Nunc) precoated with 50µg/ml fibronectin, and grown to confluence. During this time some were pretreated with 10µg/ml Brefeldin A (BFA) (Sigma, Cat. No. B-7651) for 2 hours at 37°C or with cytokines as indicated. Cells were then washed with PBS and fixed (acetone 47.5%, methanol 47.5%, formaldehyde 5%) for 30 seconds at 4°C. For

non-permeabilising fixation, cells were fixed with 2% formaldehyde for 5 minutes at 4°C. After washing, cells were incubated with primary mAb or 5μg/ml biotinylated wheatgerm agglutinin (WGA) (Vector Laboratories Inc., Burlimgame, CA, USA, Cat. No. B-1025), in 10% NRS/PBA for 45 minutes at 4°C. PETA-3 was detected using mAb 11B1.G4 for all confocal microscopy experiments, unless stated otherwise. Cells were washed and incubated with sheep anti-mouse Ig FITC (Silenus) diluted 1/50 in 10% NRS/PBA for 45 minutes at 4°C. For the detection of rabbit polyclonal antibody, cells were incubated with sheep anti-rabbit IgG FITC (Silenus, Cat. No. RDAF) diluted 1/50. After washing three times, cells were fixed with 1% paraformaldehyde and mounted in 86% glycerol containing 1% propyl-gallate (Sigma, Cat. No. P-3130). Slides were stored at 4°C in the dark until use.

For 2-colour labelling, cells were fixed as described above, incubated with primary mAbs, washed, then incubated with goat anti-mouse IgG1 Tri-Color (Caltag Laboratories, Burlingame, CA, USA, Cat. No. M32006) and IgG2a FITC (Caltag Laboratories, Cat. No. M32301) diluted 1/50 in 10% NRS/PBA. For the detection of biotinylated WGA, cells were incubated with streptavidin-Tri-Color (Caltag Laboratories, Cat. No. SA1006) diluted 1/50. For the detection of rat mAbs, cells were incubated with goat anti-rat IgG Texas Red (Caltag Laboratories, Cat. No. R40003). For the 2-colour detection of mouse IgG2b, cells were incubated with goat anti-mouse IgG2b FITC (Caltag Laboratories, Cat. No. M32401). After washing three times, slides were processed as for single colour labelling.

Confocal microscopy was performed using a MRC 600 confocal microscope (Bio-Rad Microscience, Cambridge, MA, USA). Isotype matched negative control mAbs were included in all indirect immunofluorescence labelling experiments as specificity controls. For single colour labelling (FITC), the sample was excitated at 488nm and viewed through a 500nm long pass filter. For 2-colour labelling (FITC and Tri-color or Texas Red), the sample was excitated at 514nm and viewed through 527nm band pass (FITC, FL-2) or 600nm long pass

(Tri-color or Texas Red, FL-1) filters. Single fluorochrome (FITC, Tri-color or Texas Red) labelled controls were included in all 2-colour labelling experiments to ensure no overlap of signal occurred between channels.

2.7 Immunoelectron Microscopy

2.7.1 Preparation of EC and Platelets

EC monolayers were fixed in 8% paraformaldehyde (BDH) in 0.1M phosphate (15.6g/L $NaH_2PO_4.2H_2O$ in MQ-water) buffer, pH 7.35, for 1 hour at room temperature. Cells were then washed with 0.2M phosphate buffer, scraped from the culture dish and pelleted at 13,000xg in a microfuge. Cells were resuspended in warm gelatin (10% in phosphate buffer), then pelleted at maximum speed in the microfuge. After cooling, the gelatin-embedded cells were infiltrated with polyvinylpyrrolidine/sucrose (15% polyvinylpyrrolidine, 2M sucrose) overnight at 4°C. For the preparation of platelets, whole blood was collected using EDTA as anticoagulant. Platelet rich plasma (PRP) was prepared by centrifugation of whole blood at 200xg for 11 minutes. PRP was then allowed to equilibrate at 37°C for 15 minutes before being pelleted at 13,000xg for 5 seconds. Plasma was then aspirated and the platelet pellet immersed in 8% paraformaldehyde (BDH) in 0.1M phosphate buffer as mentioned above. After incubation for 45 minutes at room temperature, the fixed platelet pellet was washed in 0.2M phosphate buffer and overlayed with warm 10% gelatin. After cooling, the gelatin-embedded cells were infiltrated with polyvinylpyrrolidine/sucrose overnight at 4°C. Polyvinylpyrrolidine/sucrose infiltrated material was cut into 1-2mm cubes, mounted on aluminium stubs (Leica, Germany) and snap frozen in liquid nitrogen.

2.7.2 Cryoultramicrotome Sectioning

Ultrathin frozen sections (60-80nm) were cut using a diamond knife by Drs R. Parton or M. Lindsay (University of Queensland, Brisbane, Australia) using a Leica Ultracut UCT cryomicrotome (Leica), onto formvar or carbon coated hexagonal copper 200 mesh grids (Proscitech, Australia) and stored under methylcellulose/sucrose (1% methyl cellulose, 1.15M sucrose) at 4°C until use.

2.7.3 Immunogold Labelling

For immunolabelling, grids were incubated in blocking buffer (PBS supplemented with 0.1% BSA (Sigma), 0.2% fish skin gelatin (Sigma), 50mM glycine) for 15 minutes. Grids were then incubated with appropriate primary antibody, diluted in blocking buffer, for 30 minutes at room temperature. After washing five times in blocking buffer, grids were incubated for 30 minutes with Protein-A conjugated 10nm gold (University of Utrecht, The Netherlands) diluted 1/55 in blocking buffer. Grids were then washed five times in PBS followed by five washes in water before staining with 3% uranyl acetate (Ajax Chemicals, Australia)/2% methyl cellulose at a ratio of 1/9 for 7-10 minutes on ice in the dark. Labelled grids were viewed at 80kV on a JEOL 1010 (JEOL, Japan) electron microscope at the Centre for Microscopy and Microanalysis, University of Queensland, according to published techniques (Parton *et al.*, 1997).

2.8 Immunoprecipitation and Western Blotting

2.8.1 Preparation of Protein Lysates

Confluent EC monolayers in 75cm^2 tissue culture flasks were washed twice in cold PBS, before addition of 1ml of 1% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) lysis solution (1% CHAPS, 10mM Tris pH 7.4, 140mM NaCl,

0.5mM $CaCl_2$, 0.5mM $MgCl_2$). CHAPS lysis solution was supplemented with 1/100 volume of 100x protease inhibitors (4mg/ml each of, L-[1-tosylamido-2-phenyl] ethyl chloromethylketone, 1-Chloro-3-tosylamido-7-amino-L-2-heptanone and p-Nitrophenyl-p'-guanidino-benzoate-HCl (all from Sigma) dissolved in dimethylformamide and stored at -20°C until use) and 1/100 volume of 100x phenyl methyl sulfonyl fluoride (PMSF) (4mg/ml PMSF dissolved in ethanol and stored at -20°C until use) or 1%NP 40 lysis solution (1% NP 40 (Fluka, Buchs, Switzerland), 50mM Tris-HCL, 150mM NaCl, 1mM EDTA) supplemented with protease inhibitors and PMSF as above. Cells were then scraped off the flask using a teflon Cell Lifter (Corning) and incubated for 30 minutes at 4°C. Lysate was then centrifuged at 800xg for 10 minutes at 4°C to pellet nuclei. Nuclei free lysate was then taken off and insoluble material pelleted by centrifugation at 150,000xg for 30 minutes at 4°C. Lysate was then aspirated and transferred to 2ml eppendorf tubes for immunoprecipitation experiments.

2.8.2 Biotin Surface Labelling

Cell surface biotinylation was performed as previously described (Cole *et al.*, 1987). Cells were washed twice with PBS before being incubated with 0.5mg/ml NHS-LC-Biotin (Pierce, Rockford, IL, US) for 30 minutes at room temperature while rocking. Cells were then washed twice in cold PBS to remove unreacted biotin. CHAPS lysis buffer was then added to cells and the lysate processed as above (Section 2.8.1).

2.8.3 Immunoprecipitations

Prior to immunoprecipitation, all lysates were incubated with 10µl of a 50% slurry of goat anti-mouse Ig-Sepharose rotating for 1 hour at 4°C in order to remove non-specific binding. Specific molecules were immunoprecipitated by incubation of lysate from 10^7 cells with 2-5µg of purified antibody (or goat anti-mouse Ig-Sepharose "pre-armed" for 2 hours

with hybridoma culture supernatant, for 1 hour at 4°C) followed by addition of 15µl of a 50% slurry of goat anti-mouse Ig-Sepharose for a minimum of 2 hours at 4°C. All incubations were performed on a rotator. Goat anti-mouse Ig-Sepharose was then pelleted by centrifugation at 13,000xg for 30 seconds, then washed five times in CHAPS lysis buffer by pulsing. Immunoprecipitated proteins were eluted by the addition of 15µl 2x SDS-PAGE loading buffer (a solution of 2ml 0.5M Tris-HCl, pH 6.8, 1.6ml glycerol, 3.2ml 10% SDS in H_2O, 0.4ml 0.05% bromophenol blue and 2ml H_2O) followed by boiling for 4 minutes. Where indicated, loading buffer was supplemented with 5% β-mercaptoethanol. After pelleting the goat anti-mouse Sepharose, eluted proteins were either loaded onto PAGE gels for immediate separation or stored at -20°C until use.

2.8.4 Re-Precipitation Experiments

For reprecipitation experiments, surface biotinylated lysates were prepared as described (Section 2.8.2). 11B1.G4 immunoprecipitates were then carried out as described (Section 2.8.3), however instead of eluting proteins with loading buffer, washed 11B1.G4 immunoprecipitates were re-solubilised in 500µl NP 40 buffer (1% NP 40, 50mM Tris-HCL, 150mM NaCl, 1mM EDTA) supplemented with 0.02% SDS for 2 hours rotating at 4°C. Eluted proteins were diluted 1/2 with NP 40 buffer then precleared with 10µl goat anti-mouse Sepharose. Re-precipitations with integrin or other mAbs were then carried out as described (Section 2.8.3).

2.8.5 Separation of Proteins by Gel Electrophoresis

12% SDS polyacrylamide mini-gels were set up using the multiple gel electrophoresis apparatus (Hoefer Scientific Instruments, CA, USA, Model SE 200/ SE 250/ SE 260). The gel mix was prepared (a solution of 1.7ml MQ-water, 2ml 30% acrylamide (Boehringer

Mannheim, Cat. No. 1685 821, Lot No. 84586420), 1.3ml 1.5M Tris-HCl pH 8.8, 50µl 10% SDS (Sigma, Cat. No. L-4509, Lot No. 75H0493), 50µl 10% ammonium persulfate (Sigma, Cat. No. A-9164, Lot No. 65H0750) and 4µl N,N,N',N'-tetramethylethylenediamine (Sigma, Cat. No. T-8133)). For 8% gels, the mixture consisted of 2.3ml MQ-water, 1.3ml 30% acrylamide, 1.3ml 1.5M Tris-HCl pH 8.8, 50µl 10% SDS, 50µl 10% ammonium persulfate and 3µl N,N,N',N'-tetramethylethylenediamine. After mixing, gel solutions were immediately poured between two plates (8 x 10cm) kept apart by 1mm spacers placed in a multiple gel caster. When the gel had polymerised, combs were placed over the top and freshly made 5% polyacrylamide stacking gel mix (a solution of 1.15ml MQ-water, 30% acrylamide, 0.5ml 0.5M Tris pH 6.8, 0.02ml 10% lauryl sulfate, 0.02ml ammonium persulfate, 0.002ml N,N,N',N'-tetramethylethylenediamine) was poured over the gel to create loading wells. When the stacking gel had polymerised, plates were placed in a multiple gel electrophoresis unit and filled with running buffer (3.03g/L Tris, 1g/L SDS and 14.41g/L glycine dissolved in MQ-water). Immunoprecipitated proteins in loading buffer were boiled for 5 minutes immediately before being loaded onto the mini-gel. After loading, proteins were electrophoresed through the stacking gel at 15mA, followed by 20mA through the 12% separating gel until the dyefront of loading buffer approached the end of the gel (1.5-2 hours).

Proteins were then transferred from the gel to a filter using a Tranphor electrophoresis system (Hoefer Scientific Instruments, CA, USA, Model TE 22) according to the manufacturer's instructions. Polyvinylidenedifluoride membrane (Micron Separations Inc., Westborough, MA, USA) was cut to size (8 x 5cm), placed in methanol and soaked in transfer buffer for at least 15 minutes prior to use. Two pieces of Whatman 5mm paper were cut to the same size and wet in transfer buffer. After removal of the stacking gel, the 12% resolving gel was placed over the filter with Whatman paper on either side of the two. After removal of any bubbles, these layers were layed between two sponges and placed into a plastic case that

was immersed into transfer buffer within the transfer apparatus. Proteins were transferred either overnight with a current of 30mA or for 2 hours at 250mA. The filter was then removed and probed.

2.8.6 Detection of Immunoprecipitated Proteins by Western Blotting

For the detection of PETA-3, the membrane was blocked for 2 hours at room temperature in 5% (w/v) skim milk powder in TBS (0.5M Tris-HCl, pH 7.6 and 1.5M NaCl in H_2O) while rocking. The filter was probed with biotinylated 11B1.G4 diluted 1/2500 in TBS-Tween (TBS supplemented with 0.1% Tween-20, ICN Biomedicals Inc., Aurora, OH) for 2 hours at room temperature. The filter was then washed once for 15 minutes, and twice for 5 minutes each in TBS-Tween at room temperature. For each subsequent step the filters were probed for 1 hour at room temperature and washed as above. Bound primary antibody was detected with streptavidin-alkaline phosphatase diluted 1/5000 in TBS-Tween and detected using a Western Blotting Kit (Amersham Life Sciences, Cat. No. RPN 5780). The filter was then scanned with a Fluoro-Imager using a 570nm bandbass filter (Molecular Dynamics, Sunnyvale, CA, USA). Alternatively, biotinylated 11B1.G4 was detected by streptavidin-biotinylated horseradish peroxidase (Amersham Life Sciences, Cat. No. RPN 1051, Lot No. 95060) diluted 1/2000 and proteins visualised using the enhanced chemiluminescence Western blotting detection system (Amersham Life Sciences, Cat. No. RPN 2106) according to the manufacturer's instructions. For the detection of biotinylated immunoprecipitates or mouse Ig/$F(ab')_2$ fragments, filters were processed as above, with the exception that incubation with biotinlyated 11B1.G4 was not performed, and detection was performed with streptavidin-alkaline phosphatase (as above) or sheep anti-mouse Ig alkaline phosphatase (Silenus, Cat. No. 985034010, Lot No. WC13B) each diluted 1/4000.

2.9 Platelet Aggregometry

2.9.1 Preparation of Platelets

Blood was collected with ethics approval from the antecubital vein of volunteers who had abstained from medication known to affect platelet function for at least 10 days prior to blood collection. Blood was collected into one-tenth volume 0.1M trisodium citrate, pH 7.35 as anticoagulant. PRP was prepared by centrifugation at 200xg for 11 minutes and separated from the cellular fraction. Platelet depleted plasma was prepared by centrifugation of remaining PRP depleted blood at 1600xg for 20 minutes at room temperature. The platelet count of PRP was then calculated using a Technicon H2 Analyser (Bayer Diagnostics, New York, NY, USA) and standardised to 300,000 platelets/μl using autologous platelet depleted plasma. Standardised PRP was stored in an air-free plastic syringe (Terumo Medical Corporation, Elkton, MD, USA) to control pH and kept at room temperature for up to 3 hours until use, as previously described (Vanags *et al.*, 1992).

2.9.2 Platelet Aggregometry

Platelet aggregation measurements were performed using siliconised glass cuvettes (0.312 inch, Paton Scientific, Adelaide, Australia) in three dual-channel platelet aggregometers (Payton, Ontario, Canada). Changes in light transmission were measured on omniscribe recorders (Houston Instruments, Austin, TX, USA), at an input sensitivity of 0.01 volts. For all experiments platelet aggregation was carried out at 37°C with stirring (via a siliconised stir bar) at 500rpm.

Aggregometers were calibrated using PRP (set at 0% light transmission) and platelet free plasma (PFP) (set at 100% light transmission) using the zero and output controls. PFP was prepared by centrifugation of platelet depleted plasma at 8000xg for 3 minutes in order to remove any remaining platelets. Aggregation was calculated as a percentage of the final

height of the trace over height of plateled depleted plasma, both relative to resting PRP (Figure 2.3). Lagtime$_{50}$ (LT_{50}) was defined as the time in minutes required to achieve 50% maximal aggregation (Figure 2.3). Platelet responses to mAb were analysed for up to 15 minutes, as has been recommended by previous studies investigating platelet-mAb interactions (Looney *et al.*, 1988; Tomiyama *et al.*, 1992).

2.10 Endothelial Cell Functional Assays

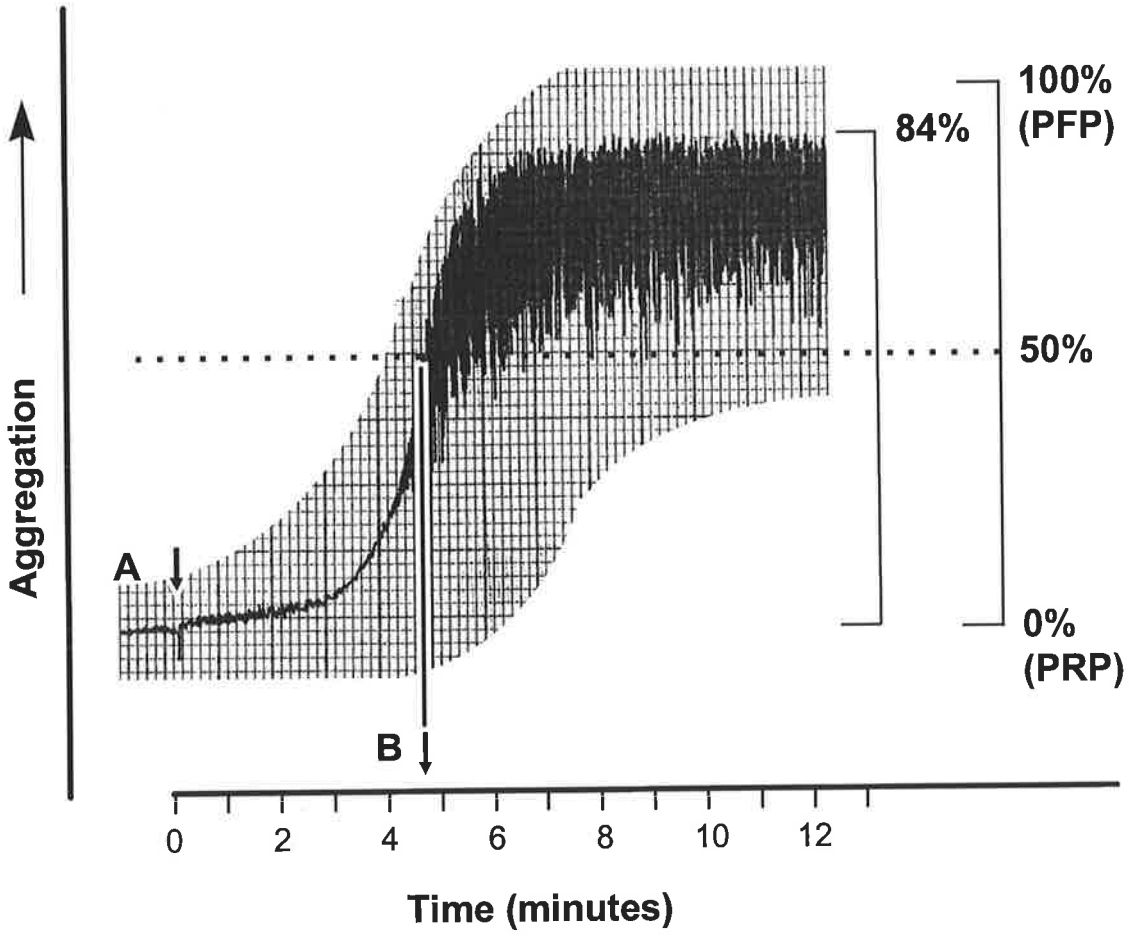
2.10.1 EC Motility Assay

Third passage HUVEC were aliquotted into gelatin coated 6 well plates (Corning) at $5x10^5$ per well in M199 medium with growth factors and heparin as described. After culture for 3 days, confluent monolayers were "wounded" with a 4mm latex scraper and the edge of the wounds marked. Monolayers were immediately washed to remove debris and complete medium with or without mAbs, as indicated, was added. Five randomly selected points along each wound were marked and the horizontal distance of migrating cells from the initial wound were measured using 200x magnification with a 50μm grid in the eyepiece at the times indicated. For each timepoint, measurements were taken from duplicate wells with three wounds per well (n=30). Perturbation of EC migration by mAbs was statistically analysed by 2-way analysis of variance (ANOVA). Treatment with mAbs 11B1.G4, 14A2.H1, 1AA2.H9 or 8A2 were compared with untreated, isotype matched binding and non-binding control mAbs. $P<0.05$ was considered statistically significant.

In order to determine whether mAb induced proliferation contributed to the movement of cells into the wound, total protein content of all wells was measured. At the conclusion of the experiment, wells were washed twice with cold PBS and the cells lysed with lysis buffer (1% NP 40, 50mM Tris-HCl, 150mM NaCl, 1mM EDTA, pH 8.0) for 2 hours at 4°C. Total protein content of all lysates was then measured using a Bradford Protein Determination Kit

Figure 2.3 Calculation of Percentage Platelet Aggregation and LT_{50}

Panel shows a representative platelet aggregation trace. The point at which agonist was added is shown by arrow (A). Aggregation was measured as increased light transmission in the vertical axis. Percentage aggregation was calculated as the height of the trace divided by the height of PFP (maximum transmission), in this case 84%. Both measurements were relative to PRP. LT_{50} was calculated as the time required for the trace to reach 50% of the height of PFP, as shown by arrow (B).


Aggregation
A
B
100%
(PFP)
84%
50%
0%
(PRP)
0
2
4
6
8
10
12
Time (minutes)

(Bio-Rad Mircoscience) in accordance with the manufacturer's instructions. Statistical analysis was performed by 2-tailed unpaired T-tests to compare the protein content of all mAb treated wells with that of the isotype matched control wells. $P<0.05$ was considered statistically significant.

2.10.2 Neutrophil Transendothelial Migration

Transendothelial migration assays were carried out using the methodology of Smith *et al.*, (1993). Transwells, 3μm pore size (Corning) were precoated with fibronectin (Boehringer Mannheim) at 50μg/ml for 30 minutes at room temperature before seeding with $5x10^4$ HUVEC per well in 150μl of M199 medium supplemented as described (Section 2.1.1). Transwells were then placed into 24-well trays (Lab Tek Nunc) containing 700μl of supplemented M199 medium. Cells were then cultured for 3 to 4 days at 37°C/5%CO_2 in air. Prior to assay, some HUVEC monolayers cultured in transwells were incubated with 100U/ml recombinant human TNF-α for 4 hours.

To prepare neutrophils, peripheral blood from normal healthy donors was collected into one-tenth volume 0.1M trisodium citrate, pH 7.35 as anticoagulant. Red blood cells were removed by the addition of 1/5 volume 5% dextran (Pharmacia, Cat. No. 17-0340-01). After incubation in dextran for 30 minutes, the upper layer was removed from sedimented red blood cells, placed into fresh 10ml tubes and underlayed with 4ml Lymphoprep (Nycomed Pharma). After centrifugation at 400xg for 25 minutes at room temperature, the plasma and buffy coat were aspirated leaving the pellet. The pellet was gently resuspended in 10ml 0.2% NaCl for 1 minute before the addition of 10ml 1.8% NaCl to remove any remaining red blood cells by hypotonic lysis. After washing twice in assay medium, purified neutrophils were counted using a haemocytometer and resuspended at $2x10^7$ cells/ml in assay medium.

Transwells were then washed with assay medium (M199 supplemented as described in Section 2.1.1, but containing only 2% FBS) and transferred to a 24-well tray precoated with gelatin and containing 600µl of assay medium. HUVEC were then treated with $F(ab')_2$ fragments for 10 minutes at 37°C in 5% CO_2 before addition of 10^6 neutrophils, purified as above. After 1 hour incubation at 37°C in 5%CO_2, migrated neutrophils were counted microscopically using a haemocytometer. Statistical analysis on the number of migrating neutrophils was performed by 2-tailed unpaired T-tests. 14A2.H1 and 11B1.G4 $F(ab')_2$ treatments were compared to untreated and isotype matched negative $F(ab')_2$ treatments. The same analysis was performed for EC pretreated with TNF-α. $P<0.05$ was considered statistically significant.

2.10.3 Matrigel Capillary Assay

In order to determine the optimum conditions for capillary formation, Matrigel, (Collaborative Biomedical Products, Bedford, MA, US, Cat. No. 40234) was titrated 1/3, 1/2, 1/1.5, 1/1 or undiluted in cold FCS-free M199 medium supplemented as described, but with the omission of EC growth factors and heparin. 100µl aliquots were then plated into flat bottomed 96 well microtitre plates and allowed to gel for 1-2 hours at 37°C. EC were then plated into each well at concentrations ranging from $2\text{-}6x10^4$/well in 150µl of complete M199 medium. The extent of tube formation was then assessed at 10 and 20 hours post plating. From these observations, it was concluded that Matrigel diluted 1/1.5 and EC plated at $2.5x10^4$/well were the optimum conditions for capillary formation. Therefore, all subsequent experiments were carried out using these concentrations. Matrigel, diluted 1/1.5 was prepared as described and EC were plated into each well at $2.5x10^4$/well in 150µl of complete M199 medium supplemented with 1-10µg/ml mAb as indicated. Tubes were defined as cellular extensions linking cell masses or branch points. These were quantitated from photographs of

duplicate wells at 10 and 20 hour time points. Both tube length and number of tubes were quantitated from photographs of the whole well by measuring tube lengths and counting tube numbers, as previously described (Gamble *et al.*, 1993). Statistical analysis was performed by 2-tailed unpaired T-tests for both tube length and number. MAb treatments were compared to both untreated and isotype matched non-binding controls for each time point and $P<0.05$ was considered statistically significant.

CHAPTER 3: DISTRIBUTION OF PETA-3 IN NORMAL HUMAN TISSUES

3.1 Introduction

Previous studies using mAb 14A2.H1 had demonstrated expression of PETA-3 on platelets, endothelium, some myeloid leukaemia cells and tonsil epithelium (Ashman *et al.*, 1991). Furthermore, investigation of PETA-3 mRNA distribution by northern blotting analysis showed expression in all tissue homogenates and cell lines examined with the exception of several lymphoid cell lines and the brain (Fitter *et al.*, 1995). However, since PETA-3 is expressed by endothelium it was unclear as to whether the vascular component of the tissues examined accounted for their positivity by northern analysis. Therefore the aim of this Chapter was to further investigate the cellular distribution of PETA-3 in normal tissues by immunohistochemical techniques.

Characterisation of the cell types expressing PETA-3 may provide valuable insight as to the potential function of this molecule as restriction to a particular lineage would indicate a cell specific role, whilst a broad distribution throughout many cell types would imply a fundamental role in cell biology. Based on the Northern analysis data, the latter appeared more likely, however the possibility of ubiquitous expression of PETA-3 by endothelium needed to be assessed. Finally, the subcellular localisation of PETA-3 within individual cells might also provide evidence towards a possible function.

Earlier studies have shown PETA-3 and several other members of the TM4SF, CD9, CD63 and CD81 to associate with each other and β1 integrins to form tetraspan/integrin and tetraspan/tetraspan/integrin complexes (Fitter, Sincock, Jolliffe and Ashman, submitted; Rubinstein *et al.*, 1994; Berditchevski *et al.*, 1996; Berditchevski *et al.*, 1995). These immunoprecipitation studies had shown PETA-3 to associate with β1 integrins, CD9 and CD63 in both megakaryocytic (M-07e) and human erythroleukaemic (HEL) cells (Fitter, *et*

al., submitted). Additionally, CD9 was found to be associated with α4β1 and α5β1 in the pre-B cell line NALM-6, while HEL cells, the association was observed for α4β1 only (Rubinstein *et al.*, 1994). CD63 has been reported to associate with α3β1 and α6β1 integrins in HT1080 fibrosarcoma cells and in transfected K562 cells expressing either the α3 or α6 chain (Berditchevski *et al.*, 1995). The specificity of TM4SF interactions with β1 integrins remains unclear as many TM4SF members investigated showed co-immunoprecipitation with α3β1, yet also specifically interact with other αβ heterodimers. Also, during the course of this study, several reports demonstrated TM4SF associations with integrins of both β1 and other β subfamilies, including β2, β3 and β7 integrins (Section 1.2.5).

Most of the studies to date have examined the association of TM4SF members with integrins using cell line and transfectant models. Therefore the characterisation of these interactions in primary cells and tissues is required to determine the *in vivo* significance of such complexes. Recently it was shown that CD9, CD63, CD81 and α3β1 both co-immunoprecipitated from cell lines and co-localised in some cells in tissue sections of skin, breast, tonsil and colon (Berditchevski *et al.*, 1996). Colocalisation of TM4SF members with integrin chains in primary tissues strongly supports the existence of TM4SF/integrin complexes *in vivo*.

Thus the association of PETA-3 with β1 integrins, CD9 and CD63 demonstrated by biochemical studies in cell lines, suggests that these antigens should be co-expressed in particular tissues. Therefore mAbs against CD9, CD63 and the integrin β1 chain were also examined in parallel to PETA-3. This Chapter reports both the localisation of PETA-3 and the extent of this molecules co-localisation with CD9, CD63, and the β1 integrin chain in normal human tissues.

3.2 Immunohistochemical Analysis of PETA-3, CD9, CD63 and β1 Integrin Distribution

3.2.1 Negative Control Staining of Normal Tissues

All tissue sections were examined for expression of PETA-3 using mAbs 11B1.G4 and 14A2.H1 by immunohistochemistry. Adjacent sections were also stained with mAbs against CD9 (1AA2.H9), CD63 (12F12) and the β1 integrin chain (61.2C4). Isotype matched negative control mAbs 1B5 (IgG1) and 1D4.5 (IgG2a) were also included in all experiments as specificity controls. (For description of mAbs used in these experiments see Section 2.3.1)

Tissue sections, prepared as described (Section 2.2), were fixed in cold 95% ethanol and stained using the indirect immunoperoxidase technique as described (Section 2.4.1). Apart from the endogenous peroxidase activity of eosinophils, alveolar macrophages and Kupffer cells, no cells in any of the tissues examined stained with the negative control mAbs using the indirect immunoperoxidase technique. One exception was tonsillar follicular dendritic cells which did show low levels of binding of the IgG2a control mAb, 1D4.5 (see below).

Due to the endogenous peroxidases of haemopoietic cells, the indirect immunoperoxidase technique was not suitable for staining of these cell types. Therefore, to study the expression of TM4SF members and β1 integrin in these cells the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used (Section 2.4.2). Again, isotype matched negative control mAbs 1B5 (IgG1) and 1D4.5 (IgG2a) were included as specificity controls. No staining of peripheral blood or bone marrow cells was observed with these negative control mAbs using the APAAP technique.

The following descriptions are of PETA-3, CD9, CD63 and β1 integrin localisation within the tissues examined, the results obtained are summarised in Table 3.1.

Table 3.1 Localisation of PETA-3, CD9, CD63 and the β1 Integrin Chain in Normal Human Tissues

Tissue sections and blood smears were prepared and stained by immunohistochemical techniques as described (Section 2.4). The level of staining was scored from + (weakly positive) to +++++ (strongly positive).

Abbreviations used; MNC: Mononuclear Cells, IEL: Intraepithelial Lymphocytes, HEV; High Endothelial Venules

Tissue	PETA-3 (11B1.G4)	PETA-3 (14A2.H1)	CD9 (1AA2.H9)	CD63 (12F12)	β1 Integrin (61.2C4)
Vasculature					
arteries	+++++	++	++++	++	+++++
veins	+++++	++	++++	++	+++++
capillaries	++++	+++	+++	++	++++
perivascular smooth muscle	+++++	++++	+++++	+/-	+++++
Peripheral Blood					
red blood cells	-	-	-	-	-
lymphocytes	-	-	+/-	-	+
neutrophils	-	-	-	+++++	+++
platelets	++++	+/-	+++++	+++	+++++
Bone Marrow MNC					
megakaryocytes	+++++	+	+++++	++++	++++
Tonsil					
squamous epithelium	++	-	+++++	++	+++++
germinal centre	++++	++	+++	+++	++
HEV	++++	+++	++++	+++	+++++
Spleen					
white pulp	+	-	+	+	+
red pulp	+++	+/-	+++	+++	+++++
central arteries	+++++	++++	+++++	+/-	+++++
Skin					
stratum basale	++++	+	++++	-	+++++
stratum spinosum	+/-	-	+++++	+/-	+
stratum granulosum	-	-	+++++	+/-	-
Connective Tissue					
fibroblasts	-	-	++++	+++	++
Gut					
epithelium	+++	+/-	+++	++	+++
lamina propria	+++	++	+++++	+	+++++
IEL	-	-	+	++	+
Kidney					
glomeruli	+++++	++	++	+++	+++++
proximal/distal tubules	++	-	-	+++	+++
collecting ducts	+	+/-	+++++	+++	++++
Peripheral nerve					
perineurium	+++++	+++	+++++	+	+++++
endoneurium	-	-	-	-	-
Schwann cells	++++	+++	++++	+	++
Skeletal Muscle	+++	+/-	++	+	++++
Cardiac Muscle	+++++	+++	-	++	+++++
Cerebral Cortex					
vasculature	++++	++	++++	+++	+++++
grey matter	-	-	+	-	-
white matter	-	-	+++++	-	-
Lung	+++++	+++	++++	++	+++++
Liver					
hepatocytes	+	-	+	-	-
bile ducts	++++	+++	++++	+++	++++
sinusoids	+++++	++	+	++++	+++
Adrenal					
cortex	++	-	++	+++++	++++
Pancreas					
acini	+	-	-	+++++	-
ducts	+++	+	+	++	++
Thyroid					
follicular cells	+	+/-	++	++++	-
parafollicular cells	+++	+/-	++	+++	+++

3.2.2 Vasculature

The vascular endothelium was stained strongly with both mAbs against PETA-3 (Figure 3.1.A, and see below). Perivascular smooth muscle and several connective tissue components were also positive (Figure 3.1.A). PETA-3 was also expressed by specialised endothelium, including tonsil high endothelial venules (HEV) (Figure 3.1.B), central arterioles of the spleen (Figure 3.1.C), and the blood brain barrier (see below). CD9 and β1 integrin were also ubiquitously expressed by vascular endothelium, perivascular smooth muscle and connective tissue components. CD63 was also identified on endothelium and connective tissue components, however it was not readily detected on smooth muscle or central arterioles of spleen.

3.2.3 Peripheral Blood

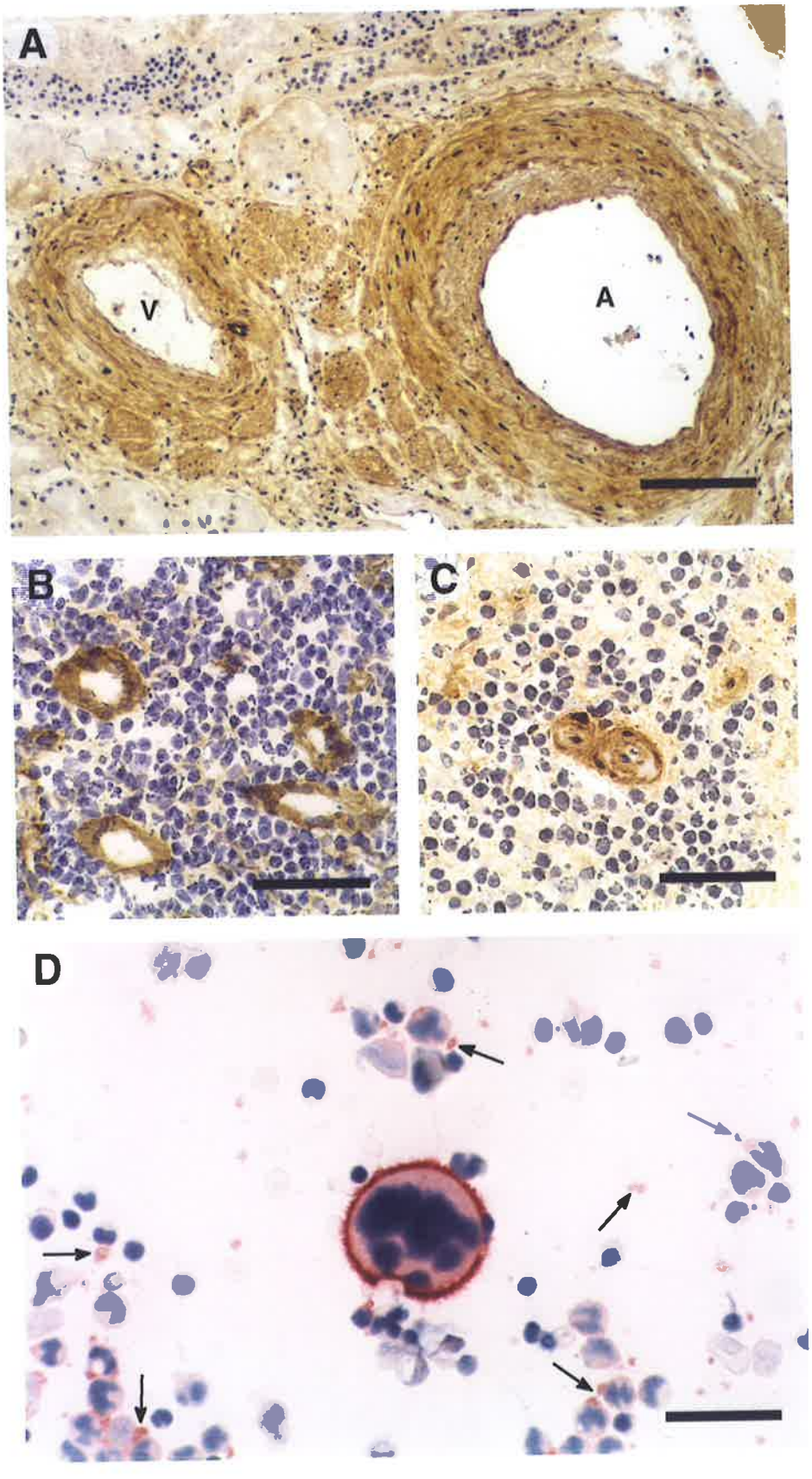
Staining of peripheral blood smears showed PETA-3 expression to be restricted to platelets. The 11B1.G4 mAb showed very strong staining, whilst 14A2.H1 stained platelets very weakly. Other cells such as lymphocytes and neutrophils failed to stain with either of the anti-PETA-3 mAbs used in this study. MAbs against CD9, CD63, and β1 integrin also stained platelets. Additionally, several other haemopoietic cell types including neutrophils, (see Table 3.1) were stained by mAbs against CD9, CD63 and β1 integrin, as previously described (Barclay *et al.*, 1991).

3.2.4 Bone Marrow Mononuclear Cells

Staining of the bone marrow mononuclear fraction demonstrated PETA-3 expression on cells of the megakaryocytic lineage. Differentiated megakaryocytes stained very strongly with 11B1.G4 (Figure 3.1.D) and weakly with 14A2.H1. As expected, platelets were also stained by both anti-PETA-3 mAbs. Immature cells of the neutrophil lineage, also showed

Figure 3.1 PETA-3 Expression by Endothelium, Perivascular Smooth Muscle, Megakaryocytes and Platelets

Panel (A) shows 11B1.G4 staining of a small artery and vein within kidney using the indirect immunoperoxidase technique (Section 2.4.1). 11B1.G4 stained the endothelium of both vessels. Furthermore staining was associated with perivacular smooth muscle. Weaker staining of epithelium associated with tubules was also apparent. (B) 11B1.G4 staining of tonsil HEV. (C) Staining of a central arteriole within spleen by 11B1.G4. Panel (D) shows APAAP staining (Section 2.4.2) of BMMNC with mAb 11B1.G4. The representative megakaryocyte shown in the centre of the field stained very strongly. Platelets (arrows) were also stained with 11B1.G4. Several other BMMNC showed lower levels of staining with 11B1.G4 which, based on their morphology, appear to be of the polymorphonuclear lineage. Bars; (A) 400μm, (B) 80μm, (C) 80μm, (D) 40μm. A - Artery, V - Vein.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weak staining with mAb 11B1.G4. Infrequent small cells were also weakly stained and further studies would be required to determine their nature. All of the other mAbs tested stained cells of the megakaryocytic lineage as well as many other cell types which were not further characterised. The expression of CD9, CD63 and β1 integrin by haemopoietic cells has been described elsewhere (Barclay *et al.*, 1991).

3.2.5 Tonsil

Squamous epithelium, germinal centres and high endothelial venules (HEVs) were stained by 11B1.G4 (Figure 3.2). Follicular dendritic cells within germinal centres were also stained by all other mAbs tested (Figure 3.3). Staining of these cells was weak for CD63 and stronger for PETA-3, CD9 and the β1 integrin chain. Although some staining of follicular dendritic cells was seen with the IgG2a negative control, 1D4.5, the staining by anti-PETA-3 mAb 11B1.G4 (IgG2a) was much stronger. Additionally the IgG1 anti-PETA-3 mAb, 14A2.H1, stained tonsillar follicular dendritic cells, thus verifying the specificity of staining. Expression of PETA-3, CD63 and β1 integrin by squamous epithelial cells was restricted to the basal layers, while strong staining for CD9 was observed by epithelial cells throughout the epithelium (Figure 3.4). Lymphocytes within both T and B cell zones were not stained by any of the mAbs tested. HEVs were stained moderately by anti-CD63 and were strongly positive for PETA-3 (Figure 3.1.B), CD9 and the integrin β1 chain.

3.2.6 Spleen

Within adult spleen, lymphocytes in the white pulp were unstained by all mAbs. Low level staining was seen with anti-PETA-3, CD9, CD63 and β1 mAbs in the region of periarteriolar lymphoid sheaths and white pulp, which may have been staining of interdigitating cells. Central arterioles were stained strongly by anti-β1 mAb and less strongly

Figure 3.2 Localisation of PETA-3 in Tonsil

(A) Low power view of 11B1.G4 staining in human tonsil. (B) Isotype matched negative control staining of tonsil. For comparison with other field, morphological features are labelled as follows; F - follicle, C - connective tissue, E - squamous epithelium. Bar; 400μm.

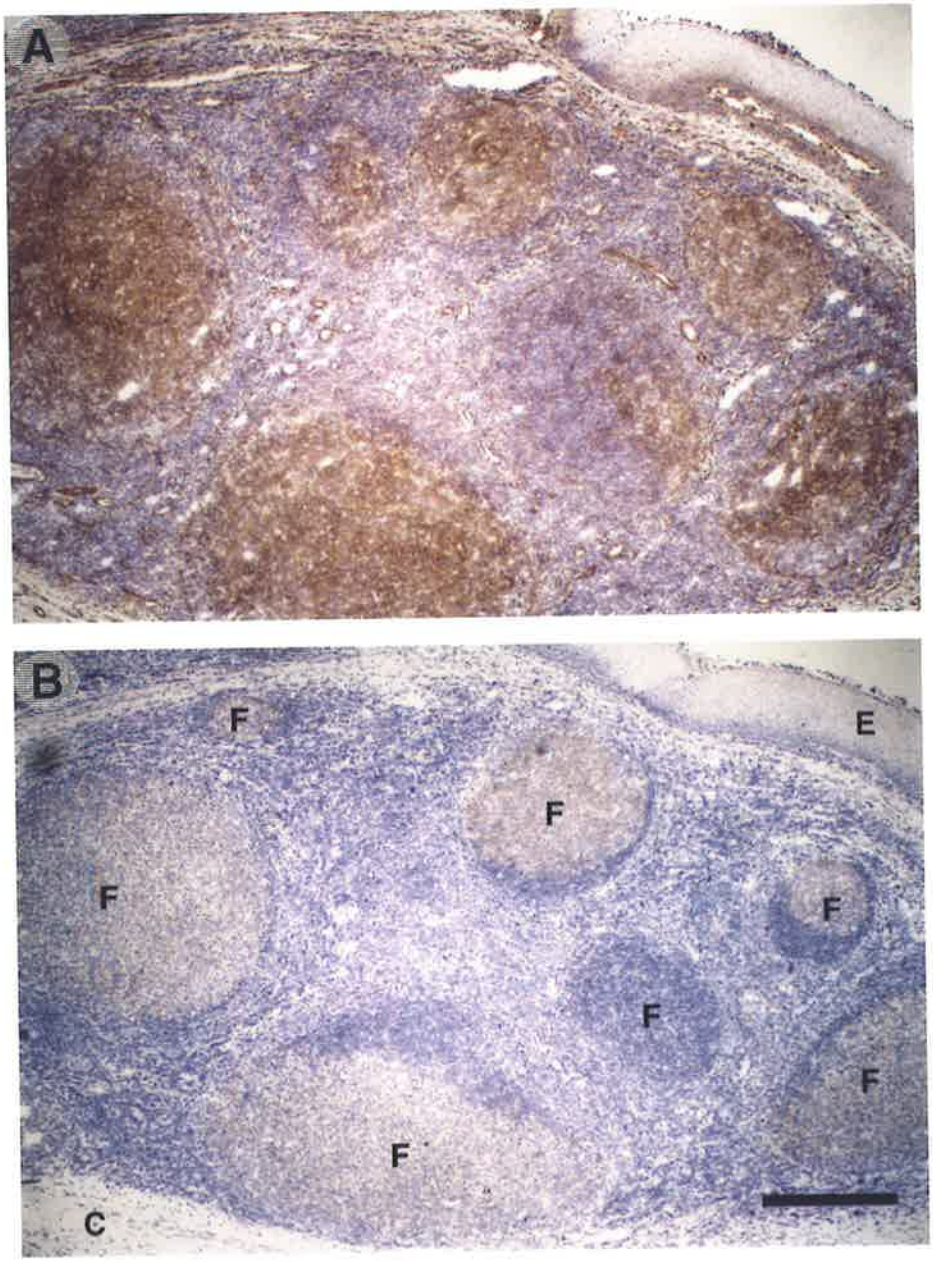
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Figure 3.3 Localisation of TM4SF Members and β1 Integrin in Tonsil Follicles

(A) High power view of 11B1.G4 and (B) 14A2.H1 staining of a B cell follicle within tonsil. (C) CD9 and (D) CD63 antibodies stained tonsil follicles. (E) The anti-β1 mAb also stained B cell follicles. (F) The IgG2a negative control mAb showed weak staining of cells within tonsil follicles. Bar; 40μm.

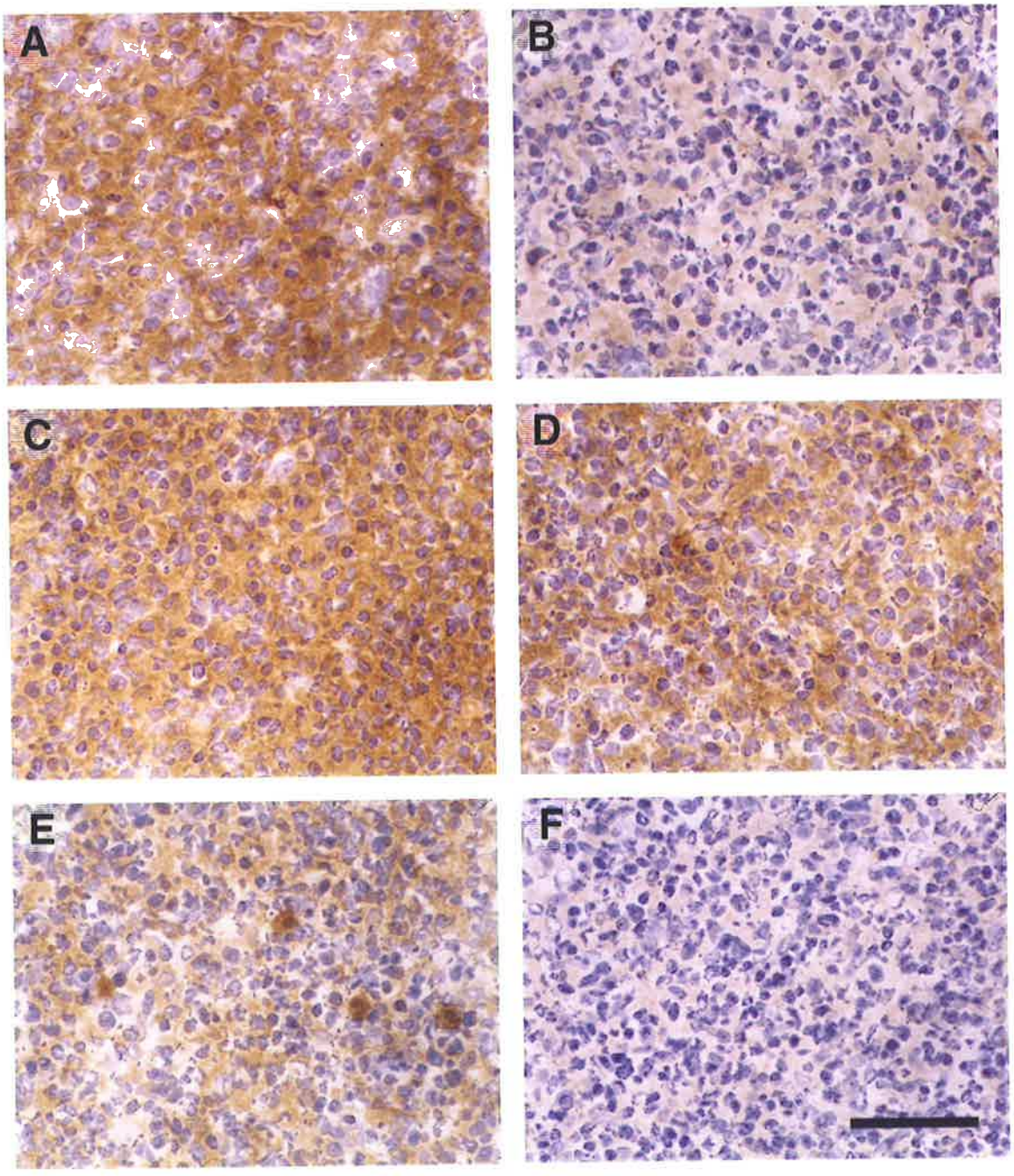
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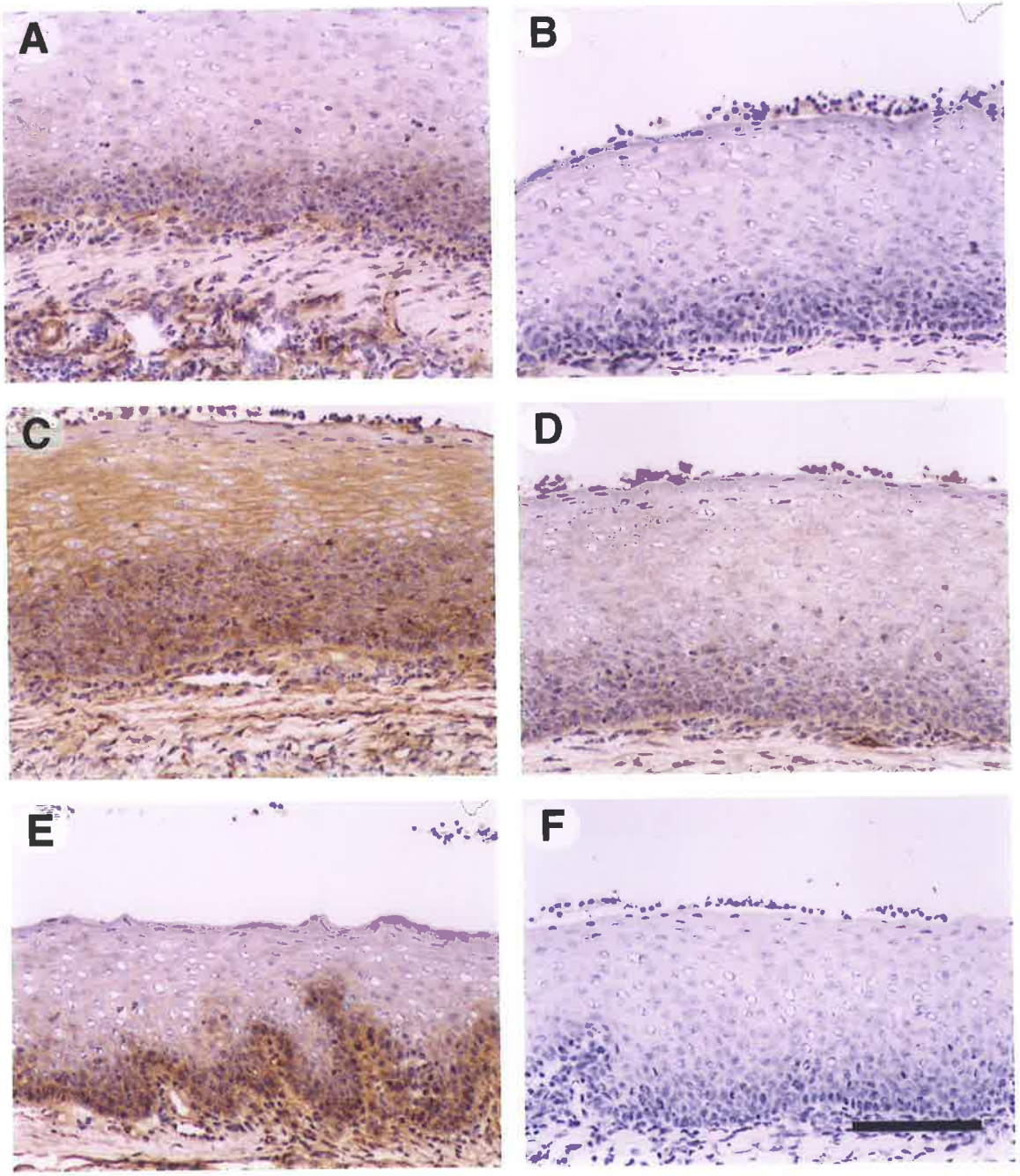
Figure 3.4 Localisation of TM4SF Members and β1 Integrin in Squamous Epithelium of Tonsil

Lower power view showing staining of squamous epithelium with the respective mAbs, (A) 11B1.G4, (B) 14A2.H1, (C) CD9, (D) CD63, (E) β1 integrin. (F) Isotype matched negative control. Note the basal distribution of 11B1.G4, CD63 and β1 integrin staining. CD9 was expressed throughout the epithelium, while staining with the anti-PETA-3 mAb 14A2.H1 was not readily detected. Bar; 80μm.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by mAbs against PETA-3 (Figure 3.1.B) and CD9. Staining of central arterioles by anti-CD63 was barely detectable. Red pulp sinusoids and splenic cords were also stained by mAbs against PETA-3, CD9, CD63 and the β1 chain (data not shown).

3.2.7 Skin

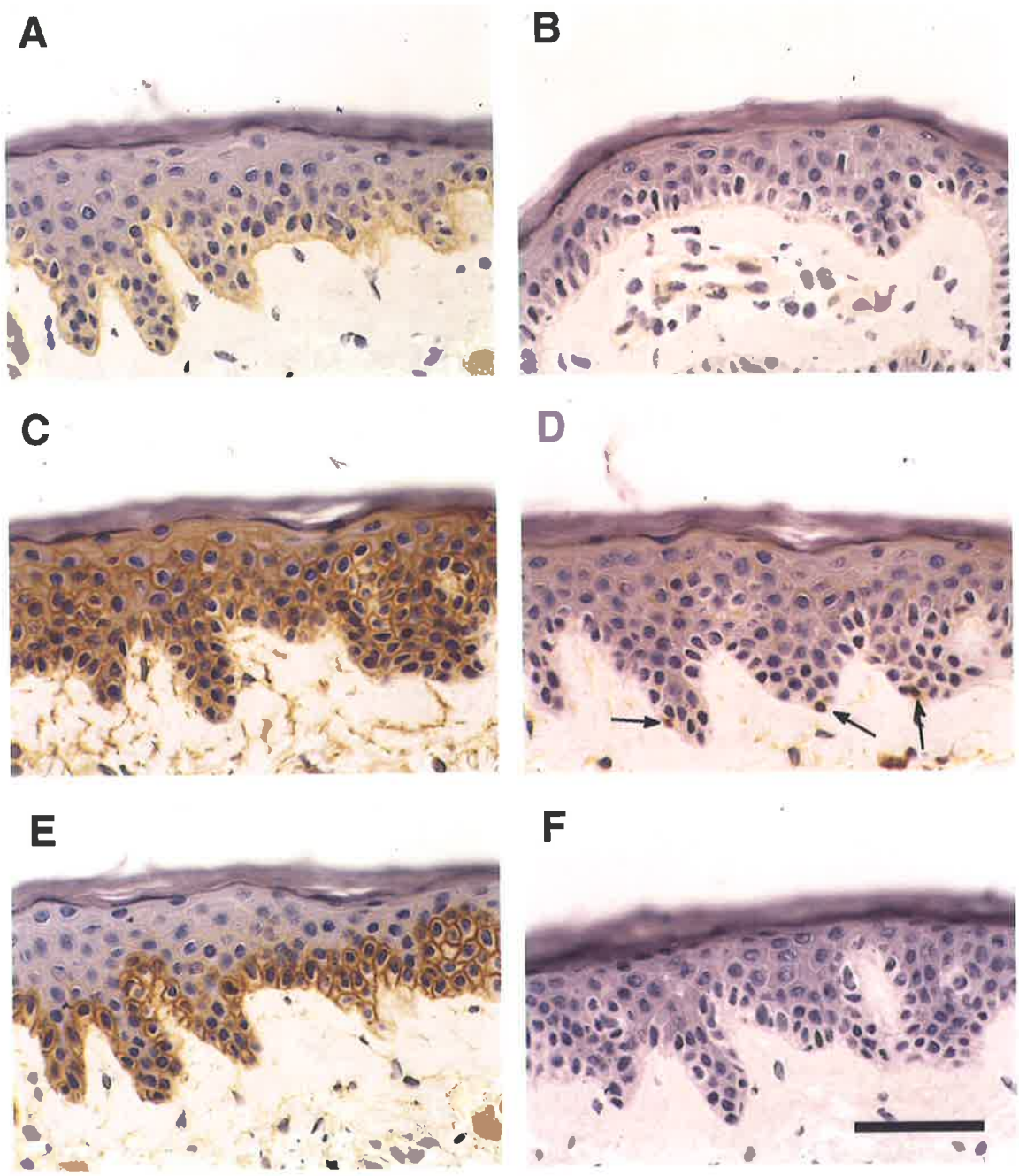
PETA-3 was expressed on the cell membrane of basal keratinocytes. Expression was strongest on the membrane adjacent to the basal lamina and was detected by 11B1.G4 (Figure 3.5.A) and weakly by 14A2.H1 (Figure 3.5.B). Anti-CD9 mAb produced intense staining of keratinocytes throughout all layers of the epidermis (Figure 3.5.C). Furthermore, unlike PETA-3, CD9 staining of basal keratinocytes was not detected on the cell membrane of that contacts the basal lamina. Anti-CD9 mAb also stained components of the dermis. Anti-CD63 stained keratinocytes throughout the epidermis very weakly, although melanocytes stained strongly as has been previously described (Hotta *et al.*, 1988) (Figure 3.5.D). The β1 integrin chain was also highly expressed on the membrane of basal keratinocytes with stronger staining of lateral and apical membranes (Figure 3.5.E).

3.2.8 Connective Tissue

Within the dense connective tissue of the deep dermis, fibroblasts were stained by mAbs to CD9, CD63 and the β1 integrin chain (data not shown). PETA-3 was not detected on fibroblasts. In the superficial dermis, cells with irregular processes were stained strongly by anti-CD63 mAb and at lower levels by mAb against PETA-3, CD9 and β1. Although these cells may be macrophages and/or mast cells other histochemical techniques would be required to confirm their identity.

Figure 3.5 Localisation of TM4SF Members and β1 Integrin in Skin

(A) Staining of basal keratinocytes by anti-PETA-3 mAb 11B1.G4. Note the more intense staining adjacent to the basal lamina. Lateral and apical membranes of basal keratinocytes were also stained. (B) Staining of the basal membrane of basal keratinocytes was also seen using 14A2.H1, although much weaker. (C) In contrast, CD9 was strongly expressed by keratinocytes throughout the epidermis and was not readily detected on the membrane in contact with the basal lamina. Additional staining for CD9 was observed within the dermis. (D) CD63 was not detected on keratinocytes, however melanocytes (arrows) displayed intense staining. (E) MAb against the β1 integrin chain strongly labelled the apical and lateral membranes of basal keratinocytes. β1 integrin was not detected within differentiated cells. For comparison, panel (F) shows a representative field of isotype matched negative control mAb staining. Bar; 40μm.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3.2.9 Small Intestine

PETA-3 was expressed on the basolateral membranes of the columnar epithelial cells of the villi (Figure 3.6.A) and crypts, and 14A2.H1 again showed weaker staining (Figure 3.6.B). which was almost undetectable on the epithelia of the crypts. Smooth muscle cells within villi and the muscularis mucosa were also positive for PETA-3 and showed a similar level of staining for both mAbs. CD9 was expressed on the basolateral membrane of immature enterocytes in the crypts and on the bases of the villi, however expression was lost upon enterocyte differentiation. (Figure 3.6.C) The anti-CD9 mAb also stained smooth muscle. CD63 was detected on crypt and villous epithelium but, in contrast to PETA-3 and CD9, CD63 had a granular appearance and was localised to the apical cytoplasm below the brush border (Figure 3.6.D). No CD63 was detected on enterocyte lateral membranes, however staining was observed throughout the lamina propria. β1 integrins were present on the basolateral membranes of epithelium in crypts and on the villi, however the anti-β1 mAb also stained the basal cytoplasm diffusely (Figure 3.6.E). Smooth muscle cells within villi and the muscularis mucosa were also strongly positive for β1 integrin. Staining of some intraepithelial lymphocytes (IEL) with mAbs against CD9, CD63 and β1 integrin was also observed.

3.2.10 Kidney

Strong staining with 11B1.G4 was observed on periarteriolar smooth muscle and EC of blood vessels and glomeruli (Figures 3.1.A, 3.7.A). Although weaker, the 14A2.H1 mAb also gave a similar pattern of staining (Figure 3.7.B). Epithelium of the proximal and distal convoluted tubules was also weakly stained with 11B1.G4. Staining of glomerular endothelium was weaker for CD9, however the anti-CD9 mAb strongly stained epithelial cells of collecting ducts (medullary rays), (Figure 3.7.C). CD63 staining of glomerular

Figure 3.6 Localisation of TM4SF Members and β1 Integrin in Small Intestine

(A) Anti-PETA-3 mAb 11B1.G4 stained the basolateral membranes of enterocytes on villi. Within the lamina propria, smooth muscle and capillaries were also stained. (B) Although staining of smooth muscle within the lamina propria was observed, expression of PETA-3 by villous enterocytes was weakly detected by mAb 14A2.H1. (C) CD9 was predominantly expressed on the basolateral membranes of enterocytes and expression was lost by more differentiated epithelial cells (ascending the villus). (D) CD63 was localised in a granular distribution below the brush border. (E) β1 integrins showed a localisation similar to that of PETA-3, although staining of the basal region appeared more diffuse. (F) Representative isotype matched negative control. Note the endogenous peroxidase activity of eosinophils within the lamina propria (arrows), which is also apparent in other fields. For comparison with other field, morphological features are labelled as follows; E - Enterocytes, LP - lamina propria. Bar; 40μm.

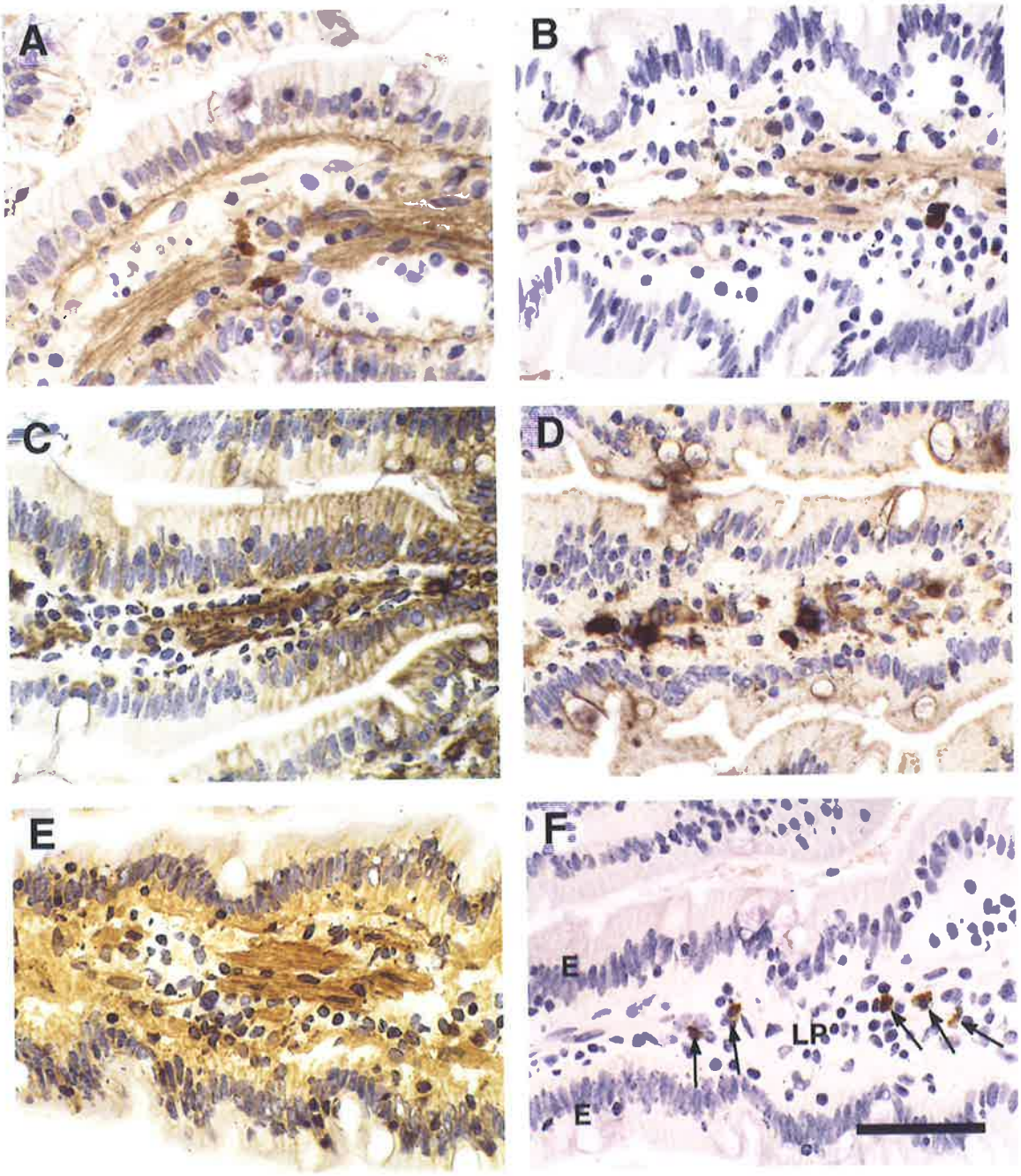
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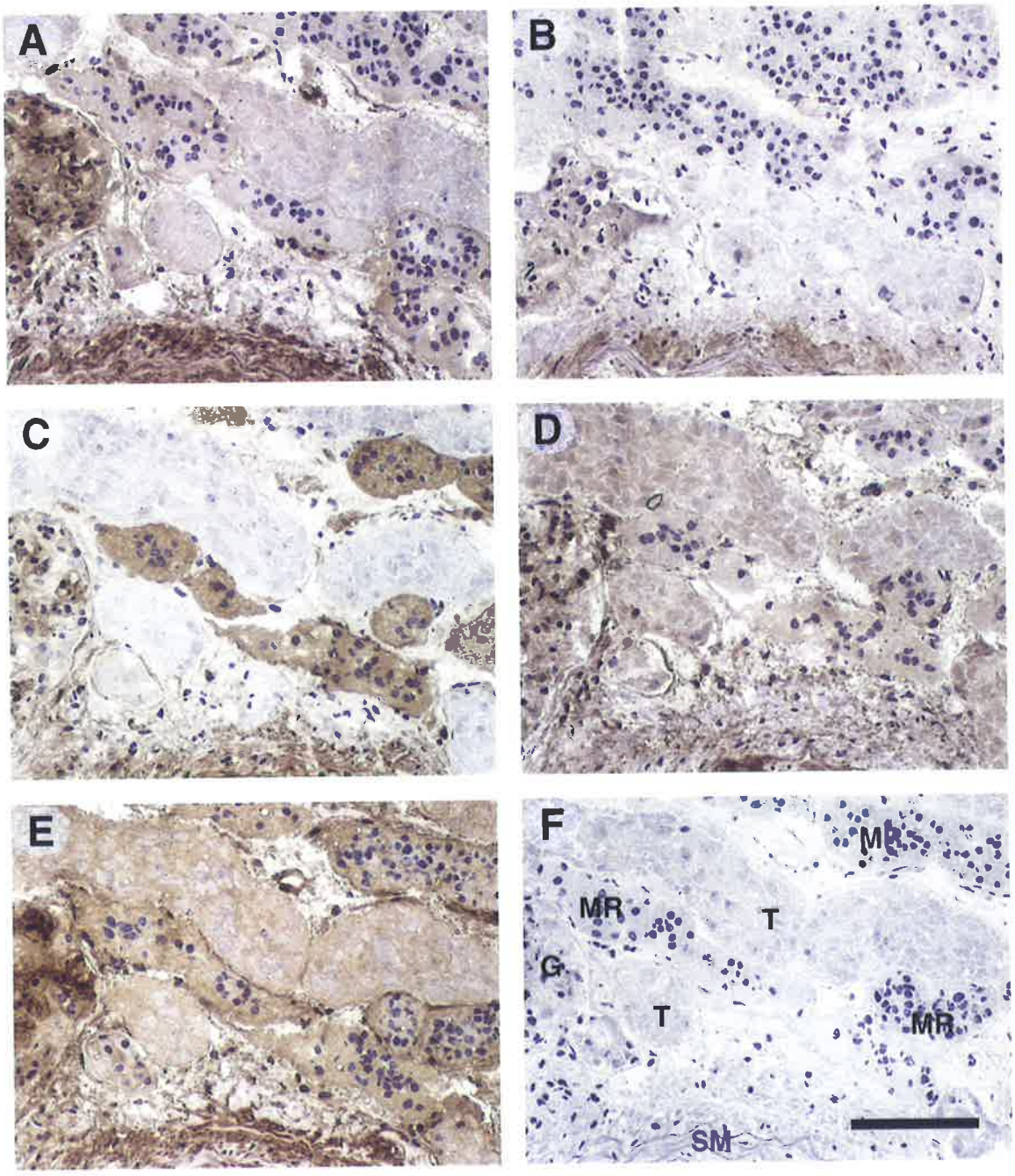
Figure 3.7 Localisation of TM4SF Members and β1 Integrin in Kidney

(A) Expression of PETA-3 in kidney glomeruli and epithelium was detected by mAb 11B1.G4. Glomeruli were strongly stained, whilst epithelium of tubules was much weaker. (B) Although 14A2.H1 also stained glomeruli, PETA-3 expression on epithelial cells of proximal and distal convoluted tubules and collecting ducts was not detected. Note the expression of PETA-3 by perivascular smooth muscle at the bottom of panels (A) and (B). (C) Staining of glomerular endothelium was weaker for CD9, and was not observed on proximal or distal tubules. However the anti-CD9 mAb strongly stained epithelial cells of collecting ducts, (medullary rays). (D) CD63 staining of glomerular endothelium was observed and epithelium of proximal/distal tubules and collecting ducts showed moderate levels of staining. (E) The Anti-β1 integrin mAb strongly labelled the endothelium of glomeruli and epithelium of the various tubules and ducts. (F) Representative field showing isotype matched negative control labelling of kidney section. For comparison with other fields, morphological features are labelled as follows; G - glomerulus, T - tubules, MR - Medullary Ray (collecting ducts), SM - Perivascular Smooth Muscle. Bar; 40μm.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endothelium was also weaker, however tubules showed a moderate staining (Figure 3.7.D). Additionally, CD63 was also detected on collecting ducts. Staining with the anti-β1 integrin mAb was strong and showed nearly ubiquitous expression throughout endothelium and epithelium of the various tubules and ducts (Figure 3.7.E).

3.2.11 Peripheral Nerve and Skeletal Muscle

Peripheral nerve fibres were identified within transverse sections of skeletal muscle. The perineurium of peripheral nerve fascicles was strongly positive for PETA-3, although 14A2.H1 staining was weaker (Figures 3.8.A, 3.8.B). CD9 (Figure 3.8.C), CD63 (Figure 3.8.D) and β1 integrin (Figure 3.8.E) mAbs also stained the perineurium, although CD63 staining was weaker. The myelin sheaths of Schwann cells were also positive for mAbs against PETA-3 and CD9, but showed weaker levels of staining for CD63 and the β1 integrin chain. Transverse sections of skeletal muscle showed that PETA-3 (Figure 3.8.A), CD9 (Figure 3.8.C), CD63 (Figure 3.8.D) and β1 integrin (Figure 3.8.E) were expressed on the sarcolemma of striated muscle cells. Staining of PETA-3 on the sarcolemma of muscle cells by 14A2.H1 was not readily detected (Figure 3.8.B).

3.2.12 Heart

Cardiac muscle showed staining for PETA-3 using both 11B1.G4 and 14A2.H1 mAbs (Figure 3.9.A, 3.9.B). Additional diffuse cytoplasmic staining of muscle cells and staining of capillaries was also observed with these mAbs. Interestingly, CD9 was restricted to the endothelium of capillaries only (Figure 3.9.C). Although weaker, anti-CD63 staining was very similar to that of PETA-3, with stronger cytoplasmic staining observed (Figure 3.9.D). Anti-β1 integrin mAb strongly stained the sarcolemma of muscle cells and capillary endothelium with diffuse cytoplasmic staining also apparent (Figure 3.9.E).

Figure 3.8 Localisation of TM4SF Members and β1 Integrin in Peripheral Nerve and Skeletal Muscle

(A) Anti-PETA-3 mAb 11B1.G4 stained capillary endothelium and the sarcolemma of muscle cells. The perineurium and myelin sheaths of nerve fibres in peripheral nerve bundles were also strongly stained. (B) 14A2.H1 failed to detect PETA-3 expression on the sarcolemma of muscle cells. (C) Anti-CD9 mAb strongly stained the perineurium and myelin sheaths of nerve fibres. Although weaker than the staining observed with 11B1.G4, CD9 was also detected on the sarcolemma. (D) CD63 was detected on the sarcolemma of muscle cells, however staining of perineurium and myelin sheaths was weak. (E) β1 integrin mAb strongly stained perineurium and the sarcolemma of muscle cells. In contrast to CD9 and PETA-3, β1 integrin was not strongly detected on Schwann cells. (F) Isotype matched negative mAb staining of skeletal muscle. For comparison with other fields, morphological features are labelled as follows; P - perineurium, E - endoneurium, M - muscle cell, Arrows indicate myelin sheaths of nerve fibres. Bar; 40μm.

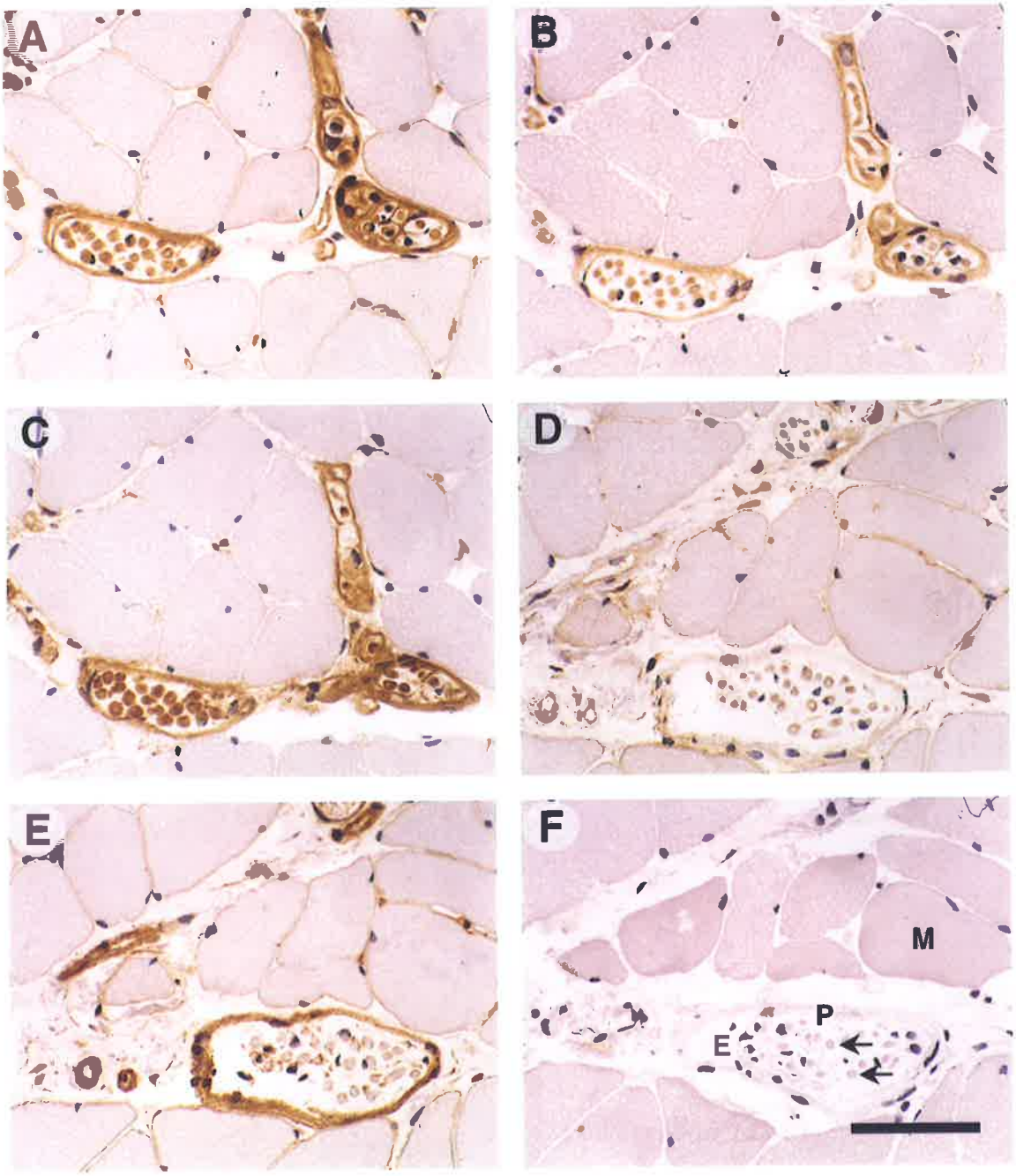
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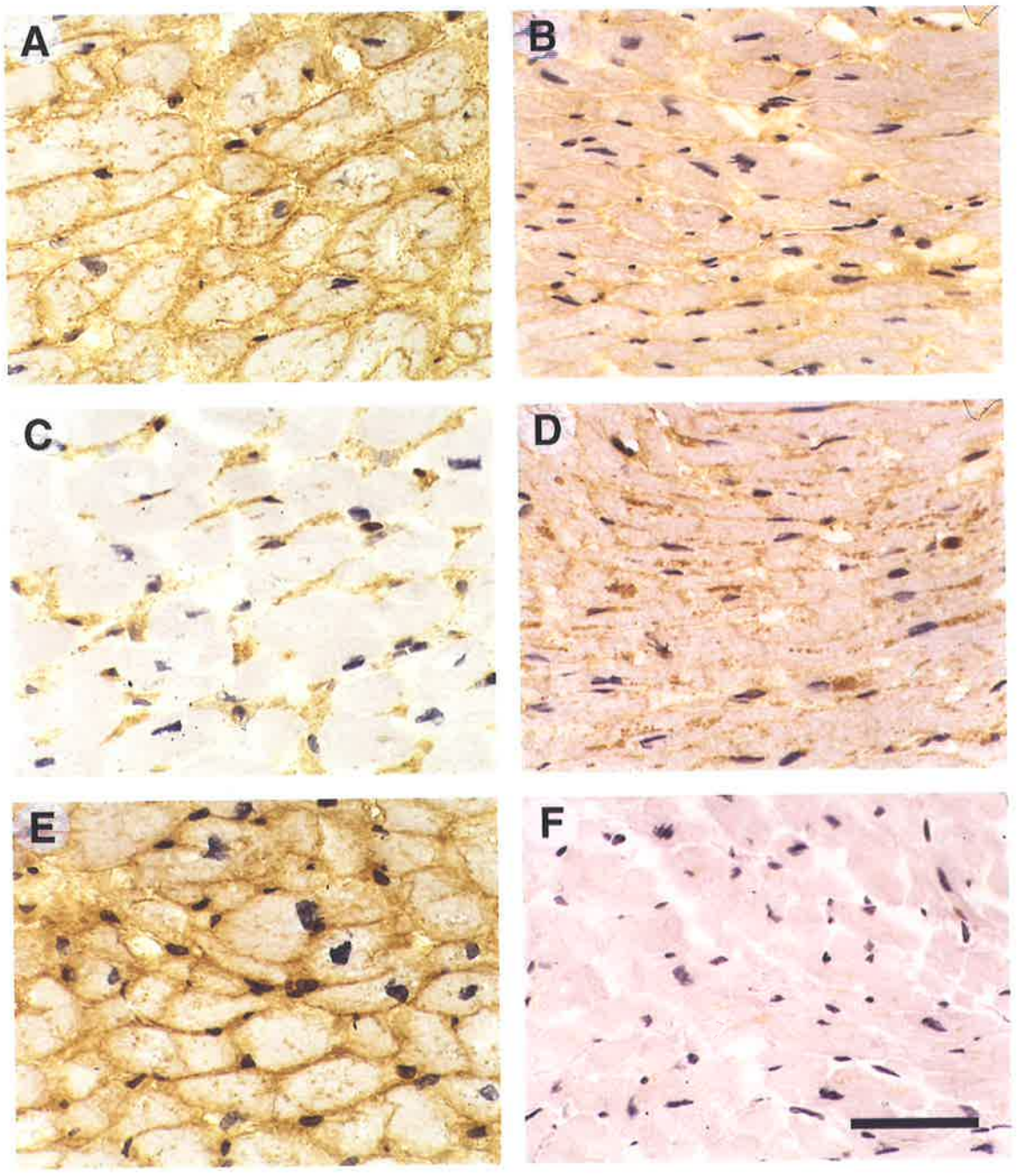
Figure 3.9 Localisation of TM4SF Members and β1 Integrin in Cardiac Muscle

(A) PETA-3 expression by cardiac muscle was detected by mAbs 11B1.G4 and (B) 14A2.H1. Capillaries were also stained by both these mAbs. (C) CD9 was restricted to the endothelium of capillaries only. (D) Staining of muscle cells by anti-CD63 mAb was granular and appeared perinuclear. (E) β1 integrin was strongly expressed by capillary endothelium and cardiac muscle cells. (F) Isotype matched negative mAb staining of cardiac muscle. Bar; 40μm.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3.2.13 Cerebral Cortex

Vascular endothelium was positive for PETA-3, although 14A2.H1 appeared to stain larger vessels only (Figures 3.10.A, 3.10.B). Nerve bodies in the grey matter and myelinated fibres in the white matter were not stained. Anti-CD9 mAb also stained endothelium and in addition, the white matter showed strong diffuse staining of the neuropil as has been previously described (Rossler *et al.*, 1992) (Figure 3.10.C). Anti-CD63 (Figure 3.10.D) mAb stained blood vessels only. The anti-β1 integrin (Figure 3.10.E) mAb strongly stained the endothelium of all blood vessels.

3.2.14 Lung

Within the lung parenchyma, smooth muscle associated with bronchi, bronchioles and blood vessels were stained strongly by mAb against PETA-3 (Figure 3.11.A), CD9 and the β1 integrin chain. Similarly epithelium and alveolar pneumocytes were also stained by these mAbs. CD63 was also present on pneumocytes and airway epithelium but staining was less intense than with the other mAbs. EC of alveolar capillaries and larger blood vessels were stained by all mAbs tested, with the exception of negative control mAbs. Alveolar macrophages showed some staining with negative control mAbs, thus the specificity of staining on these cells could not be determined.

3.2.15 Liver

Endothelium in hepatic sinusoids stained strongly positive for PETA-3 (Figure 3.11.B), CD63 and β1 integrin chain whilst CD9 was much weaker. Columnar epithelium of intrahepatic bile ducts was stained with all mAbs examined, with the exception of negative controls. The cell membranes of hepatocytes also showed weak staining by mAbs against

Figure 3.10 Localisation of TM4SF Members and β1 Integrin in Cerebral Cortex

PETA-3 expression was limited to endothelial cells of blood vessels in both grey and white matter of the cerebral cortex. (A) 11B1.G4 stained all vessels. (B) In contrast, 14A2.H1 staining was restricted to larger vessels. (C) CD9 was broadly expressed throughout the neuropil of white matter. Inset shows CD9 staining of extravascular cellular processes within grey matter. (D) Staining for CD63 was restricted to blood vessels. (E) The β1 integrin chain was strongly expressed by endothelial cells of all vessels within the cerebral cortex. (F) Representative isotype matched negative control staining of cerebral cortex. Bar; 80μm.

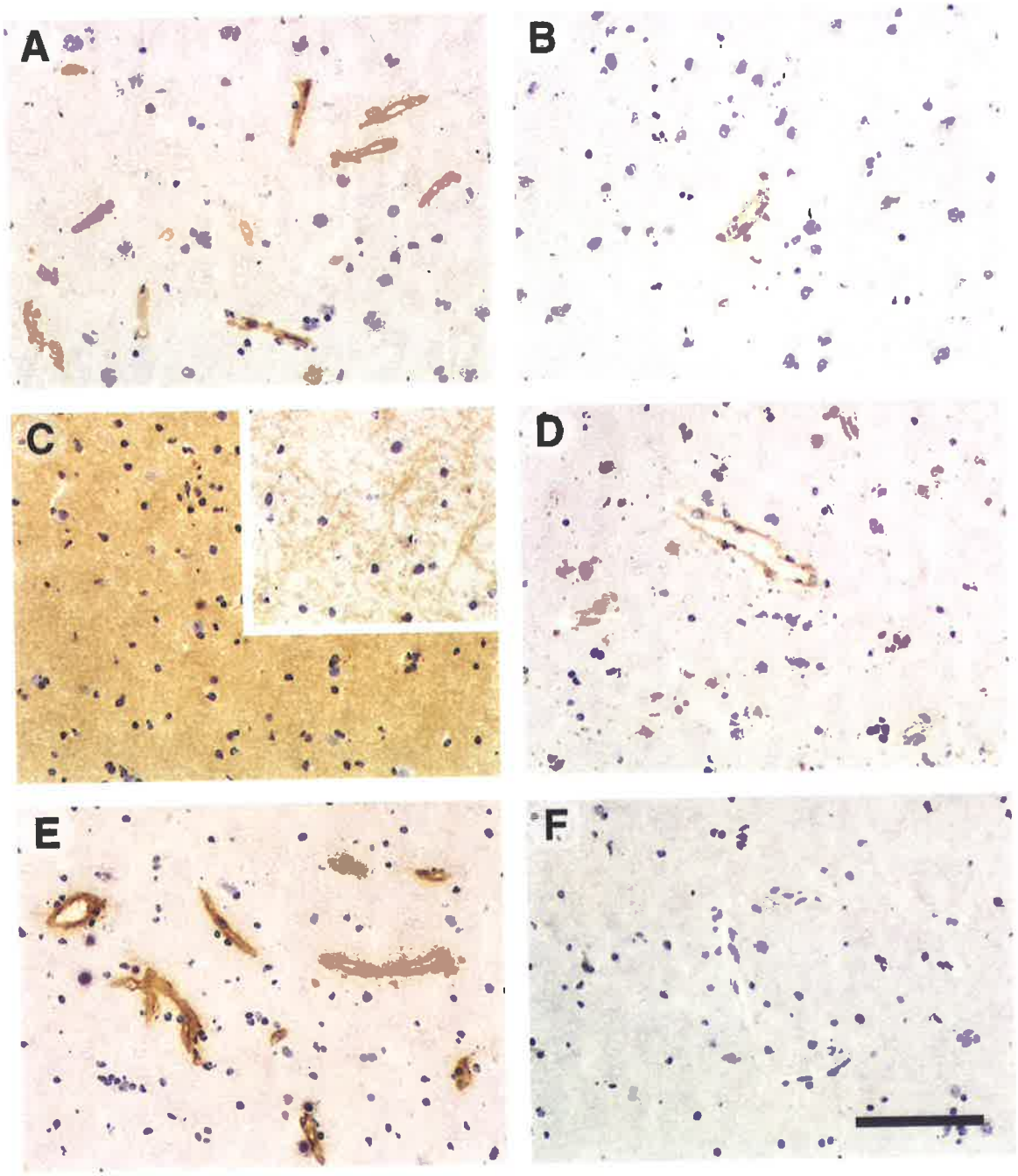
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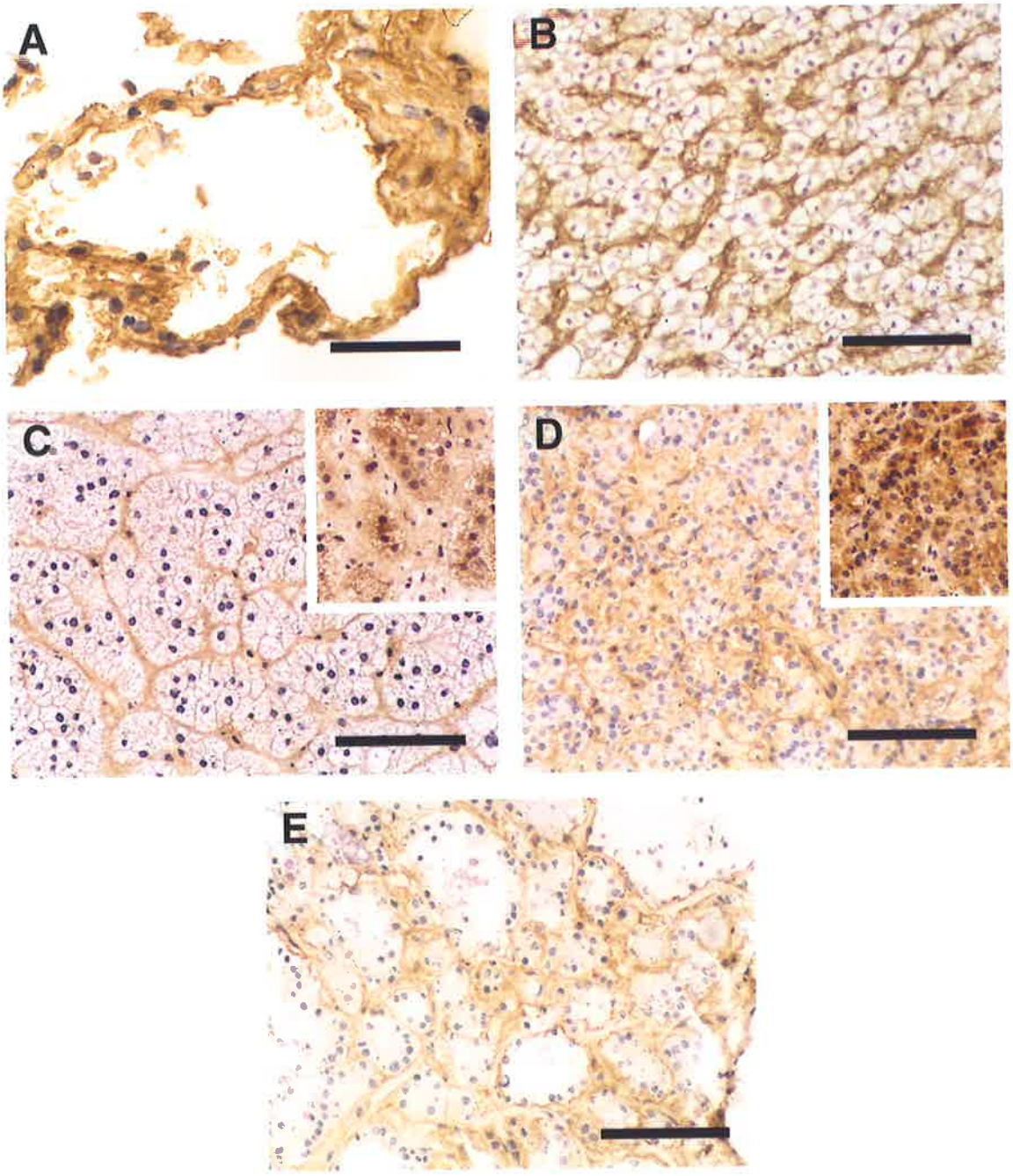
Figure 3.11 Localisation of PETA-3 in Lung, Liver, Adrenal Cortex, Pancreas and Thyroid

All panels show staining using mAb 11B1.G4. (A) Epithelium and alveolar pneumocytes of lung stained strongly for PETA-3. (B) Hepatic sinusoids were stained strongly for PETA-3. Some staining of hepatocytes with 11B1.G4 was also detected. (C) Parenchymal cells within the zona glomerulosa of the adrenal cortex were positive for PETA-3. Inset shows intense labelling of CD63 on parenchymal cells of the adrenal cortex. (D) Acinar cells within pancreas were moderately stained by 11B1.G4. Inset shows strong labelling of CD63 on acinar cells. (E) Low power view of thyroid showing PETA-3 expression on follicular and parafollicular cells. Bars; A-D: 40μm, F: 80μm.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PETA-3 (this staining was undetectable using 14A2.H1), CD9 and the β1 integrin chain. Kupffer cells showed weak staining on sections treated with negative control mAbs, thus the specificity of staining of these cells could not be determined.

3.2.16 Adrenal Cortex

Parenchymal cells of the zona glomerulosa and spongiocytes within the zona fasciculata were stained by the anti-β1 mAb (data not shown). PETA-3 (Figure 3.11.C) and CD9 were also expressed by these cells. In each case the mAbs stained the cell membranes and the periphery of cytoplasmic lipid droplets. Cells within the zona reticularis were also stained by these mAbs, although the pattern of staining on these cells was diffuse and cytoplasmic. Anti-CD63 stained spongiocytes strongly and cells of the zona reticularis very strongly (Figure 3.11.C inset).

3.2.17 Pancreas

Acinar cells and pancreatic islets were either unstained or lightly stained for most mAbs tested. 11B1.G4 stained acinar cells and ducts weakly (Figure 3.11.D) whilst 14A2.H1 failed to stain acinar cells. Similarly, CD9 was not detected on acinar cells but did stain ducts. In contrast to all other mAbs tested, anti-CD63 staining of acinar cells was very strong (Figure 3.11.D inset). Staining of acinar cells for β1 was similar to that of 14A2.H1 and CD9. In addition to acinar cells and ducts, the columnar epithelium of intralobular excretory ducts showed diffuse staining by mAbs against PETA-3, CD63, the β1 integrin chain whilst CD9 was not detected (data not shown).

3.2.18 Thyroid

The follicular epithelium and parafollicular cells were stained by 11B1.G4 (Figure 3.11.E), and mAbs against CD9, CD63. 14A2.H1 showed very weak staining of follicular and parafollicular cells within the thyroid (data not shown). Anti-PETA-3 and CD9 mAbs stained the cell membranes of follicular epithelial cells, while staining with CD63 displayed a granular appearance. Follicular epithelium was not stained by anti-β1 mAb although β1 integrin was detected on the membrane of parafollicular cells with a distribution similar to that of PETA-3 and CD9.

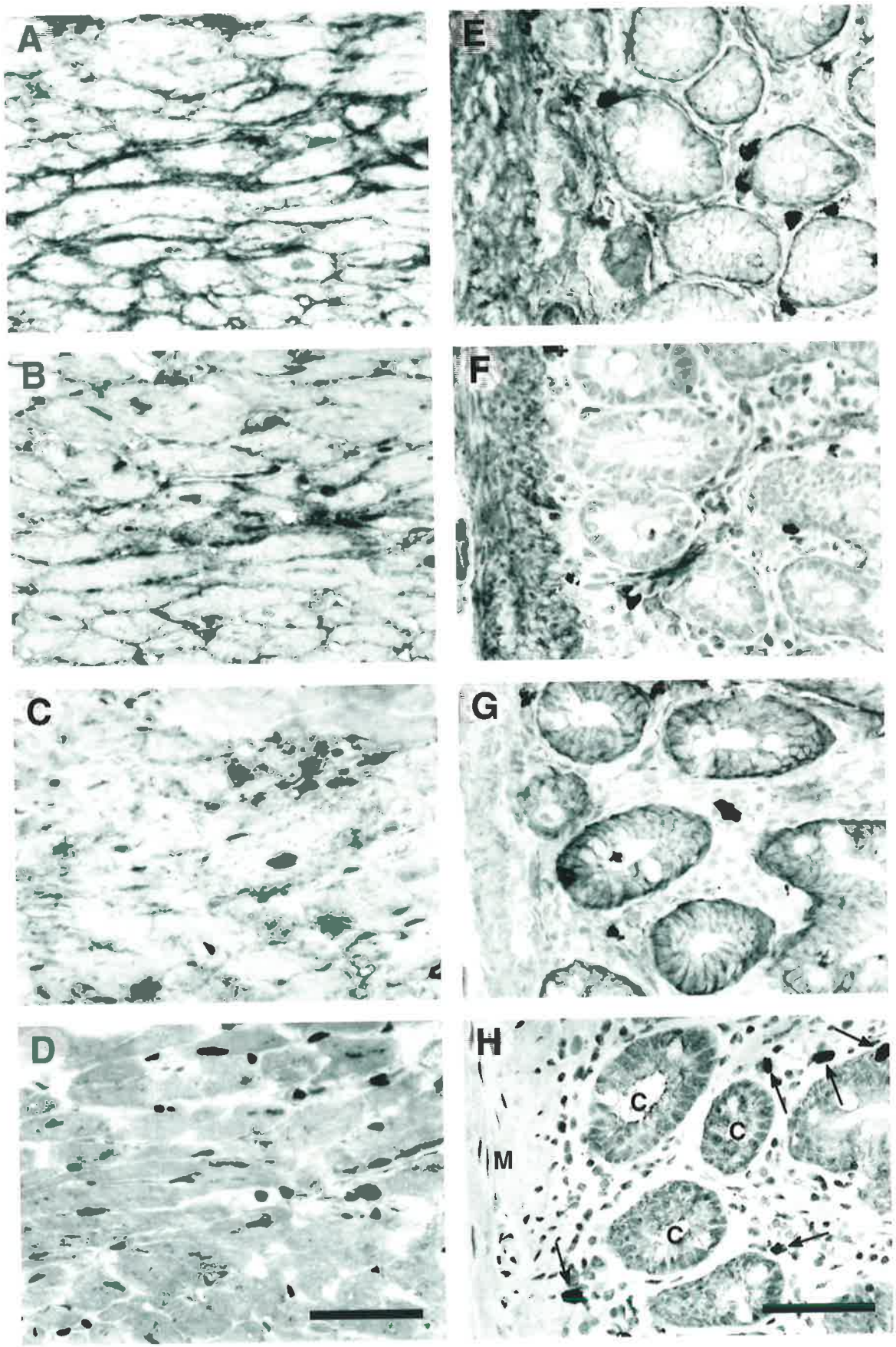
3.2.19 Comparison of the binding of Different anti-PETA-3 MAbs to Human Tissues

The data presented in this Chapter clearly demonstrated a difference in the binding of the two anti-PETA-3 mAbs 14A2.H1 and 11B1.G4. During the course of this study, another anti-PETA-3 mAb, 11G5, was identified. This mAb was purchased from Serotec, as an anti-α3 integrin mAb, for use in immunoprecipitation experiments (Section 5.2.2). However, during the course of this work, the specificity of this reagent was examined using transfected cell lines. The 11G5 mAb bound a PETA-3 transfectant, but did not bind an α3 transfectant or parent cell line, suggesting that the specificity of this mAb was against PETA-3 (S. Fitter, unpublished). Furthermore, 11G5 immunoprecipitated a band of molecular weight corresponding to that of PETA-3 (S. Fitter, unpublished). Therefore it was concluded that the specificity of this mAb was not for the integrin α3 chain, but for PETA-3.

During this time, the different binding properties of 14A2.H1 and 11B1.G4 was being investigated. Since α3 was believed to be involved in masking the epitope recognised by 14A2.H1, the localisation of 11G5 (believed at that time to be an anti-α3 integrin mAb) was investigated in cardiac muscle and gut crypts. Interestingly the binding of these three anti-PETA-3 mAbs to cardiac muscle and gut sections varied (Figure 3.12). Both 11B1.G4 and

Figure 3.12 Comparison of the Binding of anti-PETA-3 Antibodies to Human Tissues

Panels (A-C), binding of anti-PETA-3 mAbs 11B1.G4, 14A2.H1 and 11G5 to cardiac muscle respectively. Panel (D) shows binding of isotype matched negative control mAb to cardiac muscle. Note the poor staining of cardiac muscle cells by 11G5. Panels (E-G), binding of anti-PETA-3 mAbs 11B1.G4, 14A2.H1 and 11G5 to gut crypts respectively. Note the differential staining of 14A2.H1 and 11G5. Panel (H), isotype matched negative control mAb staining of gut crypts and underlying muscularis mucosa. For comparison with fields E-G, morphological features are labelled as follows; M - muscularis mucosa, C - crypt. Arrows show endogenous peroxidase activity of several eosinophils. Bars; (A-D) 40μm, (E-H) 80μm.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14A2.H1 bound well to cardiac muscle, however 11G5 preferentially stained the endothelium of capillaries (Figure 3.12.B-D). Similarly, in gut sections, both 11B1.G4 and 14A2.H1 bound the smooth muscle of the muscularis mucosa, whilst 11G5 failed to stain these cells. In contrast 11B1.G4 and 11G5 strongly stained the basolateral membranes of enterocytes within crypts. Interestingly, 14A2.H1 failed to stain enterocytes, resulting in an inverse of the pattern of staining observed for 11G5. Therefore it was concluded that different anti-PETA-3 mAbs show markedly altered binding to tissues. The 11B1.G4 mAb appeared to bind to all tissues/cell types in which PETA-3 was expressed. In comparison, 14A2.H1 preferentially bound PETA-3 expressed by smooth and cardiac muscle whilst 11G5 predominantly bound PETA-3 associated with epithelial cell types.

3.3 Discussion

The association of tetraspan molecules with each other and with β1 integrins has suggested a role for these proteins in the modulation of integrin function (Berditchevski *et al.*, 1996; Berditchevski *et al.*, 1995; Rubinstein *et al.*, 1994). PETA-3, like other members of the TM4SF, appears to share these associations. Complexes containing PETA-3 in association with CD63, CD9 and β1 integrins have been demonstrated by co-immunoprecipitation experiments using haemopoietic cell lines (Fitter *et al.*, submitted). Although much emphasis has been placed on the characterisation of these associations using cell lines, the distribution of PETA-3 *in vivo* and its co-localisation with associated antigens in various tissues has not been studied in detail. The aim of the current section was to determine the tissue distribution of PETA-3 and to compare systematically the localisation of known associated molecules including CD9, CD63 and the β1 integrin chain.

PETA-3 showed expression in a variety of different cell types. It was readily detected on vascular endothelium and smooth muscle in all tissues examined. Expression of PETA-3 by vascular endothelium was constant throughout all tissues examined including regions of specialised endothelium (eg. glomeruli, HEVs, hepatic sinusoids, alveolar capillaries and capillaries constituting parts of the blood brain barrier). The widespread expression of PETA-3 in endothelium suggests that its primary role may be in the maintenance of vascular integrity. This could be achieved through co-operation with integrins in mediating adhesion to ECM or between cells. In addition the widespread expression of PETA-3 suggests that endothelial activation, by locally produced proinflammatory cytokines, does not regulate the level of expression. Such regulation of expression was seen with CD63 a known marker of endothelial activation (Vischer and Wagner, 1993) which was not detected within central arteries of spleen, yet was expressed within HEVs of the tonsil and most other vasculature. This suggests that expression of CD63 may be regulated by factors in the local

microenvironment of the EC. Interestingly PETA-3 was expressed on the vasculature of brain tissue, although PETA-3 mRNA was not detected by northern analysis (Fitter *et al.*, 1995). CD63 and β1 were also restricted to the vasculature in brain tissue while CD9 was expressed by other tissue components as well. The expression of CD9 by in the brain parenchyma has been previously described (Rossler *et al.*, 1992) and results of the current study are similar. However the restriction of the β1 integrin chain to the vasculature in brain suggests that CD9, unlike the other tetraspans, may be predominantly associated with another integrin β chain or that it has an integrin independent function in these cells.

PETA-3 expression was also observed in a number of epithelia. In the skin and tonsil, staining was restricted to the basal layers of the stratified epithelia and exhibited polarity with brightest staining adjacent to the basal membrane. In contrast, CD9 was expressed by all keratinocytes in skin and showed an opposite polarity in cells of the stratum basale. The presence of PETA-3 in close proximity to the basement membrane suggests that it may be involved in the anchoring of cells to the basal lamina. The loss of PETA-3 expression by keratinocytes as they leave the proliferative compartment and lose contact with the basal lamina further supports this hypothesis. In contrast, CD9 may be primarily involved in interactions between keratinocytes, as suggested by its expression by keratinocytes throughout the epidermis and its undetectable expression on the basal membrane in cells of the stratum basale. β1 integrin expression was also restricted to the basal layers suggesting that association of PETA-3 with β1 may be involved in anchoring to the basement membrane. Fibroblasts and some other undefined cells within connective tissue of the dermis also expressed PETA-3, CD9, CD63 and β1 integrins. The widespread expression of these antigens throughout connective tissue probably reflects their interaction with components of the ECM such as collagen, laminin and fibronectin. Similar interactions would be expected in other cells that interface with connective tissue ECM (eg. cardiac and skeletal muscle,

Schwann cells, cells of the perineurium and various epithelial cells with basement membranes). In most cells, expression of tetraspan molecules, including PETA-3, is accompanied by expression of β1 integrin. However exceptions exist in hepatocytes, in the epithelia of the thyroid, within the pancreas and in cerebral white matter.

Expression of PETA-3 by simple columnar or cuboid epithelia also demonstrated a basolateral distribution, especially in villus and crypt enterocytes of the small intestine. In these polarised cells, PETA-3 and CD9 were excluded from the membrane domain of the brush border. The basolateral distribution of PETA-3, CD9 and the β1 chain implies that they may be involved in interactions of the cells with the basement membrane and possibly inter-cellular adhesion. The expression of CD9 by gut epithelium was restricted to immature enterocytes of the crypts and the bases of the villi. Therefore CD9 may be regulated during epithelial differentiation and its function may be limited to interactions with specific ECM components in the crypt microenvironment. In contrast to PETA-3 and CD9, CD63 expression was localised to granules or structures located in the apical cytoplasm, below the brush border. Ultrastructural studies would be necessary to determine whether CD63 is expressed on the brush border membrane. CD63 has been shown to possess a lysosomal targeting sequence (Metzelaar *et al.*, 1991) and demonstrated to localise in Weibel-Palade bodies of EC and platelet granules (Vischer and Wagner, 1993; Nieuwenhuis *et al.*, 1987). The apical region of the enterocyte cytoplasm is rich in multivesicular bodies that express MHC class II (Mayrhofer and Spargo, 1990). Therefore CD63 may be expressed in the early endocytic pathway in enterocytes. Furthermore, other members of the TM4SF, CD37, CD53, CD81 (TAPA-1) and CD82 (R2/C3) have been shown to associate with MHC class II (Angelisova *et al.*, 1994; Schick and Levy, 1993), suggesting that CD63 may also be involved in these complexes. Since β1 integrins were present on the basolateral membranes of

enterocytes and possibly in the basal cytoplasm, CD63 does not appear capable of association with β1 integrins in the gut epithelium due to its distinct compartmentalisation.

Simple epithelia in several other tissues also expressed very high levels of PETA-3. Such structures included bile ducts, terminal bronchioles and pancreatic intralobular ducts. CD9, CD63 and β1 integrins also showed similar localisation in these tissues although CD9 was not detected on pancreatic ducts. Interestingly staining of bile duct and pancreatic duct epithelia for these antigens was diffuse, suggesting cytoplasmic localisation. However, studies on biopsy material would be required to eliminate postmortem degeneration as a cause for this appearance.

Lymphocytes in blood and organised secondary lymphoid tissues (spleen and tonsil) did not express PETA-3 or the other tetraspans investigated. An interesting exception was the population of IEL in the small intestine. These cells, which appear to be extra-thymically derived T cells have been shown to express several unique antigens including the αEβ7 integrin (Yuan *et al.*, 1991). This study appears to be the first report describing expression of tetraspan molecules, CD9 and CD63 by IELs, and their restriction to this subset of lymphocytes may be related to the specialised microenvironment that these cells occupy.

Previous studies with mAb 14A2.H1 had characterised the expression of PETA-3 in haemopoietic cells, within bone marrow cells and peripheral blood. These studies showed expression to be restricted to megakaryocytes and platelets (Ashman *et al.*, 1991; von dem Borne, 1989). In tonsil sections PETA-3 was reported to be present on the squamous epithelium and blood vessels (Ashman *et al.*, 1991). The results of the current study are in general agreement with these earlier findings on haemopoietic cells and tonsil. However the use of mAb 11B1.G4 in parallel to 14A2.H1 has produced some interesting observations. Throughout the tissues examined, 11B1.G4 staining appeared superior to that 14A2.H1. Furthermore, gut enterocytes, immature cells within the BMMNC population, kidney epithelia

and the sarcolemma of skeletal muscle failed to stain with 14A2.H1, despite the detection of PETA-3 by 11B1.G4 at these sites. However, the poor nature of 14A2.H1 staining does not appear to be due to lower affinity of this mAb since some tissues showed strong staining with both mAbs. For example, cardiac muscle and smooth muscle, both perivascular and the muscularis mucosa of the gut, showed stronger staining for 14A2.H1 than would be expected, based on 11B1.G4 staining. Furthermore, 14A2.H1, 11B1.G4, 14B5 and 11G5 all bind equally to FDC-P1 cells transfected with PETA-3 cDNA (Figure 2.2.A; S. Fitter, unpublished). Therefore it appears that the epitope 14A2.H1 recognises may be masked by molecules, most likely integrins, associated with PETA-3. Additionally, 11G5 binding appeared to be sensitive to epitope masking in an opposite manner to that observed for 14A2.H1. This phenomenon was clearly demonstrated by the differential staining of the three anti-PETA-3 mAbs, 11B1.G4, 14A2.H1 and 11G5 (Figure 3.12).

In summary this Chapter has shown that PETA-3 co-localises with β1 integrins and the other tetraspans CD9 and CD63 in particular tissues. However it is important to note that distinct localisations of several antigens investigated occurred throughout some tissues. This may indicate that tetraspan molecules share common functions in a number of tissues but they may also have specific functions in some cell types. This is exemplified by PETA-3 in cardiac muscle, CD9 in tissue components of the brain and medullary rays of the kidney, and CD63 in pancreatic ancini cells, adrenal spongiocytes, and the apical localisation of CD63 in enterocytes. It is also important to note that co-expression of these antigens by a given cell type may not always imply that they form complexes. For example compartmentalisation of CD63 within intracellular granules in enterocytes would prevent its association with surface antigens. Therefore loss of polarity in cultured or transformed cells may lead to reports of TM4SF interactions which would not occur under physiological conditions. Whether the complexes formed by tetraspans and integrins are constitutive or inducible remains to be

investigated, however the co-localisation of these antigens in normal tissues provides the basis for such interactions to occur.

CHAPTER 4: SUBCELLULAR LOCALISATION OF PETA-3 IN PLATELETS AND ENDOTHELIAL CELLS

4.1 Introduction

The previous Chapter described the immunohistochemical localisation of PETA-3 to epithelium, muscle, Schwann cells and ubiquitous expression by endothelium *in vivo*. Although PETA-3 is expressed by EC, the role for this molecule in endothelial biology remains undefined. Within haemopoietic cells, PETA-3 is mostly restricted to platelets and megakaryocytes (Ashman *et al.*, 1991), (see also Chapter 3), where it may be involved in the recognition of subendothelial matrix during thrombus formation and in thrombopoiesis respectively. The work presented in the previous Chapter and many preliminary studies on PETA-3 have examined the expression of this molecule by various tissues and haemopoietic cells. Although basolateral staining of gut enterocytes and basal keratinocytes was evident, thorough characterisation of PETA-3 at the subcellular level has not been carried out. Determination of the tissue distribution has suggested a role for PETA-3 in cell-ECM and/or cell-cell interactions. Likewise, characterisation of PETA-3 at the subcellular level should provide valuable insight as to the possible function of this TM4SF member. Therefore the aim of this section was to examine the sub-cellular localisation and expression of PETA-3 in EC and platelets by both light and electron-microscopic techniques.

The name PETA-3 was coined since expression of this protein by platelets and endothelium was all that had been characterised at the time the cDNA was cloned (Fitter *et al.*, 1995). However, unlike PETA-3, CD9 and CD63 have been well characterised in platelets and EC. In platelets, CD9 has been shown to be abundantly expressed, at approximately 80,000 copies per platelet, and anti-CD9 mAbs act as platelet agonists (Worthington *et al.*, 1990; Sections 1.5.4 and 1.5.5). Apart from expression on the plasma

membrane, CD9 has also been localised to platelet granules (Cramer *et al.*, 1994). Similarly CD63 has also been shown to be a component of platelet granules (Nieuwenhuis *et al.*, 1987), however unlike CD9, expression of CD63 on the surface of resting platelets is low. Upon platelet degranulation however, CD63 acts as an activation marker and is rapidly mobilised to the plasma membrane (Nieuwenhuis *et al.*, 1987).

As mentioned previously, CD63 has been well characterised as a late endosomal and lysosomal marker (Nieuwenhuis *et al.*, 1987; Metzelaar *et al.*, 1991). Furthermore, this molecule possesses a putative lysosomal targeting motif in its carboxyl cytoplasmic tail (Metzelaar *et al.*, 1991). Thus the low expression of CD63 on the plasma membrane appears to be due to internalisation and trafficking to an intracellular compartment. Apart from late endosomes, lysosomes and platelet α-granules, CD63 has also been localised to the Weibel-Palade bodies of EC (Vischer and Wagner, 1993). Although the lysosomal targeting motif for CD63 has been identified, the mechanism by which this molecule is targeted to Weibel-Palade bodies, α-granules, late endosomes and lysosomes in a coordinated manner has not been elucidated.

The surface expression of many molecules expressed by EC has been shown to be regulated by a variety of stimuli. P-selectin and CD63 are components of the Weibel-Palade bodies and are rapidly mobilised to the cell surface upon treatment of EC with thrombin, histamine, phorbol esters, calcium ionophores or complement proteins (reviewed in Carlos and Harlan, 1994). Similarly, both of these molecules are present in platelet α-granules and are mobilised to the surface upon activation (McEver *et al.*, 1989; Nishibori *et al.*, 1993). In contrast to translocation to the plasma membrane, transcriptional regulation of many molecules has been demonstrated by stimulation of EC with agonists such as TNF-α, lipopolysaccharides or IL-1. Since platelcts lack nuclei, transcriptional regulation is not applicable. In EC examples of transcriptionally regulated molecules include E-selectin,

ICAM-1 and VCAM-1 (reviewed in Carlos and Harlan, 1994). Most of the molecules upregulated by EC upon activation are pro-inflammatory such that they promote platelet adhesion/activation and leukocyte adhesion and subsequent diapedisis.

The expression of integrins by EC may also be regulated by cytokines. Stimulation with TNF-α or transforming growth factor-β generally results in increased integrin expression, especially those of the β1 subfamily. One exception is α6β1, which has been consistently reported to be downregulated in TNF-α or IL-1 treated EC (Defilippi *et al.*, 1992). Studies with bFGF have reported both increased (α2β1, α3β1, α5β1, α6β1, α6β4 and αvβ5) and decreased (α1β1, αvβ1 and αvβ3) integrin expression (reviewed in Luscinskas and Lawler, 1994). Expression of αvβ3 may also be downregulated by the synergistic action of TNF-α and IFNγ, while either of these cytokines alone has little effect on this integrin.

Preliminary studies in haemopoietic cell lines had demonstrated the association of PETA-3 with CD9 and CD63 (Fitter, Sincock, Jolliffe and Ashman, submitted). Furthermore, the widespread reports of TM4SF/TM4SF associations (reviewed in Maecker *et al.*, 1997), suggest these complexes are found in a broad range of cell types. Therefore a similar or at least overlapping distribution of PETA-3, CD9 and CD63 within EC and platelets, should be observed, assuming such complexes exist in these cell types.

This Chapter examines the subcellular localisation of PETA-3 in EC by confocal microscopic analysis. The localisation of PETA-3 in EC was verified by immunogold labelling and electron microscopic techniques. This technique was also used to examine the localisation in platelets. Finally, the possible regulation of PETA-3 expression by EC and platelets activation was investigated.

4.2 Subcellular Localisation of PETA-3 in Endothelial Cells

4.2.1 PETA-3 is Localised to Perinuclear Vesicles and Endothelial Cell Margins

The subcellular distribution of PETA-3 in EC was investigated by indirect immunofluorescence and confocal microscopy. In formaldehyde-fixed confluent EC monolayers, anti-PETA-3 mAb strongly stained regions of cell-cell contact (Figure 4.1.A). However, upon permeabilisation, PETA-3 displayed granular staining of the perinuclear region in addition to cellular margins (Figure 4.1.B). At low culture density, PETA-3 showed stronger, more diffuse staining of the perinuclear region (Figure 4.1.C). Although staining of the plasma membrane at low culture density was diffuse, concentration of PETA-3 in regions of cell-cell contact was still apparent. Staining of CD9 on the EC plasma membrane showed a similar distribution as was observed for PETA-3, however no intracellular staining was observed in permeabilised cells (Figure 4.1.D).

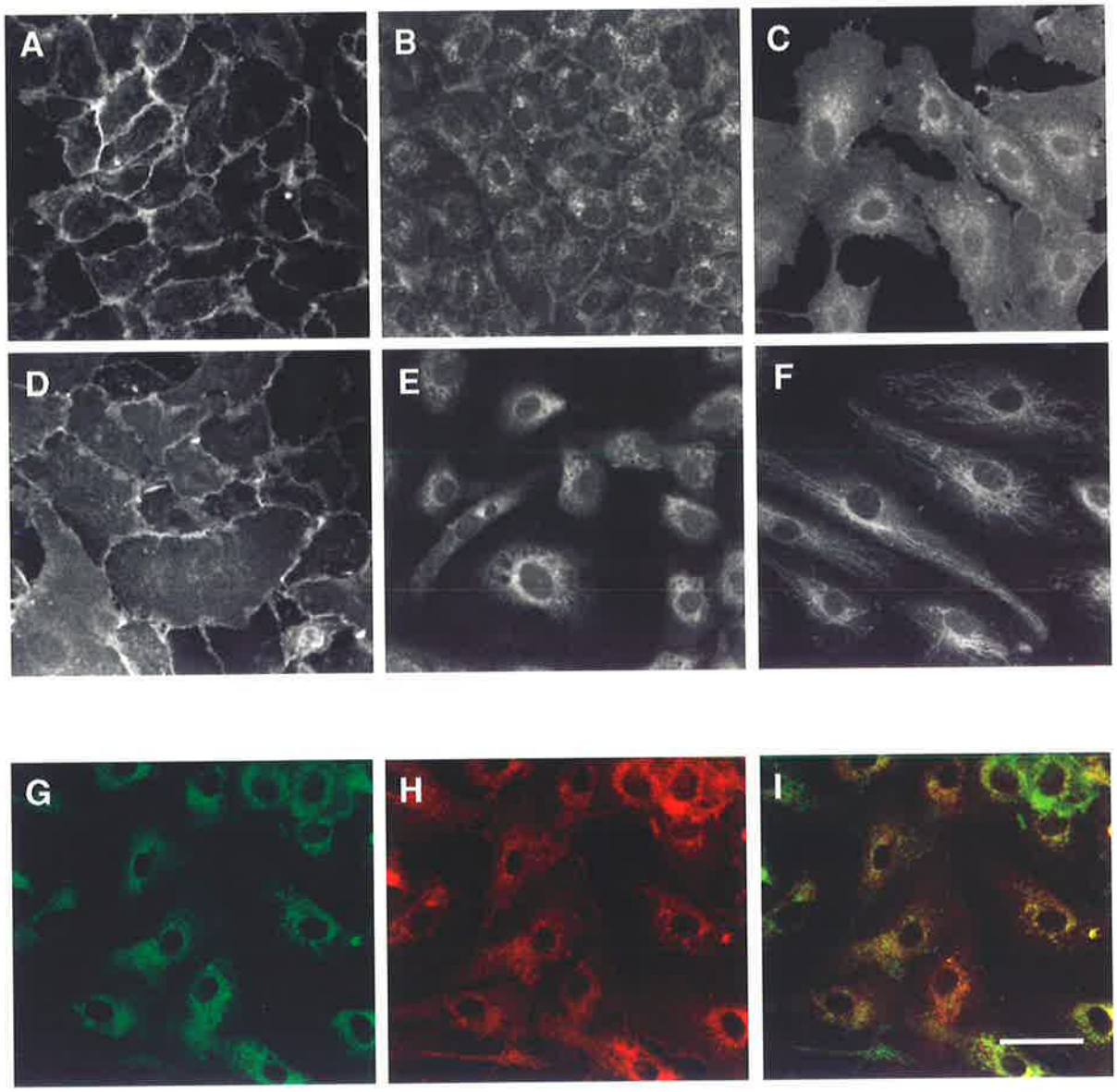
Intracellular PETA-3 did not resemble the morphology of the endoplasmic reticulum marker, gp180 (Savitz and Meyer, 1990) (Figure 4.1.E), nor the mitochondria (Figure 4.1.F), as determined by the mitochondrial specific mAb, TOM-20 (Hanson *et al.*, 1996). Since PETA-3 staining did not resemble these compartments, the possibility of an endosomal/Golgi localisation was examined. Staining of low density EC with WGA, which binds mature oligosaccharide residues on post-endoplasmic reticulum processed glycoproteins, and recognises the Golgi-apparatus, endosomes and plasma membrane (Tartakoff and Vassalli, 1983) displayed a perinuclear morphology strongly resembling PETA-3. Furthermore staining with WGA showed strong colocalisation with PETA-3 by 2-colour confocal microscopy (Figure 4.1.G-I). Therefore the majority of intracellular PETA-3 appears to reside within a post-endoplasmic reticulum compartment.

In order to quantitate the level of intracellular PETA-3, EC were labelled by indirect immunofluorescence in the presence and absence of the permeabilising agent saponin and

Figure 4.1 Characterisation of the Subcellular Localisation of PETA-3 in Cultured EC

The subcellular distribution of PETA-3 in EC was examined by immunofluorescence and confocal microscopy as described (Section2.6). (A) PETA-3 staining of formaldehyde fixed non-permeabilised cells demonstrated localisation to cellular margins. (B) Staining of PETA-3 in permeabilised EC revealed an additional intracellular pool with a granular perinuclear distribution. (C) Subconfluent EC showed strong perinuclear staining for PETA-3 while surface staining was concentrated in regions of cell-cell contact. Note the accumulation of PETA-3 at sites of cell-cell contact. (D) Expression of CD9 was restricted to the plasma membrane primarily at cellular margins in permeabilised cells. (E) The perinuclear morphology of PETA-3 staining did not correlate with the endoplasmic reticulum marker gp180, or (F) the mitochondrial marker, hMas20p (human Mitochondrial assembly protein).

(G) The morphology of intracellular PETA-3 staining strongly resembled (H) WGA, as determined by 2-colour confocal microscopy. (I) shows the merge of images (G) and (H), demonstrating colocalisation (yellow). Bar; 55μm.


A
B
C
D
E
F
G
H
I

analysed by flow cytometry (Figure 4.2). After correction for negative control binding, PETA-3 staining was enhanced approximately 3-fold in saponin treated cells, indicating a large intracellular pool which comprised approximately 66% of the total PETA-3 expressed (Figure 4.2.A). In contrast, negligible intracellular CD9 was detected (Figure 4.2.B), whilst approximately 85% of CD63 was found to be intracellular (Figure 4.2.C). Staining for the endosomal marker, transferrin receptor, was also increased upon saponin treatment, such that approximately 88% was intracellular (Figure 4.2.D). Treatment of EC with saponin did not significantly alter negative control mAb binding, although a minor decrease in cell size was observed (data not shown). The detection of intracellular antigens by saponin permeabilisation was consistent with that observed by confocal microscopy (see Figure 4.1, and below).

4.2.2 Intracellular PETA-3 Resides within an Endosomal Compartment

To determine whether PETA-3 was localised to the Golgi, EC were treated with the fungal toxin BFA (Fujiwara *et al.*, 1988). Treatment with BFA leads to resorption of Golgi components back into the endoplasmic reticulum. Furthermore, BFA also causes tubulation of endosomes (Wood and Brown, 1992). However the action of BFA may vary, depending on the target cell type, and cells resistant or partially resistant to the actions of this agent have been reported. Therefore in order to assess the sensitivity of EC to BFA treatment, the localisation of the Golgi marker γ-adaptin (Robinson and Pearse, 1986) was assessed. Treatment of EC with BFA induced rapid redistribution of γ-adaptin from the Golgi (within 10) minutes confirming the action of BFA in these cells (Figure 4.3). However, treatment of EC with BFA for 10 minutes failed to induce any significant change in the localisation of PETA-3, suggesting that this molecule was not localised to the Golgi apparatus (Figure 4.3).

Figure 4.2 Quantitation of Intracellular PETA-3

Intracellular PETA-3 was quantitated by immunofluorescence labelling of EC in the absence and presence of the permeabilising agent saponin and analysed by flow cytometry as described (Section 2.5.1). Representative non-permeabilised (———), saponin permeabilised (———), and isotype matched negative control mAb binding (- - - - -) histograms are shown from one experiment of four performed. (A) Saponin treatment increased PETA-3 staining 3-fold, indicating that intracellular PETA-3 comprised approximately 66%. (B) No detectable intracellular staining was observed for CD9. (C) Intracellular CD63 accounted for 85% of the total. (E) Approximately 88% of transferrin receptor was intracellular. Data presented are representative histograms from quadruplicate tests. Numbers in parenthesis represent mean fluorescent intensity of respective peaks; boldface = saponin treatment.

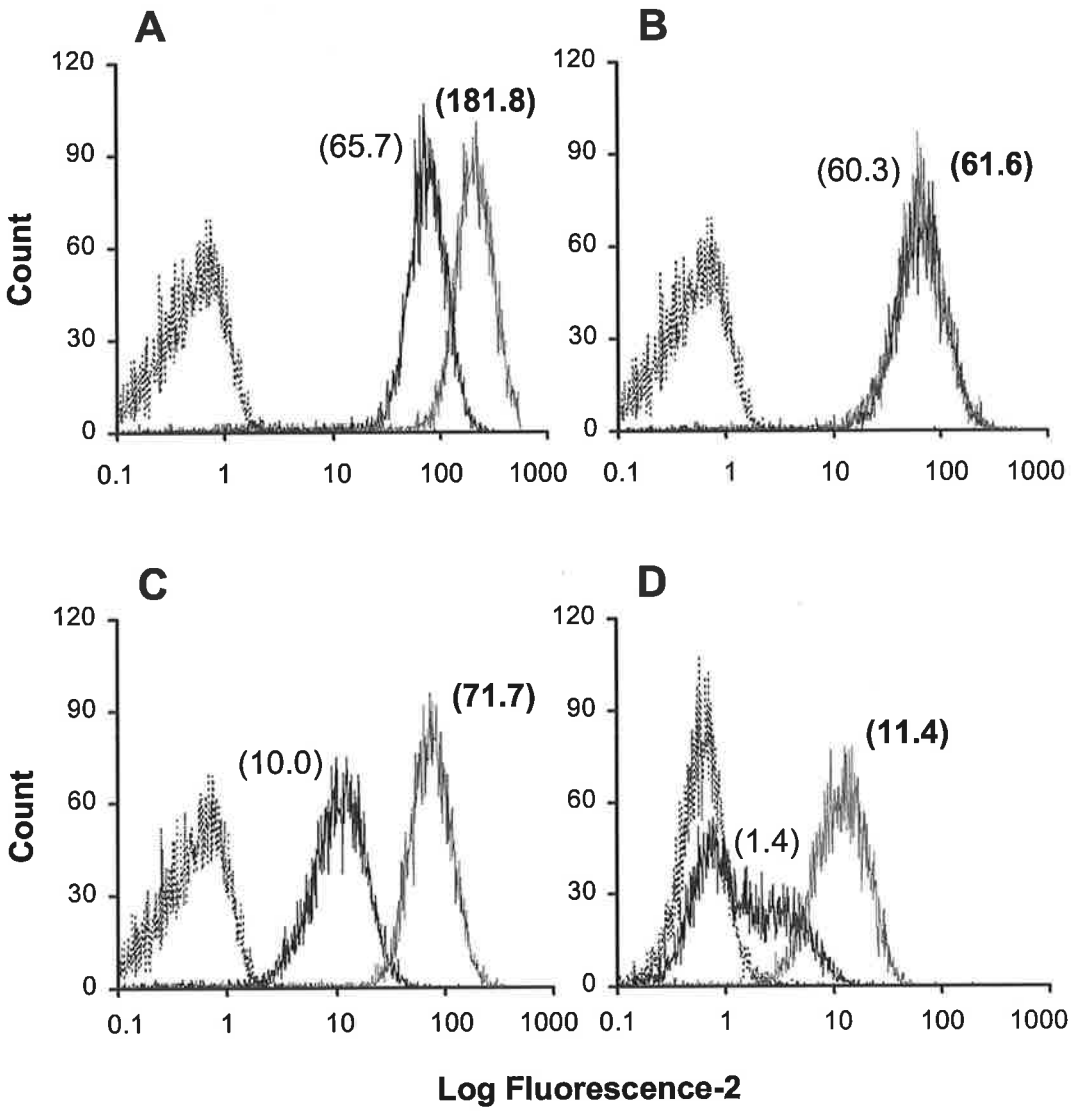
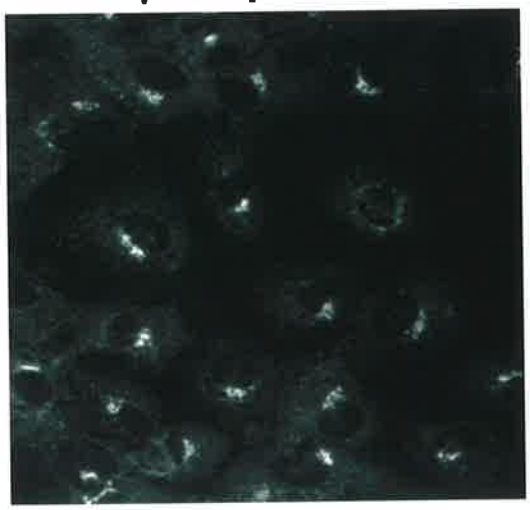

A
B
C
D
120
90
60
30
0
Count
0.1
1
10
100
1000
(65.7)
(181.8)
(60.3)
(61.6)
(10.0)
(71.7)
(1.4)
(11.4)
Log Fluorescence-2

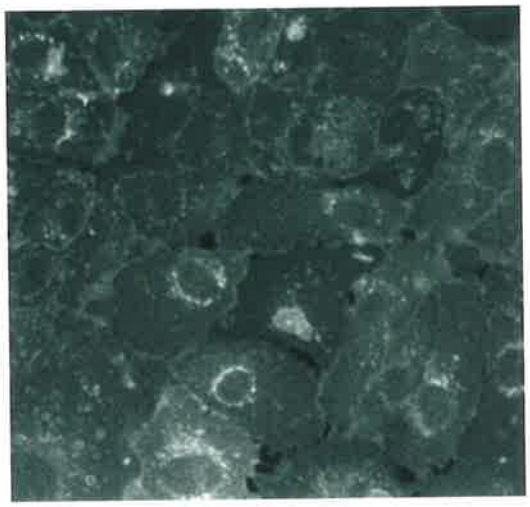
Figure 4.3 BFA Treatment of EC does Not Perturb the Localisation of PETA-3

Upper panel shows indirect immunofluorescence labelling (see Section 2.6) of the Golgi marker γ-adaptin, and PETA-3 in fourth passage HUVEC. Lower panel shows same labelling in HUVEC treated with 1.25μg/ml BFA for 10 minutes at 37°C. Note the redistribution of γ-adaptin, whilst PETA-3 staining remained unchanged. Bar; 55μm.

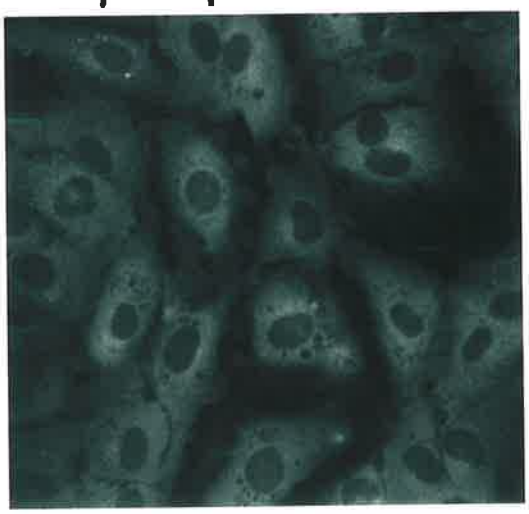
γ-Adaptin



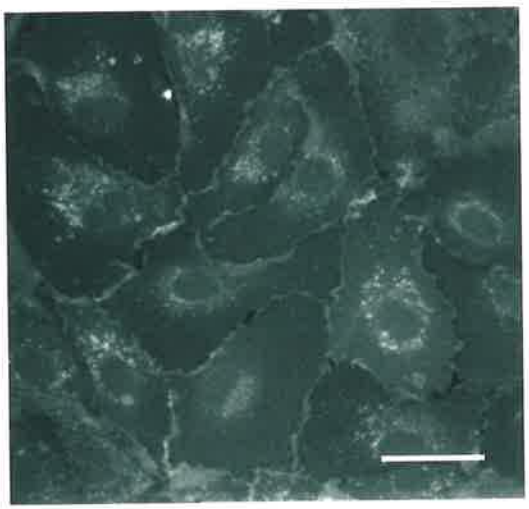
PETA-3



γ-Adaptin + BFA



PETA-3 + BFA



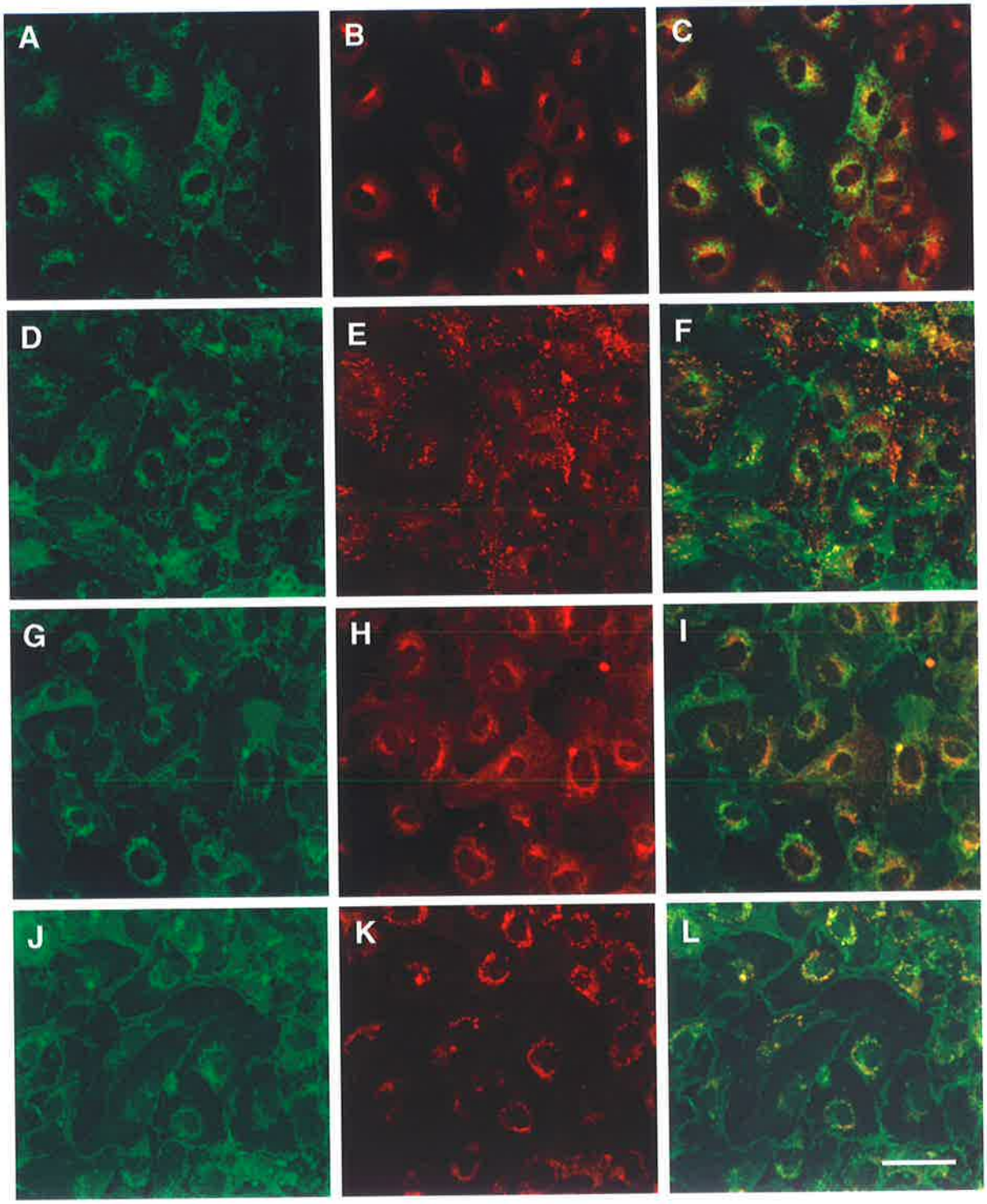
In order to further characterise the intracellular compartment in which PETA-3 resides, 2-colour colocalisation studies were carried out. Staining with mAb against the Golgi marker, γ-adaptin, did not colocalise with PETA-3 (Figure 4.4.A-C), which was consistent with the inability of BFA to perturb the localisation of PETA-3. Staining of Weibel-Palade bodies for vWF (Ewenstein *et al.*, 1987) also failed to colocalise with PETA-3 (Figure 4.4.D-F). In contrast, intracellular transferrin receptor, which is present in the early sorting endosome and the perinuclear early recycling endosome (Gruenberg and Maxfield, 1995), colocalised with PETA-3 with strongest colocalisation observed within the perinuclear region (Figure 4.4.G-I). Towards the periphery of cells, vesicles containing transferrin receptor failed to stain for with PETA-3. CD63, which is present in late endosomes, lysosomes and Weibel-Palade bodies, (Metzelaar *et al.*, 1991; Vischer and Wagner, 1993) also strongly colocalised with PETA-3 in the perinuclear region (Figure 4.4.J-L). It is interesting to note that the anti-CD63 mAb used in this study (12F12) failed to stain Weibel-Palade bodies, (compare Figures 4.4.E and 4.4.K). Taken together, these results indicate that intracellular PETA-3 is predominantly within a late endosomal and/or lysosomal compartment.

4.2.3 Ultrastructural Localisation of PETA-3 in Endothelial Cells

Due to the limited resolution of light microscopy, the distribution of PETA-3 was further examined by immunogold labelling and electron microscopy. Initial experiments were carried out to determine a fixation and labelling protocol which would provide sufficient labelling of PETA-3 in EC and platelets. Immunogold labelling experiments using whole platelets fixed with (0.1% gluteraldehyde/4% paraformaldehyde/4% sucrose in PBS), gave low levels of labelling on the plasma membrane with PETA-Rabbit (data not shown). However after embedding in LR white resin and sectioning, no antigenicity was retained. To overcome the loss of antigenicity during fixation and embedding, an attempt at pre-labelling

Figure 4.4 Colocalisation of PETA-3 with Markers of Different Subcellular Compartments

Colocalisation of PETA-3 staining (Green; panels A, D, G, and J) with γ-adaptin (panel B), vWF (panel E), transferrin receptor (panel H) and CD63 (panel K) (all Red) was visualised by indirect immunofluorescence labelling and 2-colour confocal microscopy (Section 2.6). Merges of red and green images were generated to demonstrate colocalisation (Yellow; panels C, F, I and L). Staining for the Golgi marker γ-adaptin (A-C) or Weibel-Palade bodies for vWF (D-F) did not colocalise well with PETA-3. In contrast, PETA-3 colocalised with transferrin receptor within the perinuclear region but not peripheral vesicles (G-I). The perinuclear staining of PETA-3 colocalised strongly with CD63 (J-L). Bar; 55μm.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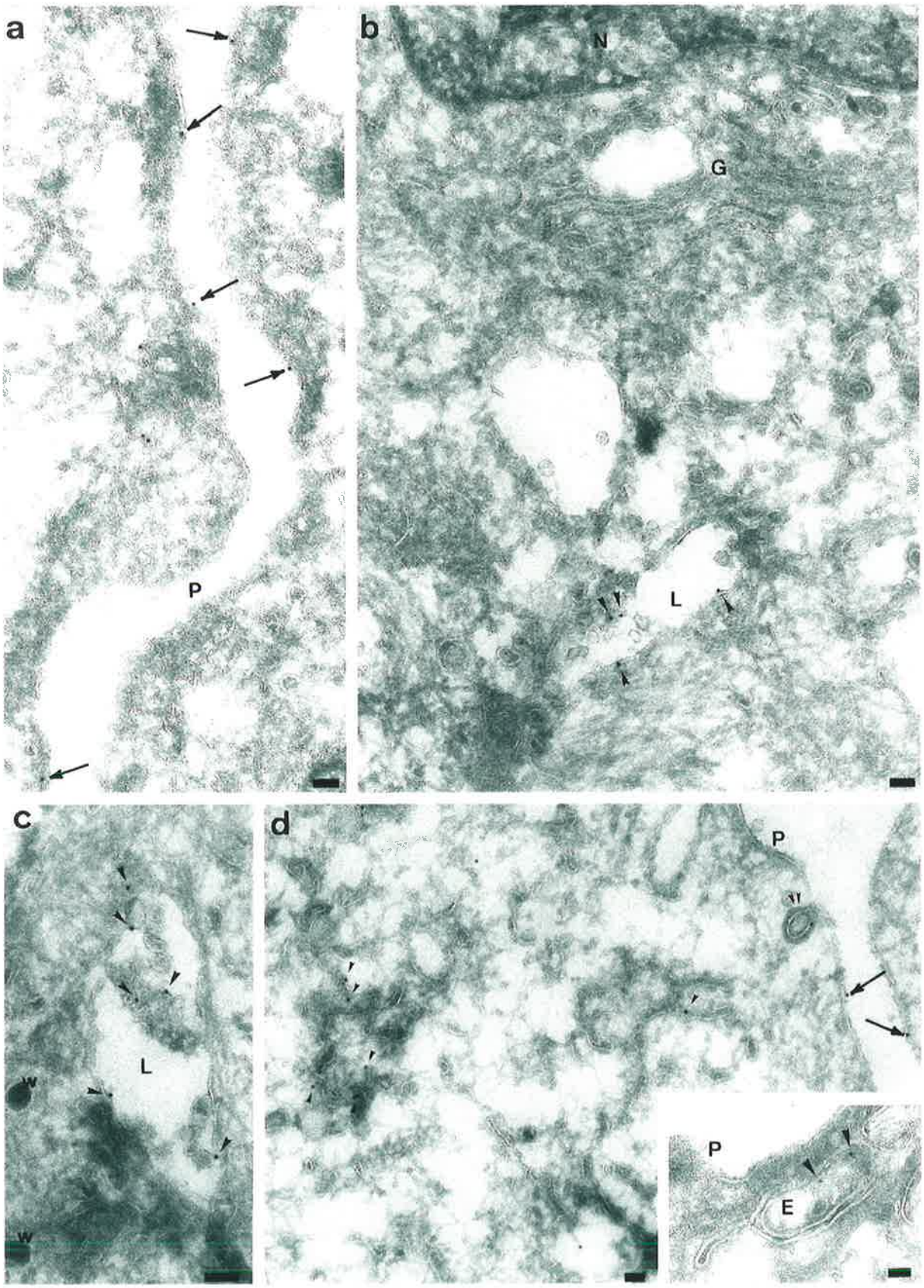
PETA-3 was carried out. In brief, saponin permeabilised EC were labelled with biotinylated 11B1.G4, fixed in 0.5% gluteraldehyde, embedded in Spurr's resin and labelled with a streptavidin-gold conjugate. Unfortunately, this protocol not only failed to detect PETA-3, but also resulted in poor ultrastructural morphology (data not shown).

An alternative method for immunoelectron microscopy was to use anti-PETA-3 antibodies on ultrathin frozen sections of EC as described in Section 2.7. Since loss of antigenicity appeared to be the major problem, the use of cryosectioned material was used to maximise the chance of maintaining protein epitopes. Indeed this was the case as low, but nonetheless specific labelling of PETA-3 in EC was observed using this protocol. Similar labelling was observed with three different antibody preparations; PETA-Rabbit (100μg/ml), purified 11B1.G4 (100μg/ml) and 11B1.G4 hybridoma culture supernatant. The specificity of affinity purified PETA-Rabbit was verified by binding to PETA-3, CD9 and CD63 transfectants, and Western blotting analysis (Section 2.3.5). Labelling was observed on the plasma membrane (Figure 4.5.a,d) and in endosomal structures (Figure 4.5.b-d). From the morphology of the labelled structures they were identified as tubulo-vesicular early endosomes (Figure 4.5.d and inset) and late endosomes (Figures 4.5.b and 4.5.c). Labelling was associated both with the internal vesicles of late endosomes and with the limiting membrane. Consistent with the endocytosis of PETA-3, low levels of labelling were also seen associated with coated pits. Negligible labelling was associated with other cellular organelles such as the endoplasmic reticulum, nucleus, mitochondria and Golgi complex (see Figure 4.5.b), although some Weibel-Palade bodies showed low but specific labelling (data not shown).

Figure 4.5 Ultrastructural Localisation of PETA-3 in EC

Ultrathin frozen sections of HUVEC were labelled with PETA-Rabbit and visualised by 10nm protein A-gold as described (Section 2.7). Panels a and b are low power overviews showing the general distribution of PETA-3 labelling. Panel a shows the plasma membranes of two closely apposed cells whereas panel b shows the juxtanuclear area of a cell. The plasma membrane (P) and a putative late endosomal structure (L, panel b) show significant labelling (labelling on plasma membrane indicated by arrows in all panels; labelling in putative endosomal structures by arrowheads). The endoplasmic reticulum surrounding the nucleus (N) as well as the Golgi complex (G) are unlabelled. Panel c shows a representative image of a putative late endosome (L). Panel d shows labelling on the plasma membrane (p), a coated pit (double arrowheads) and tubular elements, typical of early endosomal or recycling endosome tubules. Inset shows a representative early endosome (E). Bars; 100nm.


a
P
b
N
G
L
c
L
W
W
d
P
P
E

4.3 Ultrastructural Localisation of PETA-3 in Platelets

4.3.1 PETA-3 is Localised to the Plasma Membrane and Endocytic Pathway in Platelets

With the establishment of a protocol suitable for immunogold labelling, the ultrastructural localisation of PETA-3 in platelets was assessed. Since the affinity purified PETA-Rabbit proved to give the highest levels of immunogold labelling in EC, this reagent was used in the study on platelets. In contrast to EC, platelets showed very high levels of specific labelling with PETA-Rabbit (Figures 4.6.A and 4.6.B). Extensive labelling was associated with the plasma membrane (Figures 4.6.A and 4.6.B, Panel a) and, as was observed in EC, labelling of endosomal structures and clathrin coated vesicles/pits was also observed (Figure 4.6.B, Panels b-d). Lower levels of labelling were also seen in platelet granules on both the limiting membrane and multivesicular bodies within granules (Figure 4.6.C).

The high level of antibody binding observed in platelets was specific, as negligible labelling was associated with other organelles. Additionally, NRS diluted 1/500 in blocking buffer, was used as a specificity control. Although NRS did label some inter-platelet material, low levels of labelling on platelet membranes or organelles was observed (Figure 4.6.C), thus verifying the specificity of labelling observed with the anti-PETA-3 rabbit polyclonal antibody.

4.4 Regulation of PETA-3 Expression by Cellular Activation

4.4.1 PETA-3 Expression by Endothelial Cells is Not Regulated by Cytokines

Expression of several members of the TM4SF, including CD63, as mentioned above, and CD81, has been found to be dependent on the state of cellular activation (Section 1.2.2). Additionally, the association of PETA-3 and other TM4SF members with integrins suggests that these molecules may also be regulated by cytokine treatment in EC. Therefore the level

Figure 4.6.A Ultrastructural Localisation of PETA-3 in Platelets

Ultrathin frozen sections of platelets were labelled with PETA-Rabbit and visualised by 10nm protein A-gold as described (Section 2.7). Panel shows an overview of a whole platelet demonstrating the general distribution of PETA-3 labelling. The plasma membrane (P), and endosomal structures (E) showed strong labelling for PETA-3. Some labelling was also observed on the limiting membrane and within platelet granules (G). Bar; 100nm.

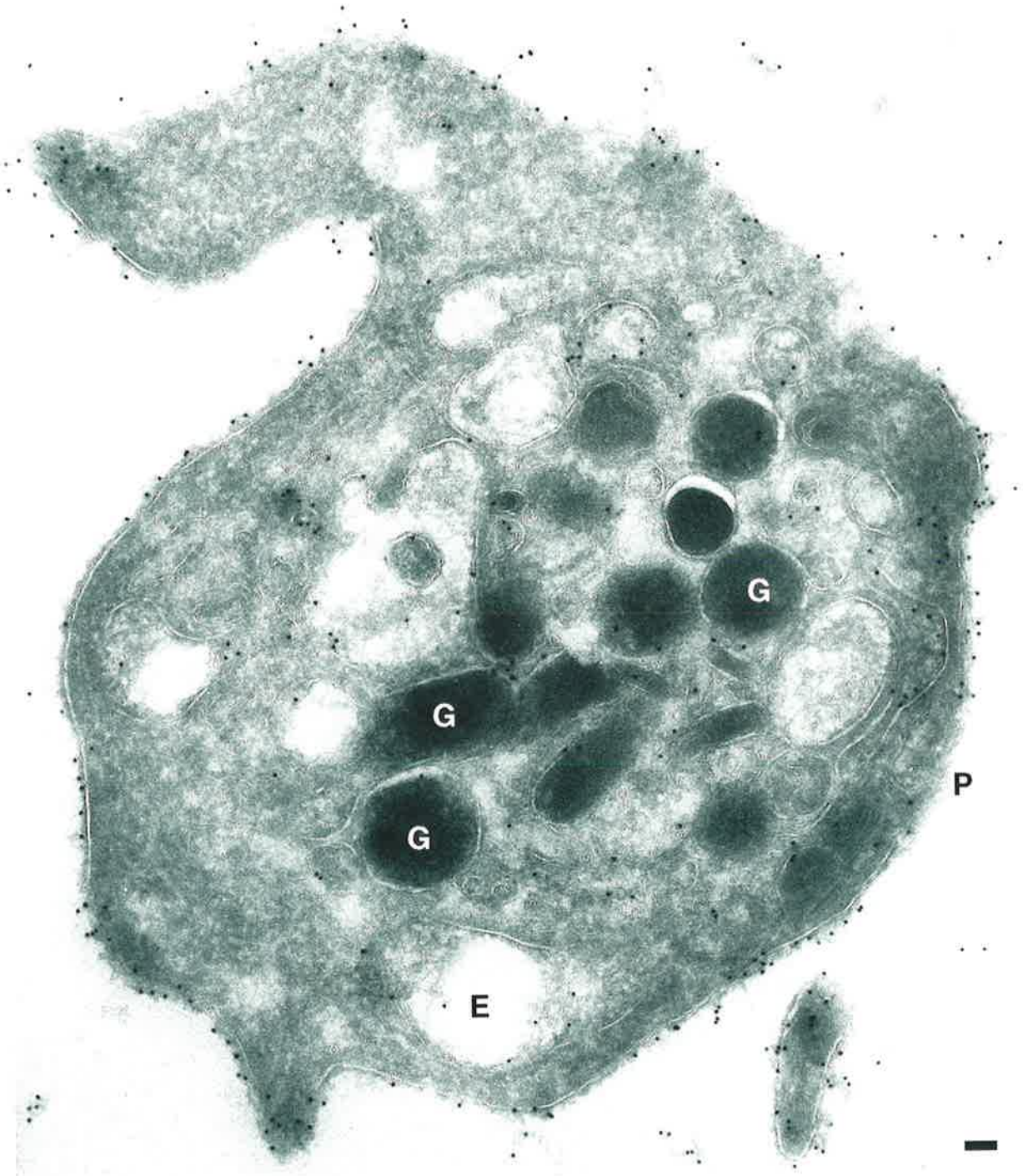

G
G
G
P
E

Figure 4.6.B Ultrastructural Localisation of PETA-3 in Platelets

Ultrathin frozen sections of platelets were labelled with PETA-Rabbit and visualised by 10nm protein A-gold as described (Section 2.7). Panel a shows strong labelling for PETA-3 on the platelet plasma membrane (P) and within a putative endosome (E). Panel b, PETA-3 labelling of a clathrin coated vesicle (double arrowheads). Panel c shows PETA-3 labelling in platelet granules (G). Bars; 100nm.

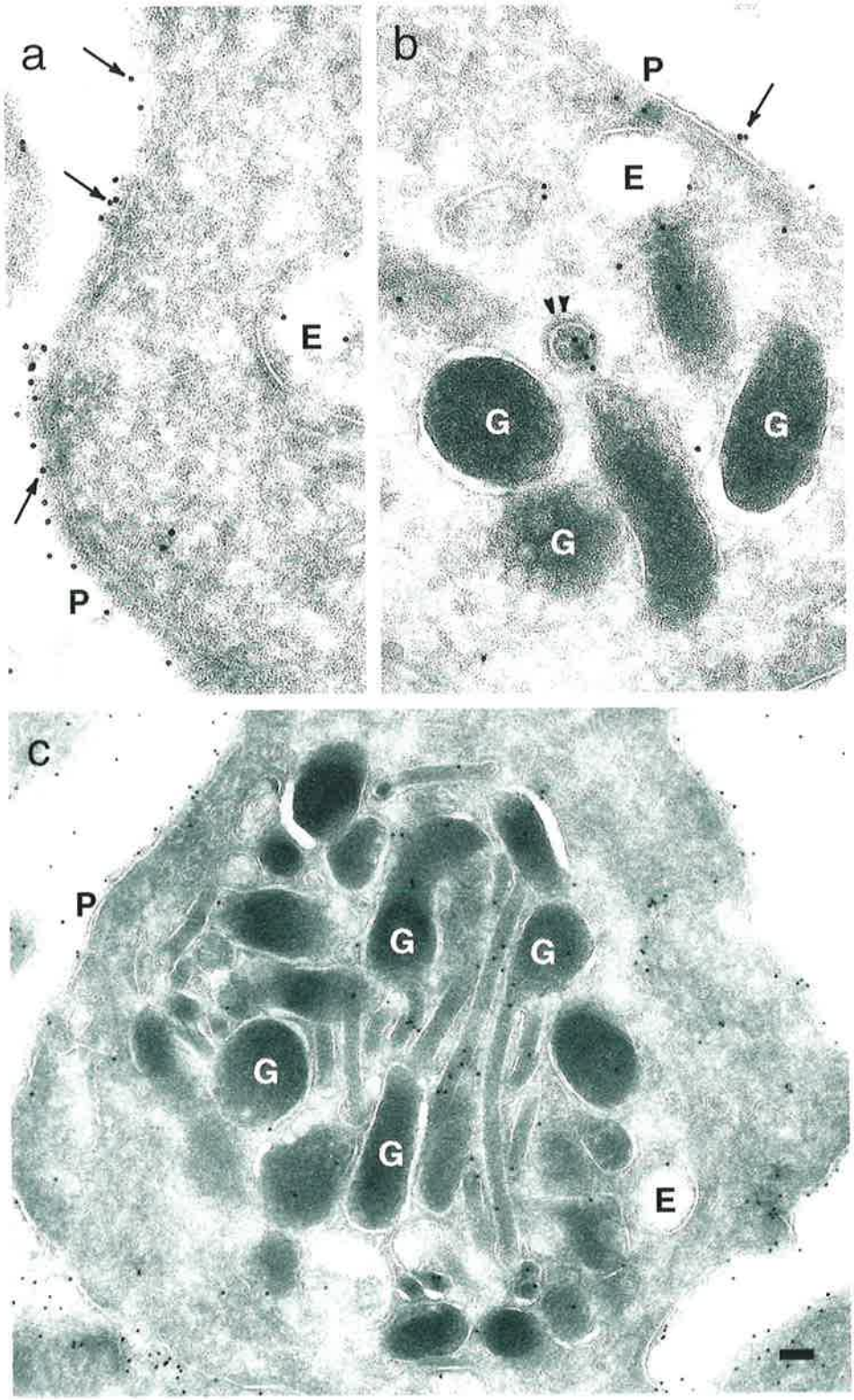
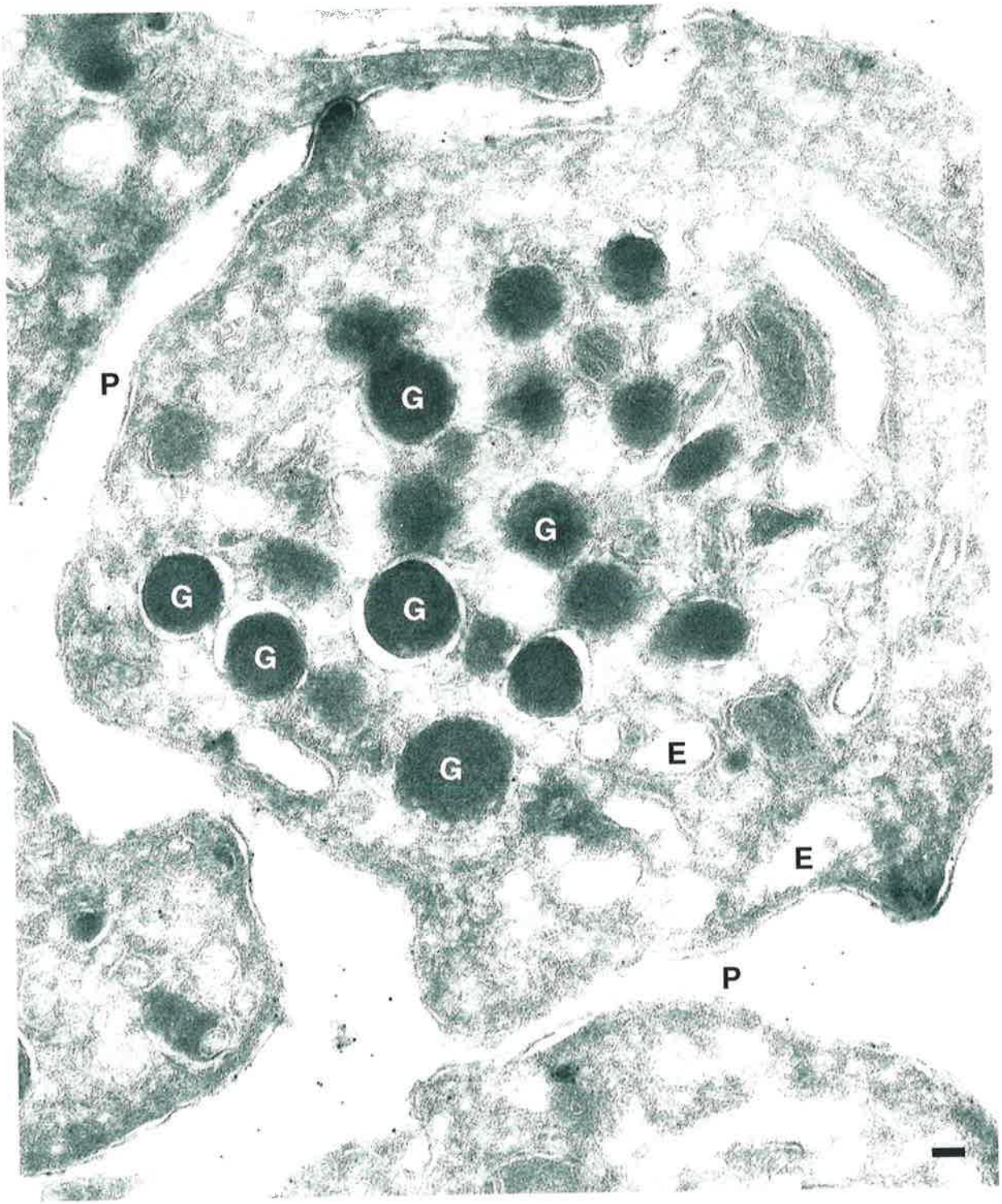

a
E
P
b
P
E
G
G
G
C
P
G
G
G
G
E

Figure 4.6.C Ultrastructural Localisation of PETA-3 in Platelets

Ultrathin frozen sections of platelets were labelled with PETA-Rabbit and visualised by 10nm protein A-gold as described (Section 2.7). Panel shows background labelling of platelets with the specificity control, NRS diluted 1/500 in blocking buffer. Negligible labelling was observed on the plasma membrane (P), endosomal structures (E) or granules (G), although some inter-platelet material was labelled. Bar; 100nm.


P
G
G
G
G
G
G
E
E
P

of surface expression and subcellular localisation of PETA-3 in resting and cytokine treated EC.

Surface expression by cytokine stimulated EC was assessed by flow cytometry as described (Section 2.5.1). Pooled data from three independent experiments are shown in Figure 4.7. Statistical analysis showed no significant change in PETA-3 surface expression for the treatments examined when compared to untreated controls ($P>0.05$). Interestingly, the synergistic action of IL-4 and IFN-γ with TNF, the latter of which has been demonstrated to downregulate integrin expression (reviewed in Luscinskas and Lawler, 1994) failed to modulate the surface expression of PETA-3.

The subcellular localisation of PETA-3 in TNF-α, bFGF or VEGF treated HUVEC was examined by confocal microscopy at 6 and 24 hours post treatment. No detectable change in the subcellular localisation of PETA-3 in cytokine treated HUVEC was observed under these conditions. Figure 4.8 shows a representative experiment using TNF-α stimulated EC. Other markers of endothelial activation, E-selectin and ICAM-1, were included as controls of endothelial activation. E-selectin showed maximal expression at approximately 6 hours post TNF-α treatment whilst ICAM expression remained elevated past the 24 hour time point as previously described (reviewed in Carlos and Harlan, 1994).

4.4.2 Is PETA-3 a Platelet Activation Marker?

CD63 has been well characterised as a platelet activation marker (Nieuwenhuis *et al.*, 1987; Nishibori *et al.*, 1993). Other molecules, including CD9 (Cramer *et al.*, 1994), P-selectin (Hsu-Lin *et al.*, 1984), β1 integrins (Wencel-Drake *et al.*, 1993) and αIIbβ3 (Wencel-Drake *et al.*, 1986), have also been shown to be rapidly mobilised to the platelet surface upon activation. These molecules reside within the membranes of platelet granules, such that upon degranulation they are rapidly exposed on the platelet surface.

Figure 4.7 Surface Expression of PETA-3 is Not Regulated by Cytokines

Cells were pretreated with cytokines, TNF-α (100U/ml), IFN-γ (100U/ml), IL-4 (10ng/ml) or IL-10 (10U/ml) for 6 ; 24 ; or 48 hours prior to staining. Cytokine treated cells were stained by indirect immunofluorescence and analysis by flow cytometry (Sections 2.5.1 and 2.5.3). PETA-3 surface expression by cytokine treated HUVEC was expressed as a percentage of that observed in untreated cells (corrected to 100% for all experiments). Data presented represents the mean ± S.D. of duplicate samples pooled from three separate experiments. Statistical analysis using the unpaired T-test, demonstrated no significant difference between untreated EC and any of the treatments examined $P>0.05$.

ND - Not Done.

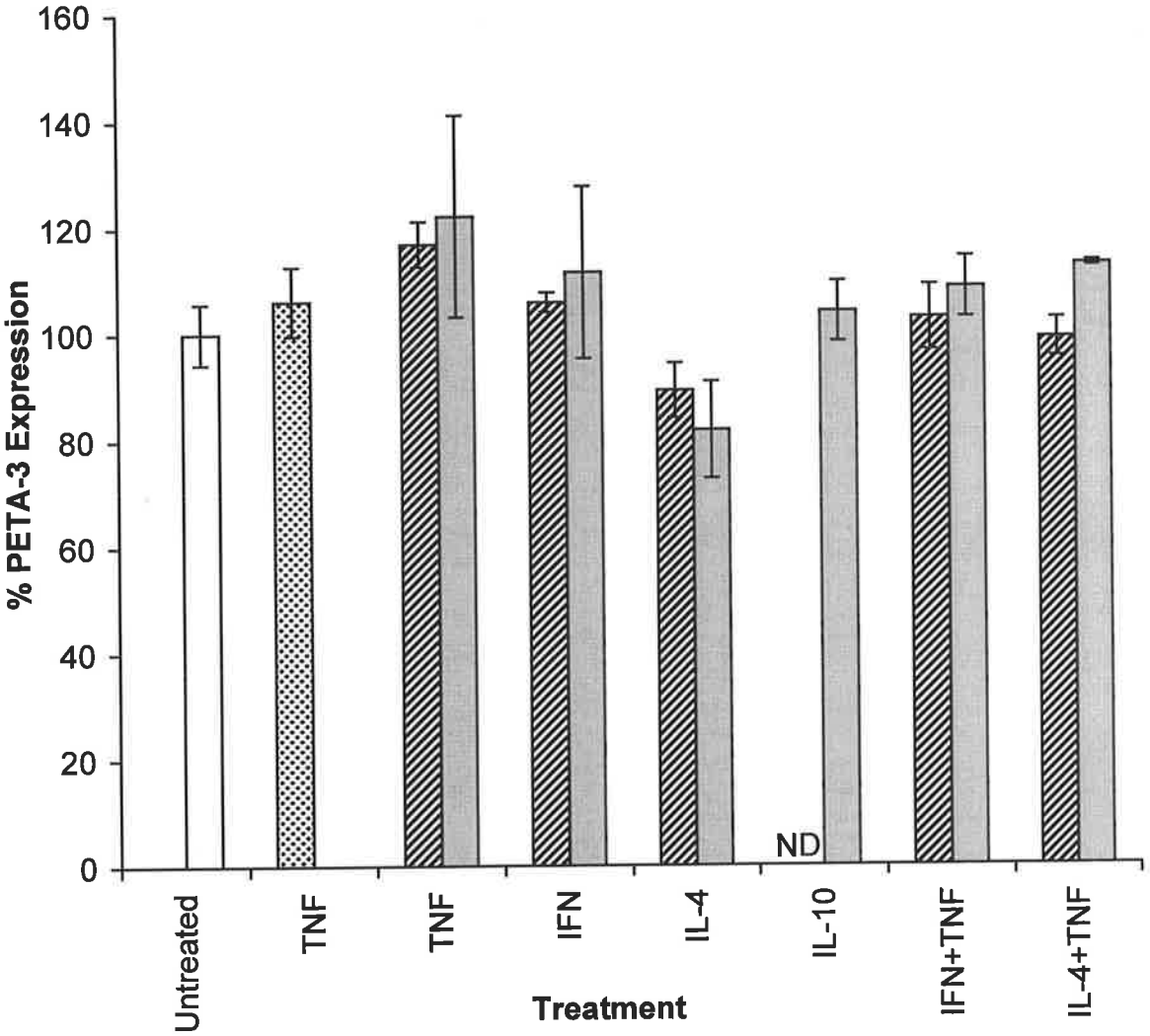
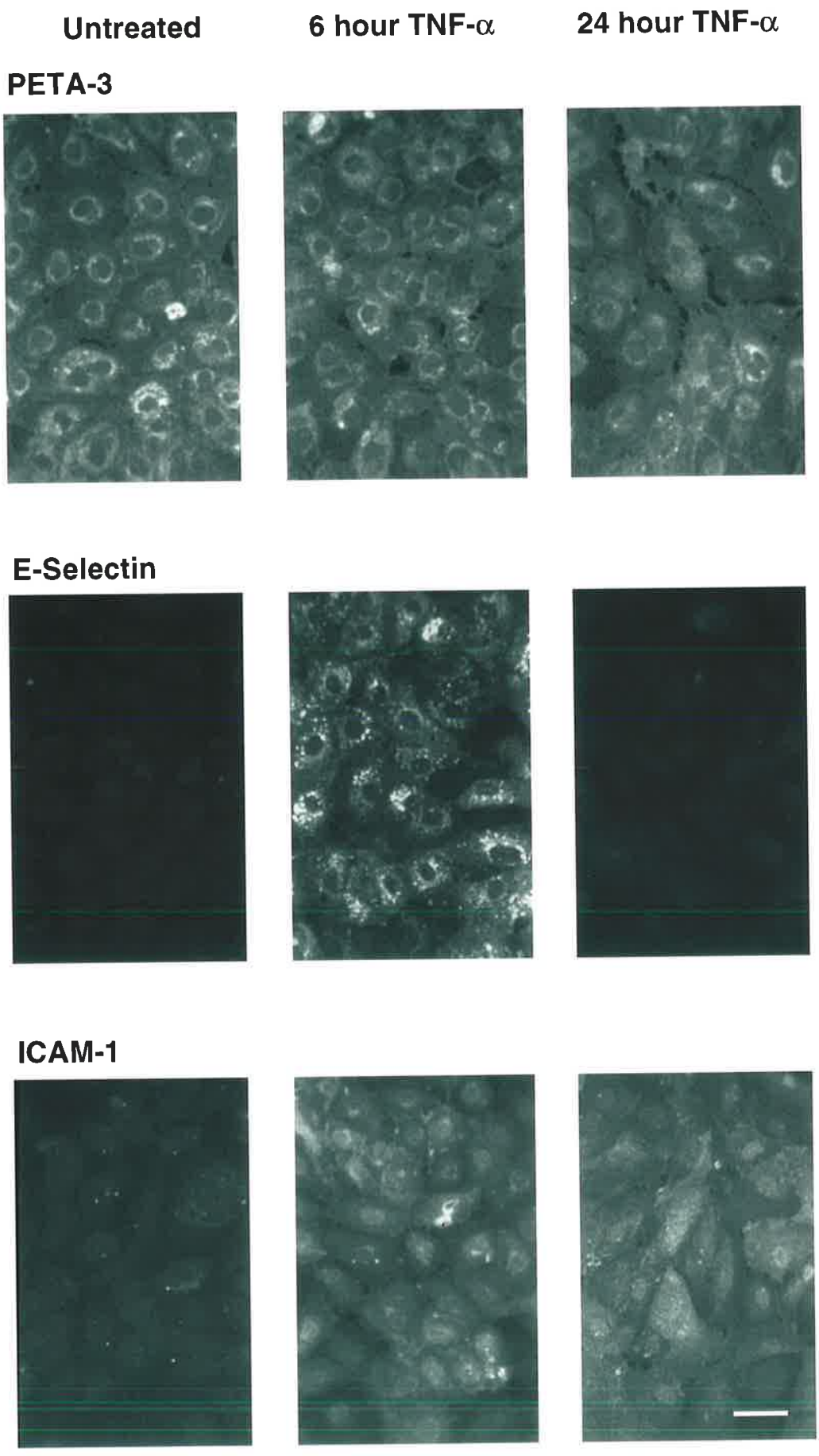

160
140
120
100
80
60
40
20
0
% PETA-3 Expression
ND
Untreated
TNF
TNF
IFN
IL-4
IL-10
IFN+TNF
IL-4+TNF
Treatment

Figure 4.8 The Subcellular Localisation of PETA-3 is Not Regulated by Cytokines

HUVEC were pretreated with TNF-α (100 U/ml) for the times indicated, before being processed for immunofluorescence (Section 2.6). At 6 and 24 hours, TNF-α treatment failed to induce any detectable change in the subcellular distribution of PETA-3. In contrast E-selectin, detected by mAb 1B11, was upregulated after 6 hours TNF-α treatment and returned to basal levels by 24 hours. ICAM-1, detected by mAb P2A4-2, also showed increased surface expression which remained elevated 24 hours post TNF-α treatment. Bar; 55μm.


Untreated
6 hour TNF-α
24 hour TNF-α
PETA-3
E-Selectin
ICAM-1

Whole blood, collected into ETDA as anticoagulant, was either untreated or stimulated with 0.12U/ml thrombin for 5 minutes at room temperature. Blood was then labelled by indirect immunofluorescence as described (Section 2.5.2). Due to the small size of platelets, flow cytometric analysis was performed using logarithmic forward and side scatter settings. Within the indicated gate (Figure 4.9), 99.4% of events were αIIbβ3 positive, thus verifying the platelet gate used for this analysis. Representative histograms, from one experiment of three, show CD63 and PETA-3 surface expression on untreated and thrombin stimulated platelets (Figure 4.10). Percentage upregulated expression was calculated as; MFI of thrombin stimulated platelets over MFI untreated platelets (corrected for negative control binding). Percentages were calculated from the pooled data of three separate experiments. Expression of CD63 on thrombin stimulated platelets was 281±45% that observed on untreated platelets (Figure 4.10.A), as has been previously described (Nieuwenhuis *et al.*, 1987). PETA-3 surface expression on unstimulated platelets was readily detected by the three anti-PETA-3 mAbs 14A2.H1, 11B1.G4 and 14B5 (Figure 4.10.B-D). Upon thrombin treatment, the MFI of 14A2.H1 binding was 143±16% that of untreated platelets (Figure 4.10.B). In contrast, both 11B1.G4 and 14B5 failed to detect any major change in PETA-3 expression on thrombin stimulated platelets, 103±1% and 97±5% respectively, the MFI of untreated platelets (Figure 4.10.C-D).

Figure 4.9 Verification of the Platelet Gate for Flow Cytometric Analysis

EDTA anti-coagulated whole blood was labelled by indirect immunofluorescence as described (Section 2.5.2). Panel (A) shows a representative plot of forward (FSC) and side scatter (SSC) using a logarithmic scale in both cases. Note the heterogeneous scatter characteristics of the red blood cell (Rbc) population. The gate used for all platelet analysis is marked in red. Panel (B) shows isotype matched negative control mAb (1B5) staining of ungated whole blood plotted against SSC. The vertical quadrant was set at the 99th percentile of 1B5 fluorescence. The horizontal quadrant was set at the upper limit of the platelet gate, showing separation of the platelet and Rbc populations. Panel (C) shows staining of whole blood for the platelet marker αIIbβ3 using mAb 10E5. Note the right shift in the low SSC (platelet) population. Panel (D) displays the same staining for αIIbβ3, but only those events lying within the platelet gate are shown (99.4% of events within the designated platelet gate were αIIbβ3 positive).

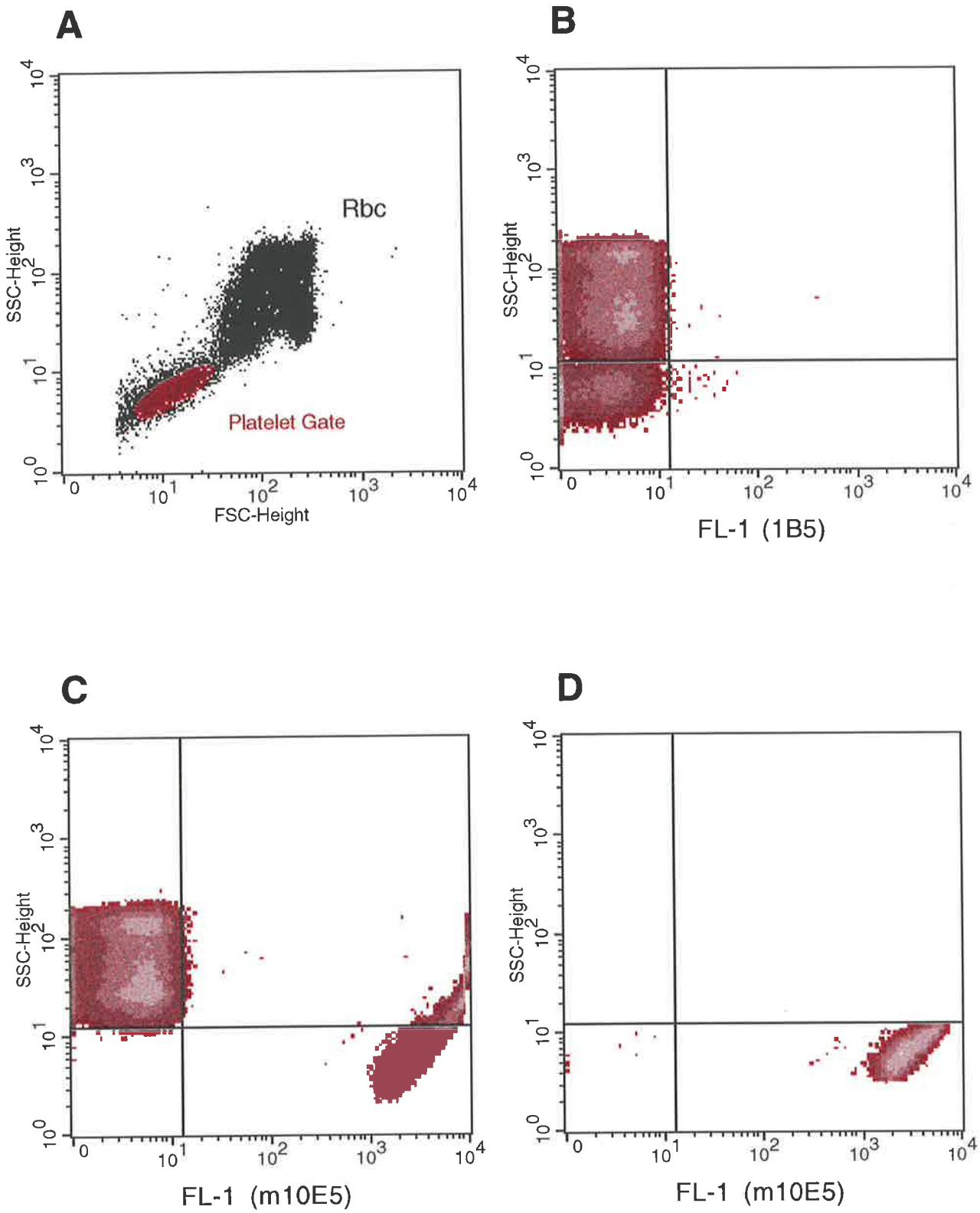

A
Rbc
Platelet Gate
SSC-Height
FSC-Height
B
SSC-Height
FL-1 (1B5)
C
SSC-Height
FL-1 (m10E5)
D
SSC-Height
FL-1 (m10E5)

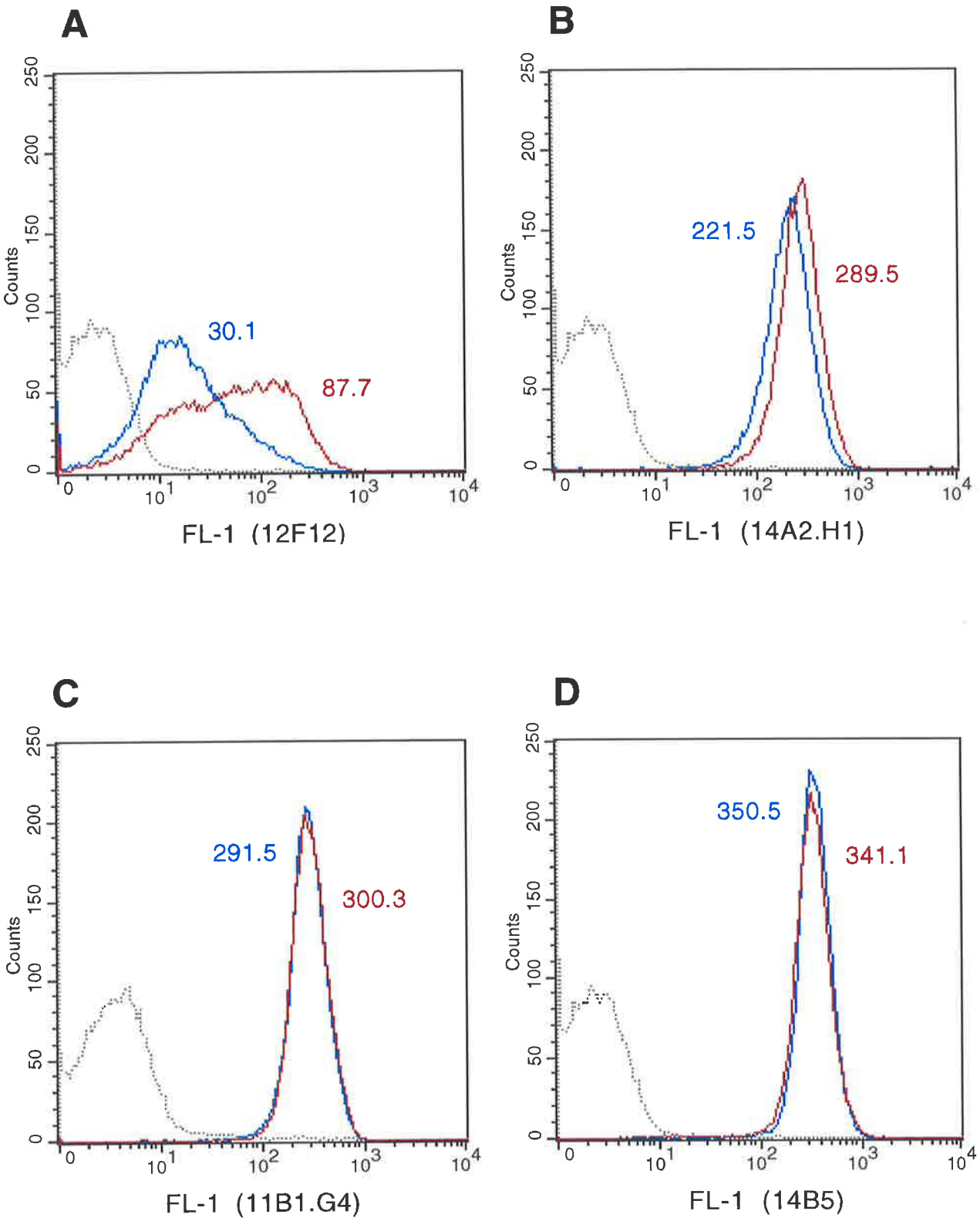
Figure 4.10 Analysis of PETA-3 Expression on Untreated and Thrombin Stimulated Platelets

Platelets (thrombin stimulated or resting) were labelled by indirect immunofluorescence and analysed by flow cytometry (Sections 2.5.2 and 2.5.3). The platelet gate was set as described (see Figure 4.9). CD63 expression was strongly upregulated on thrombin stimulated platelets (A). Expression of PETA-3 on untreated (blue) and thrombin stimulated (red) platelets was examined using the three anti-PETA-3 mAbs (B) 14A2.H1, (C) 11B1.G4 and (D) 14B5. Thrombin treatment showed no effect on negative control mAb binding (data not shown). Data presented are histograms from one representative experiment of three. Numbers represent MFI of respective peaks.

- - - Isotype matched negative control staining.

Blue: Untreated.

Red: Thrombin Stimulated (0.12U/ml for 5 minutes at room temperature).


A
Counts
250
200
150
100
50
0
30.1
87.7
0
10^1
10^2
10^3
10^4
FL-1 (12F12)
B
Counts
250
200
150
100
50
0
221.5
289.5
0
10^1
10^2
10^3
10^4
FL-1 (14A2.H1)
C
Counts
250
200
150
100
50
0
291.5
300.3
0
10^1
10^2
10^3
10^4
FL-1 (11B1.G4)
D
Counts
250
200
150
100
50
0
350.5
341.1
0
10^1
10^2
10^3
10^4
FL-1 (14B5)

4.5 Discussion

Analysis by both flow cytometry and confocal microscopy of permeabilised and non-permeabilised EC revealed both surface and intracellular staining for PETA-3. Interestingly, cell surface PETA-3 was localised to cellular margins (like CD9), but the majority of the protein was intracellular (like CD63). Therefore unlike CD9 and CD63, PETA-3 was not restricted to a single subcellular compartment. While the carboxyl terminal cytoplasmic tail of CD63 contains the putative lysosomal targeting motif, GY (Metzelaar *et al.*, 1991), the domains of PETA-3 responsible for intracellular and plasma membrane targeting remain undefined.

During the course of this project, we became aware of a similar study examining the expression of PETA-3 in EC using independently raised mAbs (Yanez-Mo *et al.*, 1998). These mAbs, LIA1/1 and VJ1/16 were originally raised and screened for their ability to recognise components of EC junctions. Subsequently, colaborative studies identified PETA-3 as the antigen recognised by these mAbs (Yanez-Mo *et al.*, 1998). Although these workers demonstrated localisation of PETA-3 and CD9 to regions of EC contact, they failed to detect intracellular PETA-3 in permeabilised EC monolayers. Interestingly, the 14A2.H1 mAb stains fixed EC monolayers poorly and does not readily detect intracellular PETA-3 (data not shown). Whether this mAb, and those used by Yanez-Mo and colleagues, are more sensitive to fixation or fail to recognise intracellular PETA-3 due to "epitope masking" by other complexed proteins remains unclear (Sections 3.2.19 and 3.3). Interestingly, immunofluorescent studies performed with mAbs LIA1/1 and VJ1/16 suggest they do indeed have binding properties similar to 14A2.H1 (Yanez-Mo *et al.*, 1998).

Intracellular PETA-3 did not colocalise with the Golgi marker, γ-adaptin (Robinson and Pearse, 1986) and its distribution was not sensitive to treatment with BFA, which targets this organelle. Additionally, as the majority of PETA-3 was fully glycosylated and showed

co-localisation with WGA, it was concluded that this molecule predominately resides within a post-endoplasmic reticulum compartment. Although three anti-PETA-3 mAbs failed to stain Weibel-Palade bodies, by light microscopy, a low level of binding was observed by electron microscopy. However, the lack of upregulated PETA-3 surface expression by treatments that induce re-localisation of other proteins, such as P-selectin, from these bodies to the plasma membrane, suggests that it is not a major component of these bodies. Based on these observations, together with the immunoelectron microscopy and 2-colour confocal analysis, the majority of intracellular PETA-3 appears to be associated with vesicles such as endosomes, late endosomes and/or lysosomes, which was confirmed by colocalisation studies using transferrin receptor and CD63. Furthermore labelling of endosomal and late endosomal structures was observed by electron microscopy. Like the recycling mannose 6-phosphate receptor in late endosomes (Griffiths *et al.*, 1988), PETA-3 was mostly present on internal membranes of these structures.

The localisation in clathrin coated pits and vesicles suggests that PETA-3 is internalised from the plasma membrane. Additionally, localisation to tubular vesicular elements of early endosomes, and the colocalisation with perinuclear transferrin receptor, which is predominantly associated with the perinuclear recycling vesicle (Gruenberg and Maxfield, 1995), imply recycling of PETA-3. Given the ultrastructural localisation of PETA-3 in early and late endosomes, this molecule may actively recycle throughout the endocytic pathway. The endosomal localisation raises the possibility that PETA-3 may be involved in vesicular transport *per se*. Antibodies against other TM4SF members, CD81 and CD82, inhibit syncitium formation (the fusion of the plasma membrane) in human T cell leukaemia virus infected cells (Imai and Yoshie, 1992; Imai and Yoshie, 1993). Thus it is possible that PETA-3 may play a role in the fusion of cellular membranes, in particular

vesicles within the endocytic pathway. Alternatively this molecule may be involved in protein-protein interactions mediating internalisation or sorting events.

Little CD63 was detected on the plasma membrane by flow cytometry and this molecule was predominantly detected within perinuclear vesicles. Based on previous characterisation of CD63, these structures were most likely late endosomes and lysosomes. In contrast, CD9 was exclusively localised to the plasma membrane and EC margins. Interestingly, PETA-3 was seen in both compartments. Therefore if PETA-3/CD9 and PETA-3/CD63 complexes exist in EC, as has been described in other cell types (Fitter *et al.*, submitted), they would be expected to be found in regions of cell-cell contact and late endosomes/lysosomes respectively. Investigation of the association of PETA-3 with CD9, CD63 and integrins in EC is described in the following Chapter (Section 5.2).

Experiments investigating the modulation of PETA-3 surface expression by cytokines found little evidence for its regulation in EC. Other studies have demonstrated increased PETA-3 expression on activated T cells and transactivation by the Human T cell Leukaemia virus-1 protein Tax (Hasegawa *et al.*, 1996). Recent studies investigating the murine homologue of PETA-3 have characterised the genomic structure and identified three splice variants of the transcript encoding mouse PETA-3 (Hasegawa *et al.*, 1997; Fitter *et al.*, 1998). These transcripts differ in their 5' untranslated regions and appear to be widely expressed. Perhaps these transcripts reflect alternative transcriptional regulatory mechanisms of PETA-3 expression between various cell types and/or resting versus activated cells. At the time experiments investigating the regulation of PETA-3 by cytokines were carried out, the association of TM4SF members with integrins had not been described. Thus cytokine modulated integrin expression was not addressed. However, the lack of PETA-3 regulation observed in these experiments, including those stimuli known to modulate integrin surface

expression (ie the synergistic effect of TNF-α and IFNγ) did not warrant further pursuit of this issue.

Although immunofluorescence studies with three anti-PETA-3 mAbs failed to detect PETA-3 expression in Weibel-Palade bodies, low level binding was observed in these structures by the anti-PETA-3 rabbit polyclonal antibody using electron microscopy. Interestingly, the anti-CD63 mAb used in this study (12F12) failed to detect CD63 in Weibel-Palade bodies, despite its demonstrated localisation to these granules by other studies (Vischer and Wagner, 1993). Therefore it may be possible that these mAbs fail to detect PETA-3 and CD63 in Weibel-Palade bodies due to epitope masking (Sections 3.2.19 and 3.3). Insufficient permeabilisation was clearly not the case as the anti-vWF mAb readily detected these bodies. Thus, based on electron microscopy studies, it appears that a little PETA-3 may be present in Weibel-Palade bodies, and the slight increase in PETA-3 surface expression in TNF-α treated EC may have arisen from these granules. Nonetheless, the data presented in this study clearly show that the majority of intracellular PETA-3 does not reside within these granules. This finding was also confirmed by the lack of PETA-3 redistribution in cytokine treated EC.

Labelling of PETA-3 was observed in clathrin coated vesicles, endosomes, other internal membranes, which may be components of the open canalicular system, and the plasma membrane of platelets by immunoelectron microscopy. Furthermore, labelling of platelets was much stronger than that observed in EC, suggesting a higher density of PETA-3 in platelets. The anti-PETA-3 rabbit polyclonal antibody also labelled platelet granules. Thus, like CD63 and CD9, PETA-3 is also a component of platelet granules. However unlike these other TM4SF members, PETA-3 does not appear to be as highly concentrated in these structures. Additionally, some PETA-3 labelling was observed in multivesicular bodies within platelet granules. Multivesicular bodies are believed to be precursor of α-granules and the internal vesicles of these structures have been demonstrated to be enriched in CD63 and

P-selectin (Heijen *et al.*, 1998). Trafficking of molecules into multivesicular bodies is thought occur by direct transport from the Golgi, or via internalisation from the plasma membrane and subsequent transport via endosomes (Heijen *et al.*, 1998). Localisation of PETA-3 to the plasma membrane, clathrin vesicles and endosomal structures supports the latter mechanism of PETA-3 transport to platelet granules. Recent studies have also demonstrated enrichment of other TM4SF members in vesicles of multivesicular endosomes and secreted exosomes of B cells (Escola *et al.*, 1998). Perhaps the mechanism by which TM4SF members are targeted to internal membranes of multivesicular structures is shared between lymphocytes, platelets and EC.

The high expression of PETA-3 on the platelet membrane, as detected by both flow cytometry and immunogold labelling, differs from the initial studies which estimated approximately 600-1200 copies per platelet using saturation binding methodology (Ashman *et al.*, 1991). Although the earlier studies used the inferior 14A2.H1 mAb, this antibody bound platelets well throughout the current study. Whether iodination of this mAb in the earlier study diminished binding or some other factor led to the initially low estimate remains unclear. However, the relative sensitivity of flow cytometric analysis used in this study may be a major factor. Studies investigating the surface expression on thrombin stimulated platelets suggest that PETA-3 is probably not upregulated on platelet activation. Furthermore, the two anti-PETA-3 mAbs which did not detect increased expression are those which appear to bind PETA-3 under all circumstances (Sections 3.2.19 and 3.3). As to why increased binding of 14A2.H1 on thrombin stimulated platelets was observed remains unclear. 14A2.H1 binding might be sensitive to a conformational change in PETA-3 induced by platelet activation. Alternatively, dissociation of integrin chains (or other associated molecules) during platelet activation might reveal the 14A2.H1 epitope (see Sections 3.2.19 and 3.3). Concurrent studies have shown that the association of PETA-3 with $\alpha 4\beta 1$, $\alpha 5\beta 1$,

and α6β1 integrins is constitutive (Fitter *et al.*, submitted). Whether the association of PETA-3 with platelet integrins α2β1 or αIIbβ3 (or another as yet identified molecule) is inducible and might contribute to increased 14A2.H1 binding remains to be investigated.

In summary,the studies described in this Chapter have characterised the localisation of PETA-3 to EC margins and the platelet plasma membrane. Additionally the presence of intracellular PETA-3 in the endocytic pathway of platelets and EC was demonstrated. However, unlike other members of the TM4SF, modulation of PETA-3 expression by cellular activation was not readily evident in platelets and EC.

CHAPTER 5: PROTEIN-PROTEIN INTERACTIONS AND FUNCTIONAL ANALYSIS OF ANTI-PETA-3 ANTIBODIES IN PLATELETS AND ENDOTHELIAL CELLS

5.1 Introduction

The vascular endothelium provides a dynamic non-thrombogenic barrier between the bloodstream and extravascular tissues. The cellular interactions required for the maintenance and function of the vascular endothelium are tightly regulated, and are mediated through soluble factors and adhesion molecules. Members of one such family of adhesion molecules, the integrins, are involved in cell-matrix and cell-cell adhesive events through their interaction with the cytoskeleton, as had been discussed (Section 1.3). Cultured EC express many integrins including several of the β1 subfamily, αvβ3, α6β4 and αvβ5 (Section 1.4). These molecules have been shown to be involved in many aspects of endothelial function including vasculogenesis, angiogenesis, wound healing and the recruitment of leukocytes to sites of inflammation (reviewed in Shattil and Ginsberg, 1997; Shattil, 1995; Luscinskas and Lawler, 1994; Smyth *et al.*, 1993). Integrins also play a central role in platelet function and the regulation of thrombosis. Platelet adhesion, spreading, activation and aggregation have all been shown to be mediated by integrin receptors. Although the major integrin expressed by platelets is αIIb/β3, members of the β1 subfamily, α2β1, α5β1 and α6β1, and αvβ3 have also been detected (Section 1.5.3).

The association of CD9, CD63, CD81 and CD82, with α3β1 (Berditchevski *et al.*, 1995; Nakamura *et al.*, 1995; Berditchevski *et al.*, 1996; Hadjiargyrou *et al.*, 1996), α4β1 (Rubinstein *et al.*, 1994; Mannion *et al.*, 1996) and α6β1 integrins (Berditchevski *et al.*, 1995; Berditchevski *et al.*, 1996; Hadjiargyrou *et al.*, 1996) has been reported (reviewed in Hemler *et al.*, 1996; and summarised in Table 1.1). Furthermore, mAbs against CD9 and CD81 have

been shown to modulate cellular motility and adhesion (Masellis-Smith *et al.*, 1990; Takahashi *et al.*, 1990; Forsyth, 1991; Masellis-Smith and Shaw, 1994; Shaw *et al.*, 1995; Yanez-Mo *et al.*, 1998), and several of the functional responses elicited by anti-TM4SF mAbs may be inhibited by anti-integrin mAbs. Consistent with these findings, TM4SF proteins have been proposed to act as molecular facilitators of integrin complexes, thus modulating integrin function (reviewed in Maecker *et al.*, 1997). Similarly, the association of PETA-3 with β1 integrins in haemopoietic cells has been described (Ashman *et al.*, 1997) and $F(ab')_2$ fragments of anti-PETA-3 mAbs have been shown to induce homotypic adhesion of haemopoietic cell lines (Fitter, Sincock, Jolliffe and Ashman, submitted). Therefore, like other members of the TM4SF, PETA-3 appears to have the potential to modulate integrin mediated cell functions (Section 1.2.5).

The first aim of the current section was to characterise possible protein-protein interactions involving PETA-3 in EC. EC express a wide range of integrins (reviewed in Luscinskas and Lawler, 1994; Smyth *et al.*, 1993), as well as a variety of other adhesion molecules (reviewed in Carlos and Harlan, 1994). The experiments described in the following sections were designed to extend the earlier studies performed in haemopoietic cells. Furthermore, several other EC adhesion molecules were included to examine the possibility that PETA-3 may be associated with other non-integrin molecules.

Additionally, the effect of anti-PETA-3 mAbs on platelet and EC function were investigated. Previous studies have demonstrated that 14A2.H1 acts as a platelet agonist, promoting platelet activation, degranulation and aggregation (Ashman *et al.*, 1991; Roberts *et al.*, 1995). This mAb synergised with other platelet agonists, including ADP, serotonin and adrenaline. However, like most other platelet activating mAbs, signalling via 14A2.H1 was shown to be mediated (at least in part) by cross-linking of the FcγRII (Section 1.5.5). Subsequently, several other anti-PETA-3 mAbs have been raised in this laboratory. Perhaps

most interesting of these is 11B1.G4. This mAb displays high affinity binding to PETA-3 and is of the IgG2a subtype. Since mAbs of the IgG2a subtype interact poorly with FcγRII (Jones *et al.*, 1985), 11B1.G4 was assessed for its ability to induce activation and aggregation of platelets.

Finally, the effect of anti-PETA-3 mAbs on EC function was investigated using cultured HUVEC. These cells provide a convenient and useful model for examining EC function *in vitro*, as several model systems have been established. Such models allow the assessment of neutrophil transendothelial migration, EC motility and EC differentiation during angiogenesis. Using these models, the role of PETA-3 in these aspects of EC function was examined.

5.2 Characterisation of PETA-3 Protein-Protein Interactions

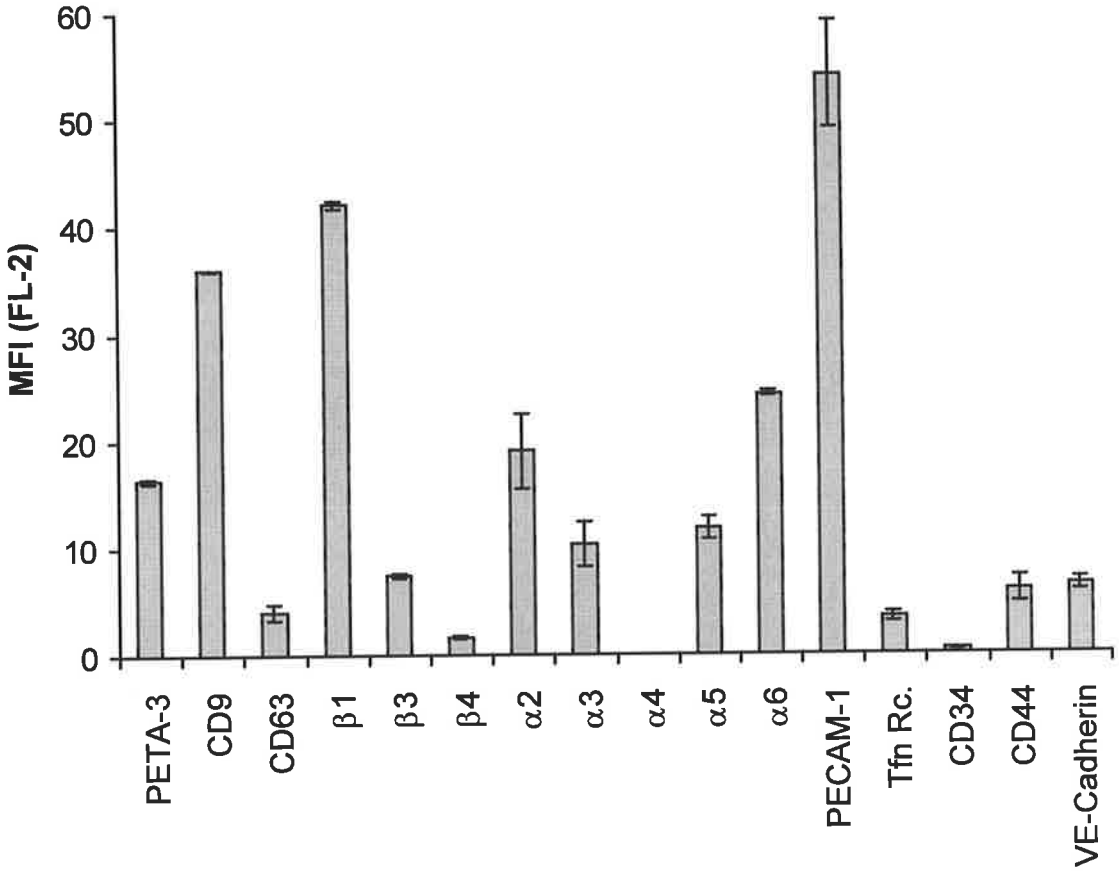
5.2.1 Surface Expression of TM4SF Members and Integrins by Cultured HUVECs

The expression of integrins, and several other molecules, by cultured EC has been shown to vary depending upon the species, vascular bed from which the cells arise, culture conditions and passage number (reviewed in Luscinskas and Lawler, 1994). Therefore preliminary flow cytometric analysis was carried out on HUVEC to characterise the surface expression of TM4SF members, integrins and other molecules that were investigated in the experiments described in this Chapter (Figure 5.1). Antibodies against members of the TM4SF varied in their level of surface binding with very strong staining of CD9, strong PETA-3, and low staining of CD63. Binding of the anti-β1 integrin mAb, 61.2C4, was the highest of the β chains examined, with lower levels of β3 and β4 detected. Expression of α2 and α6 were also high, with lower α3 and α5 levels detected. The α4 chain was not detected. The activity of the anti-α4 mAb HP2/1 was verified by binding to haemopoietic cells (Fitter *et al.*, submitted), and expression of α4 by cultured HUVEC has not been widely reported. Thus

Figure 5.1 Surface Expression of TM4SF Members, Integrins and Other Antigens on 4th Passage EC

Fourth passage HUVEC were harvested, stained by indirect immunofluorescence (Section 2.5.1) and analysed by flow cytometry (Section 2.5.3). Data presented represent the mean ± S.D. of duplicate samples, corrected for negative control mAb staining, from one experiment of three.

Tfn Rc. - Transferrin Receptor


MFI (FL-2)
60
50
40
30
20
10
0
PETA-3
CD9
CD63
β1
β3
β4
α2
α3
α4
α5
α6
PECAM-1
Tfn Rc.
CD34
CD44
VE-Cadherin

α4 was not further investigated in the subsequent analysis. By comparison, surface expression of PECAM-1 was very high, whilst VE-cadherin was low. Both of these molecules are specifically localised to regions of EC-EC contact. The surface level of transferrin receptor was comparable to that of VE-cadherin and CD63. CD44 was also present at low levels and CD34 lower still. Although the mean fluorescence intensity of anti-CD34 mAb binding was low, expression was heterogenous such that very high levels of CD34 were detected on a sub-population of cells (data not shown).

5.2.2 PETA-3 Associates with Multiple Integrins in Endothelial Cells

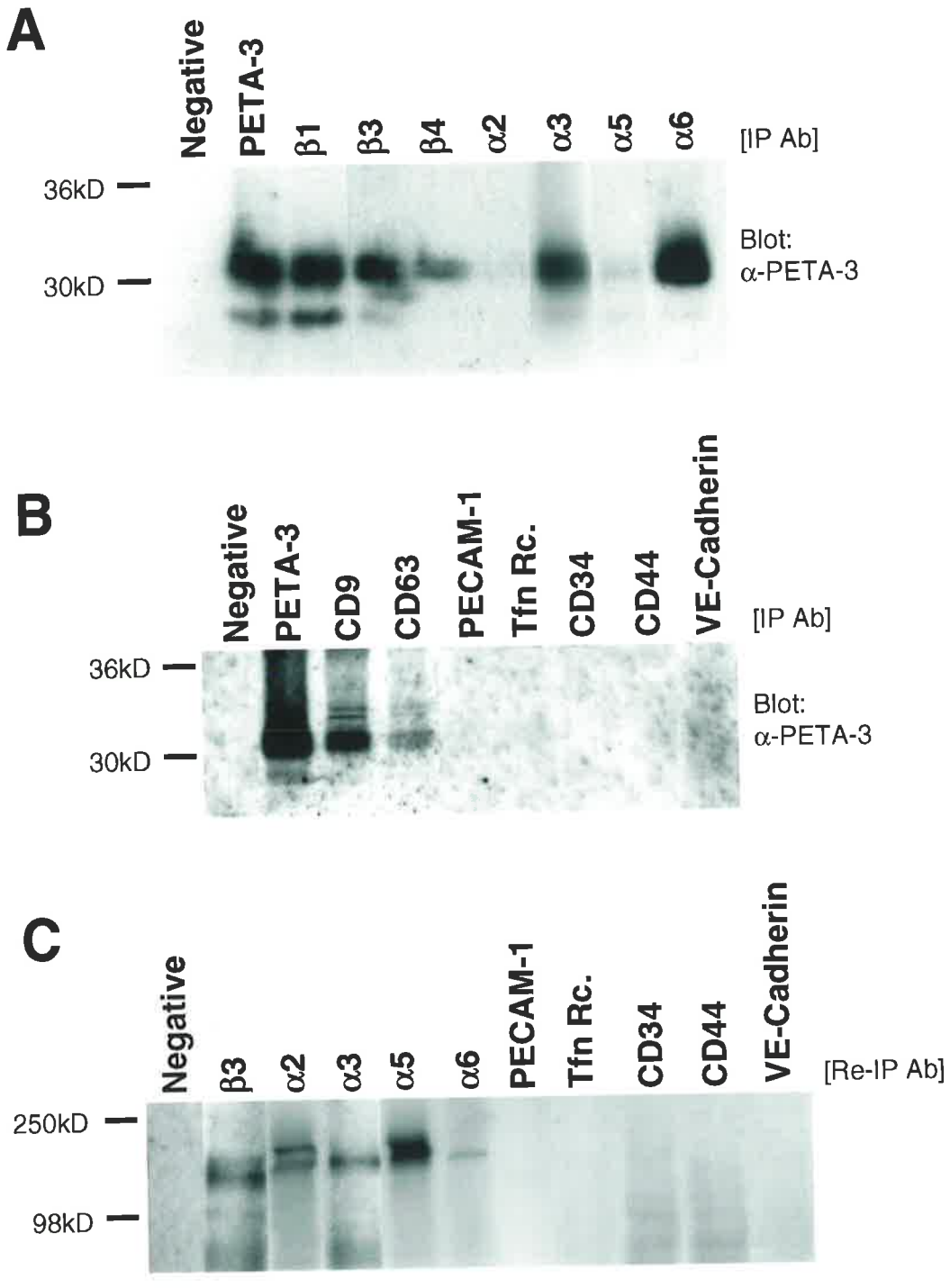
To examine possible association of PETA-3 with other proteins, confluent fourth passage EC monolayers were lysed in 1% CHAPS lysis buffer and immunoprecipitated with antibodies as described (Section 2.8). Western blotting with anti-PETA-3 mAb 11B1.G4 (Section 2.8.6) showed co-immunoprecipitation of PETA-3 with the integrin β1, β3, β4, α3 and α6 chains (Figure 5.2.A). In addition, co-immunoprecipitation was weakly observed in α2 and α5 immunoprecipitates using this methodology. The PETA-3 doublet represents the mature, fully glycosylated form (32kD) and the immature form (27kD). This was verified by treatment of the immunoprecipitates with Endoglycosidase F, which resulted in the conversion of the 32kD form to the lower molecular weight form (data not shown). In order to assess the specificity of PETA-3/integrin interactions, immunoprecipitations with several other endothelial adhesion molecules and with transferrin receptor, which was previously shown to colocalise with PETA-3 (Section 4.2.2) were carried out (Figure 5.2.B). PETA-3 was not detected in PECAM-1, transferrin receptor, CD34, CD44, or VE-cadherin precipitates but did co-immunoprecipitate with the other TM4SF members CD9 and CD63, as observed in haemopoietic cells (Fitter *et al.*, submitted).

Figure 5.2 Co-immunoprecipitation of PETA-3 with Multiple Integrins from CHAPS Lysates

(A,B) EC lysates were prepared using 1% CHAPS and immunoprecipitations were performed using the indicated mAbs. Immunoprecipitated proteins were separated on 12% PAGE gels under non-reduced conditions, transferred to nitrocellulose and Western blots were performed using biotinylated anti-PETA-3 mAb 11B1.G4 as described (Section 2.8).

(C) EC were surface biotinylated and lysed in 1% CHAPS. Immunoprecipitations with the anti-PETA-3 mAb 11B1.G4 were carried out and followed by solubilisation of immunoprecipitates in NP 40. Re-precipitation from NP 40 solubilised 11B1.G4 immunoprecipitates were then performed, separated by PAGE and detected by streptavidin-alkaline phosphatase as described (Section 2.8).

Tfn Rc. - Transferrin Receptor


A
Negative
PETA-3
β1
β3
β4
α2
α3
α5
α6
[IP Ab]
36kD
30kD
Blot:
α-PETA-3
B
Negative
PETA-3
CD9
CD63
PECAM-1
Tfn Rc.
CD34
CD44
VE-Cadherin
[IP Ab]
36kD
30kD
Blot:
α-PETA-3
C
Negative
β3
α2
α3
α5
α6
PECAM-1
Tfn Rc.
CD34
CD44
VE-Cadherin
[Re-IP Ab]
250kD
98kD

To further confirm the interaction of PETA-3 with α2 and α5 integrin chains, re-precipitations from re-solubilised 11B1.G4 immunoprecipitates were carried out as described (Section 2.8.4). Using this methodology, α2 and α5 were readily detected, thus confirming their association with PETA-3 (Figure 5.2.C). Similarly, α3, α6 and β3 were detected using this method indicating a cell surface association with PETA-3. As specificity controls, PECAM-1, transferrin receptor, CD34, CD44, and VE-cadherin re-precipitations were carried out, all of which failed to re-precipitate under these conditions. In order to verify that the mAbs used as specificity controls were capable of immunoprecipitation, EC were surface labelled with biotin and immunoprecipitations with these mAbs were carried out (Figure 5.3.A) as described (Sections 2.8.2 and 2.8.3). Immunoprecipitated proteins were separated using 12% PAGE and detected by streptavidin-HRP as described (Sections 2.8.5 and 2.8.6). Apart from negative controls, all mAbs tested immunoprecipitated proteins of predicted molecular weight (Kishimoto *et al.*, 1997) (Figure 5.3.B).

Given the hydrophobic nature of TM4SF proteins, the use of mild detergents has been suggested to lead to artifactual associations (Hemler *et al.*, 1996). Therefore, co-immunoprecipitation experiments using an alternative solubilising agent, (1% NP 40), were carried out in parallel to the studies performed with CHAPS (Figure 5.4). Under these conditions, co-immunoprecipitation with β1, β4 and α3 were observed, however α2 and α5 were not detected. Additionally the co-immunoprecipitation of β3 and α6 was much weaker than that found using 1% CHAPS. Finally the associations of PETA-3 with CD9 and CD63 were not observed under these conditions (Figure 5.4.B).

5.2.3 Colocalisation of PETA-3 with Integrins

To confirm that the associations observed in co-immunoprecipitation experiments may occur in intact cells, and to investigate whether integrin associations with intracellular

Figure 5.3 Verification of Immunoprecipitating Antibodies

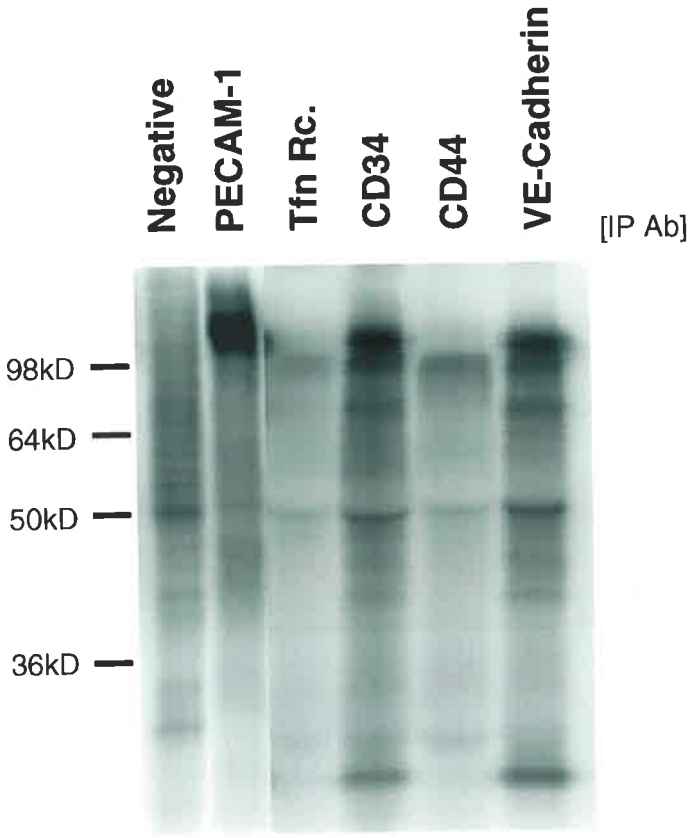
Panel (A), EC were surface biotinylated, lysed in 1% CHAPS and immunoprecipitations were performed using the indicated mAbs. Immunoprecipitated proteins were separated on 12% PAGE gels under non-reduced conditions, transferred to nitrocellulose and detected by streptavidin-alkaline phosphatase as described (Section 2.8).

Panel (B) shows the predicted molecular weights* for the immunoprecipitated antigens shown in Panel (A).

* Predicted molecular weights based on those published by (Kishimoto *et al.*, 1997).

Tfn Rc. - Transferrin Receptor

A



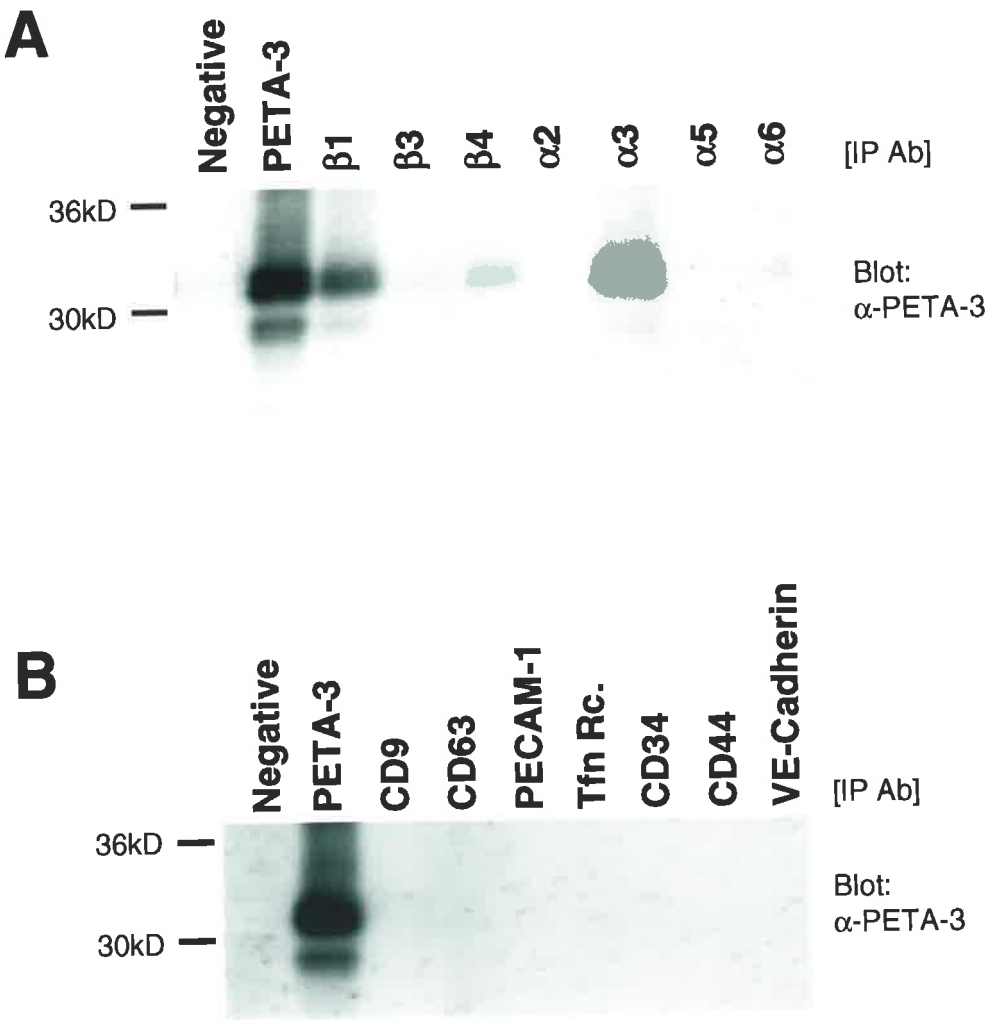
B

Antigen	Predicted Molecular Weight* (kD)
PECAM-1	140
Tfn Rc.	95
CD34	105 -120
CD44	80-95
VE-Cadherin	135

Figure 5.4 Co-immunoprecipitation of PETA-3 with Multiple Integrins from NP 40 Lysates

(A,B) EC lysates were prepared using 1% NP 40 and immunoprecipitations were performed using the indicated mAbs. Immunoprecipitated proteins were separated on 12% PAGE gels under non-reduced conditions, transferred to nitrocellulose and Western blots were performed using biotinylated anti-PETA-3 mAb 11B1.G4 as described (Section 2.8).

Tfn Rc. - Transferrin Receptor


A
Negative
PETA-3
β1
β3
β4
α2
α3
α5
α6
[IP Ab]
36kD
30kD
Blot:
α-PETA-3
B
Negative
PETA-3
CD9
CD63
PECAM-1
Tfn Rc.
CD34
CD44
VE-Cadherin
[IP Ab]
36kD
30kD
Blot:
α-PETA-3

PETA-3 may exist, the colocalisation of integrins with PETA-3 was examined by 2-colour confocal microscopy in permeabilised EC monolayers as described (Section 2.6). The integrin β1 chain showed colocalisation with PETA-3 in both cellular margins and within intracellular vesicles in confluent EC monolayers (Figure 5.5.A-C). Similarly, staining of intracellular β3 colocalised with PETA-3 (Figure 5.5.D-F). The α5β1 integrin has previously been shown to be present in cell contacts of confluent EC monolayers (Lampugnani *et al.*, 1991). Staining of confluent monolayers for α5 demonstrated expression on the plasma membrane which colocalised with PETA-3. Additionally, α5 colocalised with PETA-3 to perinuclear vesicles (Figure 5.5.G-I). The α2, α3 and α6 chains are also shown for comparison (Figure 5.5.J, K and L). The α2 chain predominantly stained lateral margins of EC as previously reported (Lampugnani *et al.*, 1991). In contrast, the intracellular staining of α3 appeared similar to that of the endoplasmic reticulum antigen gp180 (Section 4.2.1). The α6 was localised to cytoplasmic vesicles and strongly resembled staining with α5.

5.3 The Anti-PETA-3 Antibody 11B1.G4 Acts as a Platelet Agonist

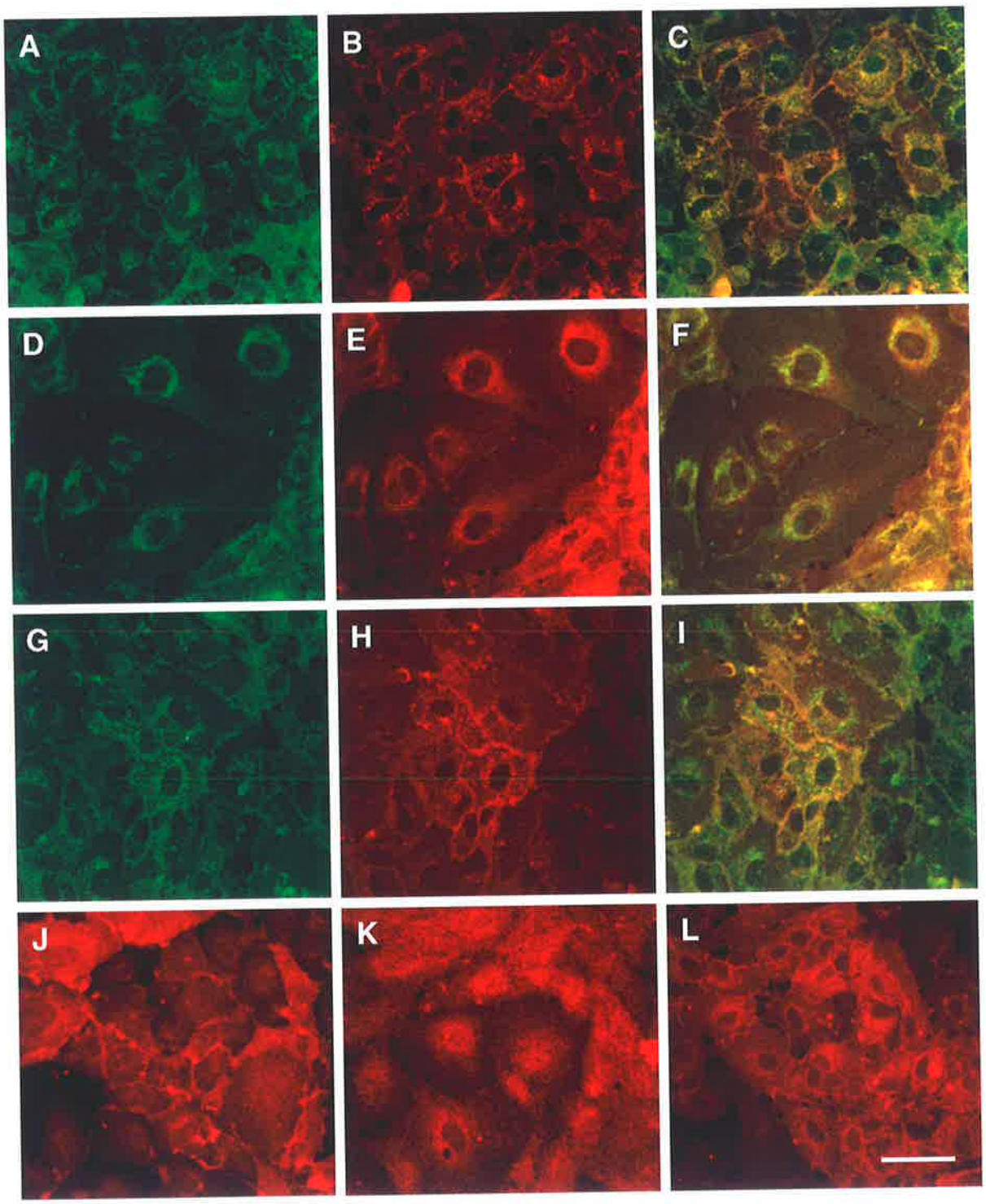
5.3.1 Investigation of Platelet Aggregation Induced by Anti-TM4SF mAbs

Previous studies have shown that treatment of PRP with whole Ig of anti-CD9 or the anti-PETA-3 mAb 14A2.H1 induces aggregation which is dependent on crosslinking of the FcγRII (Worthington *et al.*, 1990; Roberts *et al.*, 1995) (Sections 1.5.4 and 1.5.5). MAbs of the IgG2a subtype have been reported to bind less avidly to the FcγRII (Jones *et al.*, 1985), suggesting that the anti-PETA-3 mAb 11B1.G4 would not crosslink this receptor as readily as the IgG1 mAb 14A2.H1. Therefore the potential of 11B1.G4 to induce platelet aggregation was investigated.

Platelet rich plasma (PRP) was obtained by centrifugation of citrate anti-coagulated whole blood as described (Section 2.9.1). For all platelet aggregometry, PRP was

Figure 5.5 Colocalisation of PETA-3 with Integrins in EC

EC were permeabilised, labelled by indirect immunofluorescence and examined by confocal microscopy as described (Section 2.6). Staining of β1 (panel B), β3 (panel E), α5 (panel H) and α3 (panel K) integrin chains was visualised using FL-1 (Red) with PETA-3 staining visualised using FL-2 (Green; panels A, D and G) of the same field. Merges of red and green images were produced to demonstrate colocalisation (Yellow; panels C, F and I). Intracellular PETA-3 colocalised with β1 (A-C), β3 (D-F) and α5 integrin chains (G-I). Furthermore, β1 and α5 also colocalised to cellular margins. For comparison, fields showing (J) α2, (K) α3 and (L) α6 staining are also presented. Bar; 55μm.


A
B
C
D
E
F
G
H
I
J
K
L

standardised to 300,000 platelets/μl by supplementation with autologous platelet depleted plasma. Aggregometry was carried out at 37°C with stiring as described (Section 2.9.2). All experiments reported were performed at least in duplicate and on separate occasions. In order to detect weaker responders, PRP from donors was stimulated with 25μg/ml mAb, which was shown to be effective in the previous 14A2.H1 study (Roberts *et al.*, 1995). Under these conditions, whole Ig of the IgG2a anti-PETA-3 mAb 11B1.G4 was capable of inducing aggregation of PRP (Figure 5.6) from three of five donors tested. Aggregation induced by 25μg/ml 11B1.G4 was slower than that observed for a similar amount of 14A2.H1 or the CD9 mAb 1AA2.H9 (Figure 5.6). Two of the three 11B1.G4 responding donors also responded to lower concentrations of $F(ab')_2$ fragments of 11B1.G4 (Figure 5.7), prepared by Ficin digestion (Section 2.3.2), however aggregation induced by 11B1.G4 $F(ab')_2$ fragments was slower than that observed with a similar concentration of whole Ig. Subsequent experiments demonstrated that 11B1.G4 Ig and $F(ab')_2$ induced aggregation was not directly related to concentration, such that higher concentrations, usually above 6-12μg/ml retarded aggregation (Figure 5.8). Thus the concentration of 11B1.G4 used in the experiment shown in Figure 5.5, (25μg/ml) was not optimal, leading to an LT_{50} approximately twice that of 14A2.H1 induced aggregation. At optimal doses (3-6μg/ml) 11B1.G4 stimulated aggregation of PRP was as effective 14A2.H1 (Figure 5.8). These finding were in contrast to the 14A2.H1 response which was directly related to mAb concentration and was inhibited by 14A2.H1 $F(ab')_2$ fragments of 14A2.H1 (Roberts *et al.*, 1995). The responses of PRP from the five donors examined in this study to 11B1.G4, 11B1.G4 $F(ab')_2$ fragments, 14A2.H1 and 1AA2.H9 are summarised in Table 5.1. Since treatment with 6μg/ml 11B1.G4 Ig or $F(ab')_2$ fragments was optimal, responses at this concentration are shown in Table 5.1. For comparison, 14A2.H1 responses are also shown at 6μg/ml. Note however, that the optimum dose of 14A2.H1 was 25-50μg/ml (Figure 5.7). Furthermore responses of PRP from individual donors to whole Ig

Figure 5.6 Platelet Activation by Anti-TM4SF Antibodies

Platelet aggregometry was performed as described (Section 2.9). For direct comparison, all aggregometry traces shown are taken from PRP of the same donor stimulated with 25μg/ml of whole Ig. (A) Platelet activation by anti-CD9 mAb 1AA2.H9 was rapid with an LT_{50} of approximately 1 minute. (B) Stimulation with 14A2.H1 was slower than that of the anti-CD9 mAb. (C) Stimulation of PRP with the IgG2a anti-PETA-3 mAb 11B1.G4 also induced platelet aggregation. Traces (D) and (E) show treatment of PRP with 25μg/ml IgG1 and IgG2a whole Ig non-binding controls 3D3.3 and 1D4.5 respectively. Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2) and LT_{50} of treatments are shown below.

Treatment	LT_{50} (minutes)
A	1.2
B	3.7
C	9.8
D	>15
E	>15

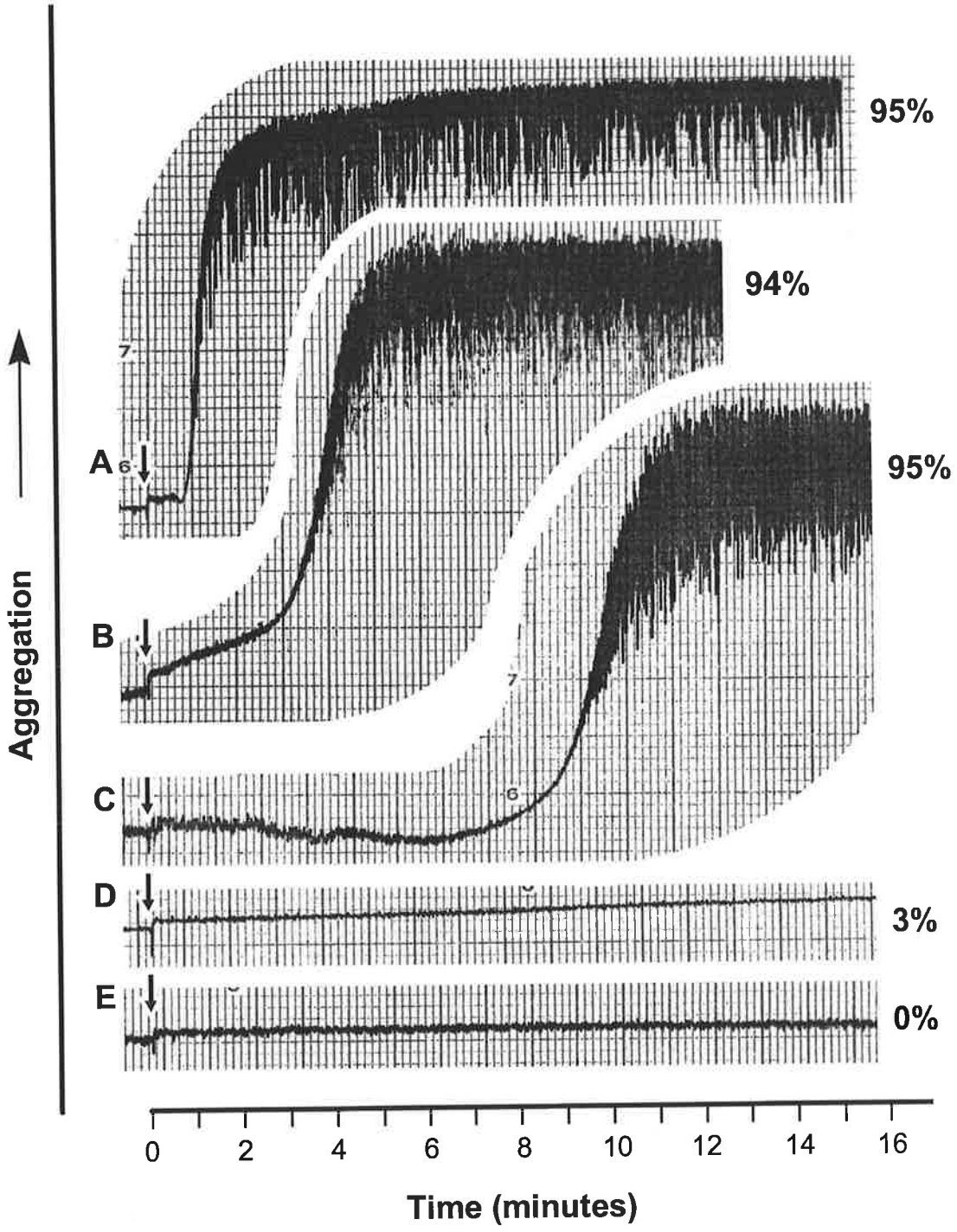

95%
94%
95%
3%
0%
A
B
C
D
E
6
7
7
6
Aggregation
0
2
4
6
8
10
12
14
16
Time (minutes)

Figure 5.7 Aggregation of PRP by 11B1.G4 F(ab')$_2$ Fragments

Platelet aggregometry was performed as described (Section 2.9). Stimulation with 6μg/ml 11B1.G4 Ig (A) or F(ab')$_2$ fragments of 11B1.G4 (B) lead to aggregation. (C,D) Representative traces showing treatment of PRP with 25 μg/ml F(ab')$_2$ fragments of the IgG2a non-binding control mAb 1D45.5 or F(ab')$_2$ fragments of 14A2.H1 respectively. No aggregation was observed with negative control or 14A2.H1 F(ab')$_2$ fragments for all concentrations examined (0.8-100μg/ml). Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2) and LT_{50} of treatments are shown below.

Treatment	LT_{50} (minutes)
A	7.2
B	12.3
C	>15
D	>15

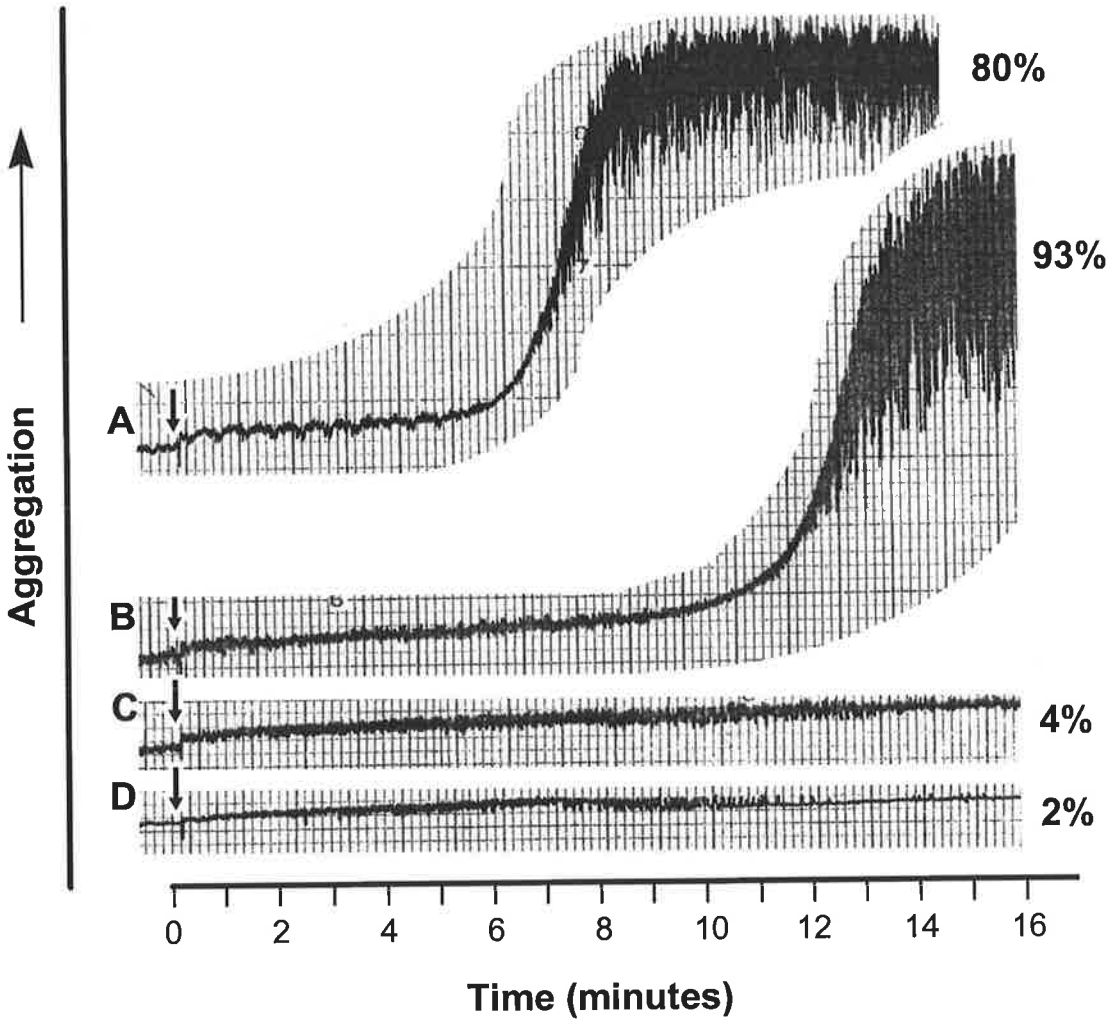

Aggregation
A
B
C
D
80%
93%
4%
2%
0
2
4
6
8
10
12
14
16
Time (minutes)

Figure 5.8 Dose Response Curves of Responding Donors to 11B1.G4 Ig or $F(ab')_2$ Fragments

Panels shows the LT_{50} versus antibody concentration for donors (A) NH, (B) OD and (C) SR. All titrations were performed within 2 hours of blood collection. Since donor SR did not respond to 11B1.G4 $F(ab')_2$ fragments, a titration with 14A2.H1 is shown for comparison.

Closed Circles	11B1.G4
Open Squares	11B1.G4 $F(ab')_2$ Fragments
Open Triangles	14A2.H1

* LT_{50}>15 minutes.

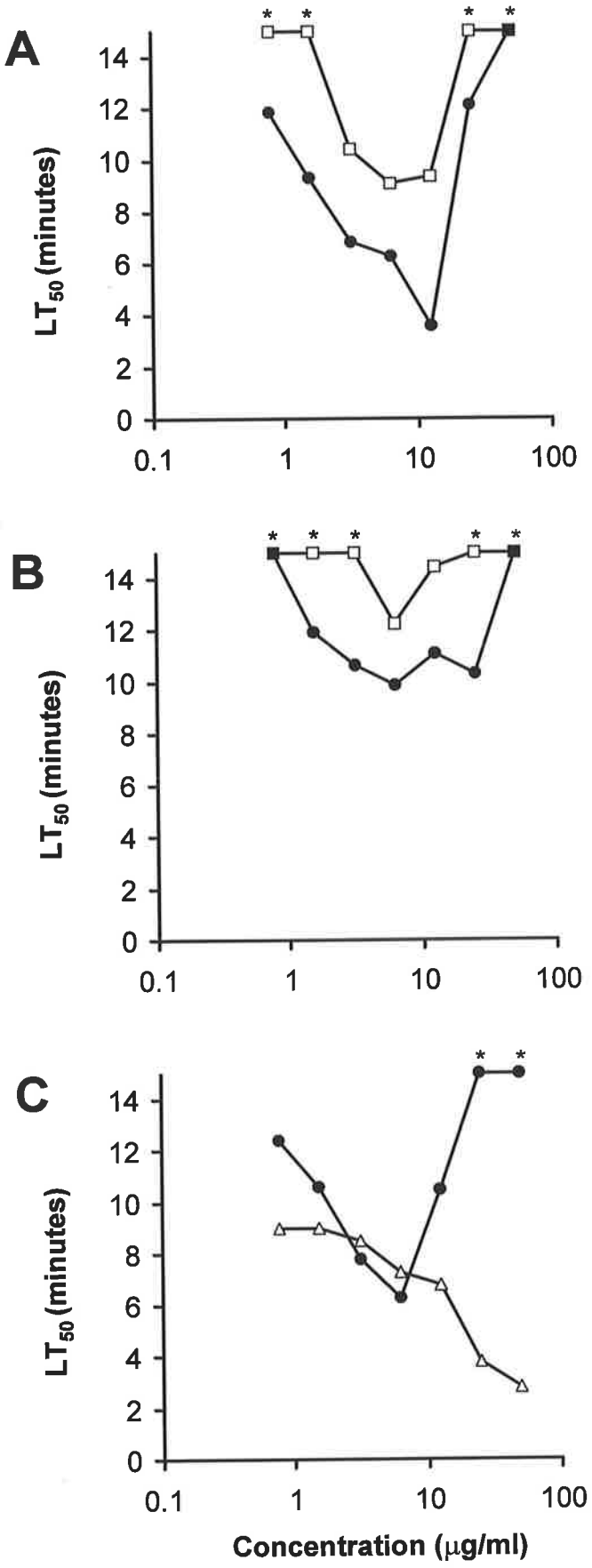

A
B
C
LT50 (minutes)
0
2
4
6
8
10
12
14
0.1
1
10
100
Concentration (μg/ml)

Table 5.1 Donor Responses to 11B1.G4, 11B1.G4 F(ab')$_2$ Fragments, 14A2.H1 and 1AA2.H9

Donor	11B1.G4		11B1.G4 F(ab')$_2$		14A2.H1		1AA2.H9
	min. conc	LT_{50} (6 µg/ml)	min. conc	LT_{50} (6 µg/ml)	min. conc	LT_{50} (6 µg/ml)	LT_{50} (25 µg/ml)
NH	<0.8	6.3	6.3	9.1	<0.8	6.7	1.0
OD	1.6	10.7	6.3	12.3	6.3	10.1	ND*
SR	<0.8	6.2	>12.5	>15	1.6	6.5	0.9
KH	>25	>15	ND	-	>25	>15	0.8
SF	>25	>15	ND	-	>25	>15	1.68

ND - Not Done.

Min. conc - refers to the minimum concentration of mAb for which aggregation was observed.

* Although not done at 25µg/ml, donor OD did respond to mAb 1AA2.H9 at concentrations ranging from 0.2-6µg/ml.

or $F(ab')_2$ fragments of all mAbs examined in this study were reproducible when examined on separate occasions.

The 11B1.G4 response was labile, such that PRP lost reactivity to stimulation with this mAb during storage at room temperature. Within 2-3 hours of blood collection, PRP no longer responded to either Ig or $F(ab')_2$ fragments of 11B1.G4, although both 14A2.H1 and 1AA2.H9 responses persisted for over 4 hours. Loss of the 11B1.G4 response was accelerated if PRP was stored at 37°C (data not shown) as was previously reported for 14A2.H1 responses (Roberts *et al.*, 1995).

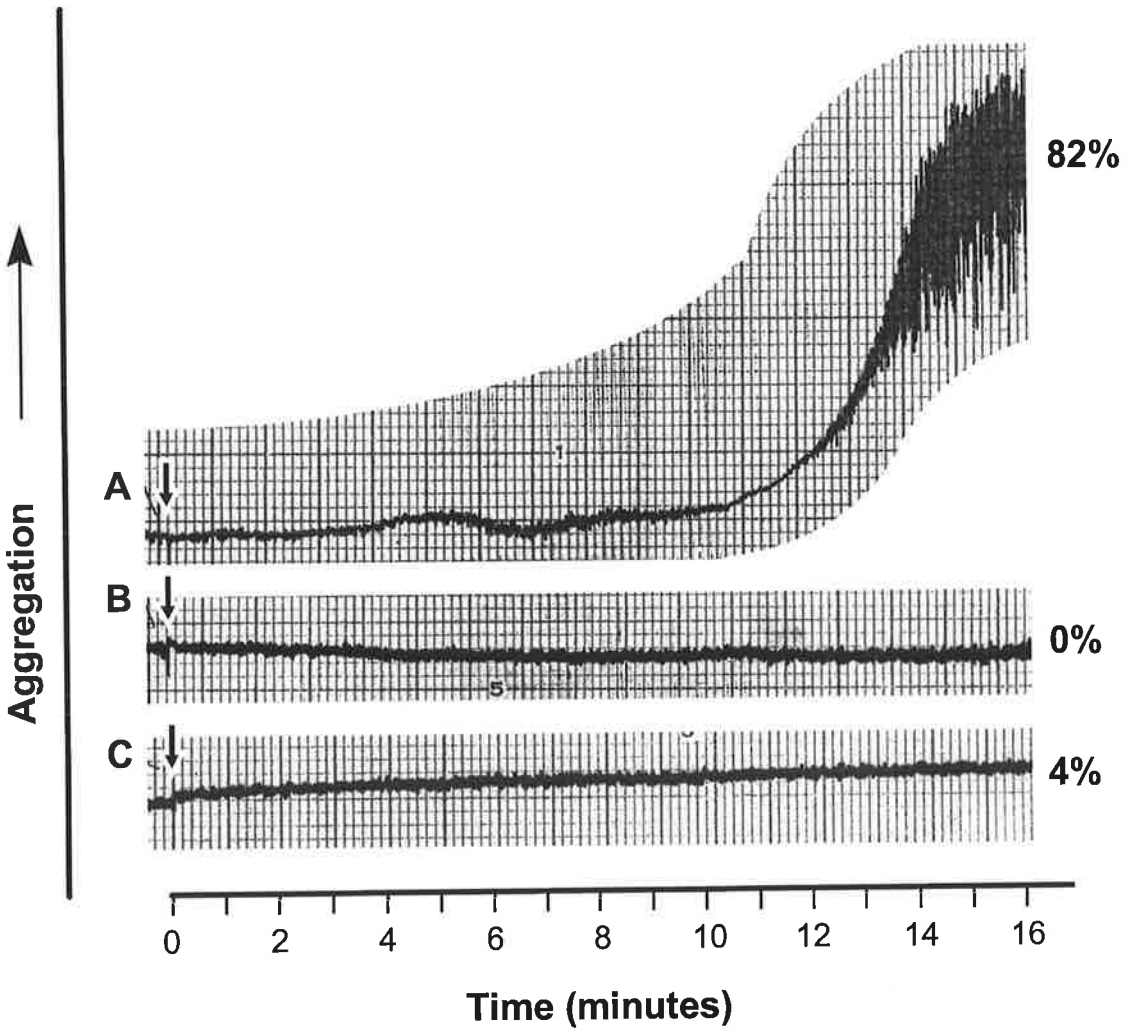
5.3.2 Metabolic Inhibitors Block 11B1.G4 Induced Platelet Aggregation

To investigate whether passive agglutination of platelets could account for the aggregation induced by 11B1.G4, PRP was pretreated with inhibitors of platelet metabolism. Pre-incubation of PRP for 5 minutes with 10^{-7}M PGE_1, which activates adenylate cyclase leading to decreased levels of ATP (Kerins *et al.*, 1991), resulted in inhibition of platelet aggregation induced by 11B1.G4 $F(ab')_2$ fragments (Figure 5.9). Similarly, pretreatment of PRP with 10^{-3}M aspirin, an irreversible inhibitor of cyclo-oxygenase (Roth *et al.*, 1975), also blocked aggregation (Figure 5.9). Since higher concentrations of 11B1.G4 were sub-optimal, inhibition by aspirin could not be overcome by increasing the dose of 11B1.G4. This finding differs from previous results, whereby inhibition of the 14A2.H1 response by aspirin could be overcome by increased 14A2.H1 doses (Roberts *et al.*, 1995). Based on the abrogation of 11B1.G4 responses by metabolic inhibitors, it was concluded that aggregation induced by 11B1.G4 was dependent on platelet activation and was therefore not due to passive agglutination.

Figure 5.9 Metabolic Inhibitors Block 11B1.G4 Induced Aggregation

Representative traces showing; (A) Stimulation of PRP, pretreated with Tyrodes buffer for 5 minutes, with 6μg/ml 11B1.G4 $F(ab')_2$ fragments. (B) Stimulation of PRP, pretreated with 10^{-7}M PGE_1 for 5 minutes, with 6μg/ml 11B1.G4 $F(ab')_2$ fragments. (C) Stimulation of PRP, pretreated with 10^{-3}M aspirin for 5 minutes, with 6μg/ml 11B1.G4 $F(ab')_2$ fragments. These experiments were performed using PRP from donor OD, and on several occasions. Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2) and LT_{50} of treatments are shown below.

Treatment	LT_{50} (minutes)
A	13.7
B	>15
C	>15


Aggregation
A
B
C
82%
0%
4%
0
2
4
6
8
10
12
14
16
Time (minutes)

5.3.3 Role of the FcγRII in 11B1.G4 Induced Platelet Aggregation

Platelet activating mAbs have been shown to induce aggregation by cross-linking and subsequent signalling through the FcγRII (Worthington *et al.*, 1990; Roberts *et al.*, 1995). A unique mAb to the FcγRII, IV.3, antagonises these responses by blocking Fc binding (Worthington *et al.*, 1990; Rubinstein *et al.*, 1991b). Therefore the importance of FcγRII ligation during 11B1.G4 induced platelet activation was examined by pretreatment of PRP with mAb IV.3. (Figure 5.10). These studies produced disparate results with PRP from the three 11B1.G4 responders. Stimulation of PRP with 11B1.G4 from donor NH could not be blocked by mAb IV.3. Also, despite not responding to IgG1 and IgG2a non-binding control $F(ab')_2$ fragments, aggregation of PRP from this donor was observed using 1AA2.H9 $F(ab')_2$ fragments (data not shown). In contrast, PRP from donor OD, which responded to 11B1.G4 $F(ab')_2$, but not to 1AA2.H9 $F(ab')_2$ fragments, showed partial inhibition by IV.3 (Figure 5.10.A,B). PRP from donor SR, which did not respond to 11B1.G4 $F(ab')_2$ fragments (see Table 5.1), appeared to be predominately mediated by FcγRII (Figure 5.10.C,D). $F(ab')_2$ fragments of 14A2.H1 were also unable to induce aggregation of PRP from this donor (Figure 5.10.E,F).

5.3.4 ADP Does Not Synergise with 11B1.G4 Induced Platelet Aggregation

As mentioned previously, not all donors responded to 11B1.G4, and the response was labile such that it was lost during platelet storage. In an attempt to potentiate the response induced by 11B1.G4, the possibility of synergy with the weak platelet agonist ADP was examined. In order to determine low and subthreshold doses of ADP for use in synergy experiments, PRP from donors was titrated with ADP (1-0.03μM) (data not shown). Such titrations were performed on each occasion synergy experiments were performed. Preincubation of PRP from donor OD with 5μg/ml 11B1.G4 Ig failed to synergise with

Figure 5.10 Role of the FcγRII in 11B1.G4 Induced Platelet Aggregation

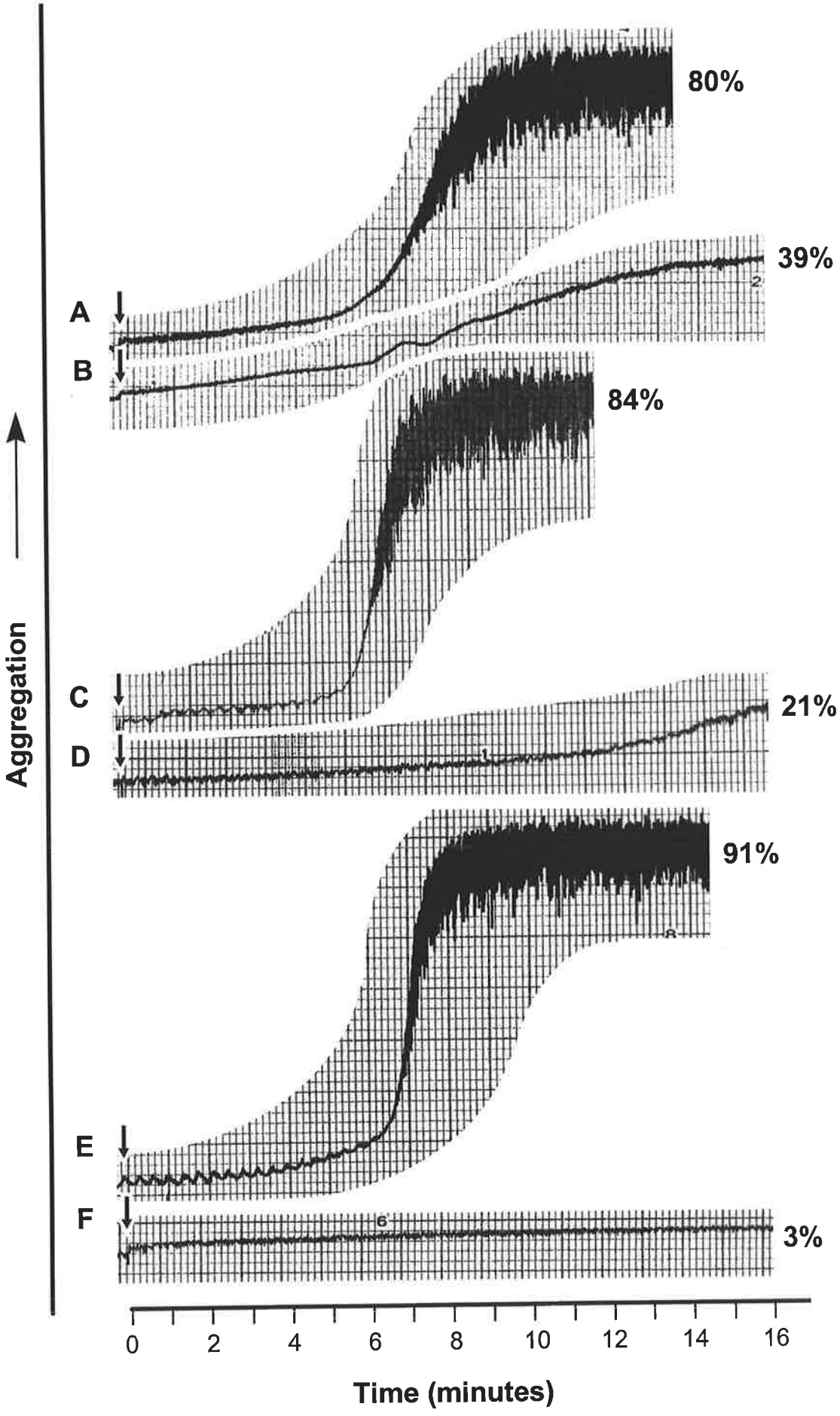
(A,B) Stimulation of PRP from donor OD, pretreated with (A) Tyrodes buffer or (B) 5 μg/ml IV.3 for 5 minutes, with 6μg/ml 11B1.G4.

(C,D) Stimulation of PRP from donor SR, pretreated with (C) Tyrodes buffer or (D) 5 μg/ml IV.3 for 5 minutes, with 6μg/ml 11B1.G4.

(E,F) Stimulation of PRP from donor SR, pretreated with (E) Tyrodes buffer or (F) 5 μg/ml IV.3 for 5 minutes, with 6μg/ml 14A2.H1.

Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2) and LT_{50} of treatments are shown below.

Treatment	LT_{50} (minutes)
A	7.2
B	>15
C	6.5
D	>15
E	6.2
F	>15


80%
39%
A
B
84%
C
21%
D
91%
E
F
3%
Aggregation
0
2
4
6
8
10
12
14
16
Time (minutes)

0.12μM ADP (Figure 5.11.A,B). Additionally, pretreatment of PRP from donor SR (a non-responding donor to 11B1.G4 $F(ab')_2$ fragments) with 5μg/ml of 11B1.G4 $F(ab')_2$ fragments failed to synergise with a sub-threshold dose (0.25μM) of ADP (Figure 5.11.C,D). Pretreatment of PRP from donor SR with 0.25μM ADP for 5 minutes before the addition of 5μg/ml 11B1.G4 $F(ab')_2$ fragments also resulted in no response (data not shown).

5.4 Anti-PETA-3 Antibodies Modulate Endothelial Cell Function

5.4.1 Anti-PETA-3 mAbs Inhibit EC Motility

It has been demonstrated that β1 and β3 integrins mediate EC migration (Leavesley *et al.*, 1993). Since PETA-3 associated with both of these integrins and TM4SF proteins have been proposed to modulate integrin function, the effect of anti-PETA-3 mAbs on EC migration was examined. Confluent fourth passage EC monolayers were wounded with a teflon scraper, treated with 2-10 μg/ml mAb, as shown, and the degree of cell migration assessed over 30 hours as described (Section 2.10.1). Results are shown in Figure 5.12.A. Incubation of cells with 10μg/ml each of isotype matched non-binding control mAbs (3D3.3 and 1D4.5), or isotype matched binding control mAbs (anti-PECAM-1, 3D2 or anti-E-selectin, 68-2A1) had minimal effect on the rate of migration when compared to untreated EC. In contrast, anti-PETA-3 mAbs 14A2.H1 and 11B1.G4 (10μg/ml) inhibited the migration of cells into the wound by 32% and 41% respectively at 30 hours post wounding when compared to negative control mAbs. Treatment of EC with 2μg/ml of 8A2, an anti-β1 mAb which induces a conformational state promoting increased integrin affinity for ligand (Kovach *et al.*, 1992), also resulted in a significantly reduced migration rate, 52% inhibition at 30 hours. Anti-CD9 mAb also inhibited EC migration, 33% at 30 hours. Analysis by two way ANOVA demonstrated a statistically significant retardation in the rate of migration of EC treated with

Figure 5.11 Platelet Activation by 11B1.G4 does Not Synergise with ADP

Platelet aggregometry was performed as described (Section 2.9). Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2). Trace (A) shows the response of PRP from Donor OD to 0.12μM ADP stimulation. (B) Pretreatment of PRP from donor OD with 5μg/ml anti-PETA-3 mAb 11B1.G4 for 5 minutes did not synergise with 0.12μM ADP. Although slight potentiation of the ADP response is evident in trace (B), this finding was not reproducible. PRP from donor SR was pretreated with (C) Tyrodes buffer, or (D) 5μg/ml 11B1.G4 F(ab')$_2$ fragments before being treated with a sub-threshold dose (0.25μM) of ADP. Numbers at ends of each trace represent percentage aggregation (calculated as described in section 2.9.2) and LT_{50} of treatments are shown below.

Treatment	LT_{50} (minutes)
A	2.7
B	2.3
C	>15
D	>15

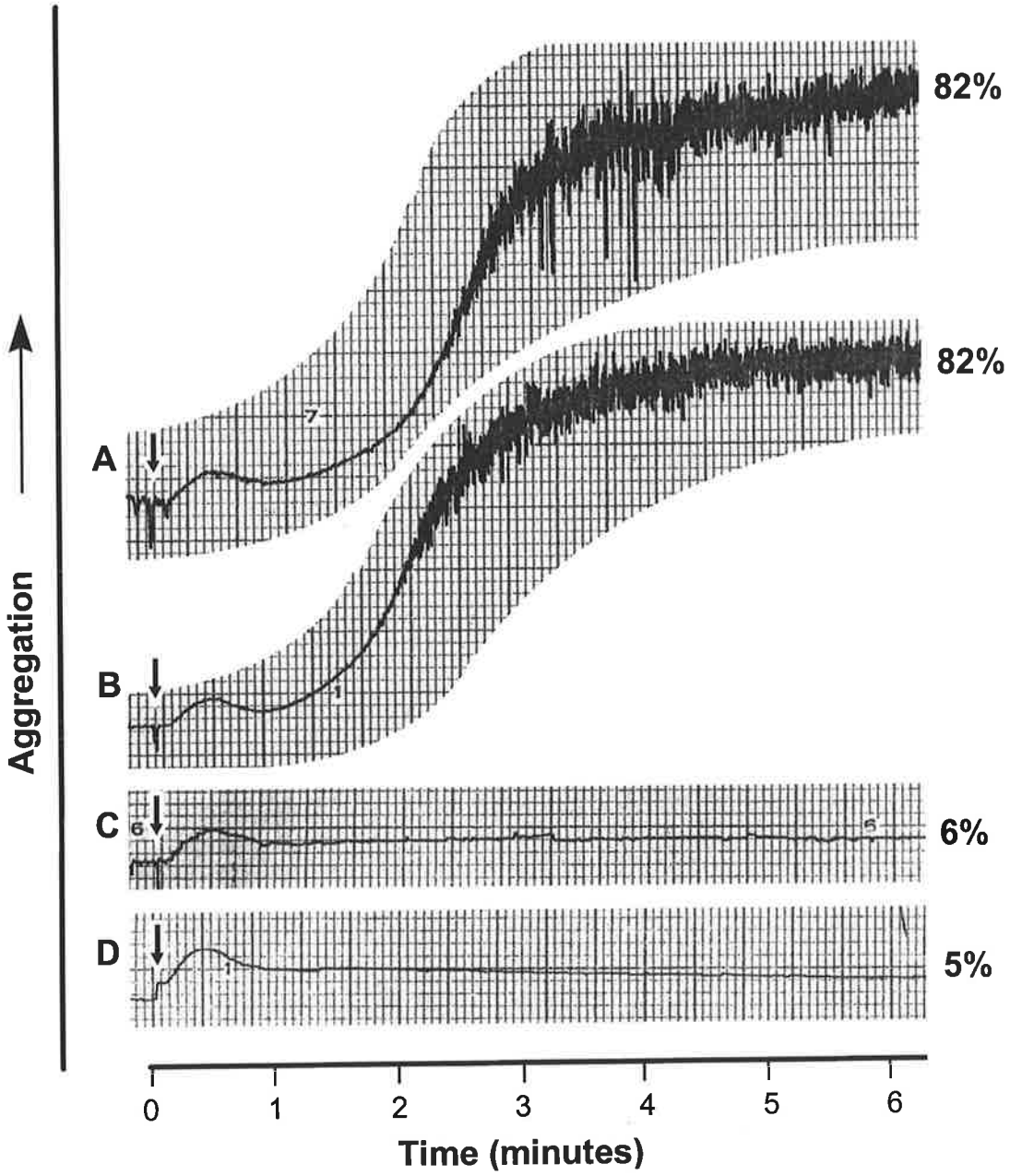

Aggregation
A
B
C
D
82%
82%
6%
5%
0
1
2
3
4
5
6
Time (minutes)

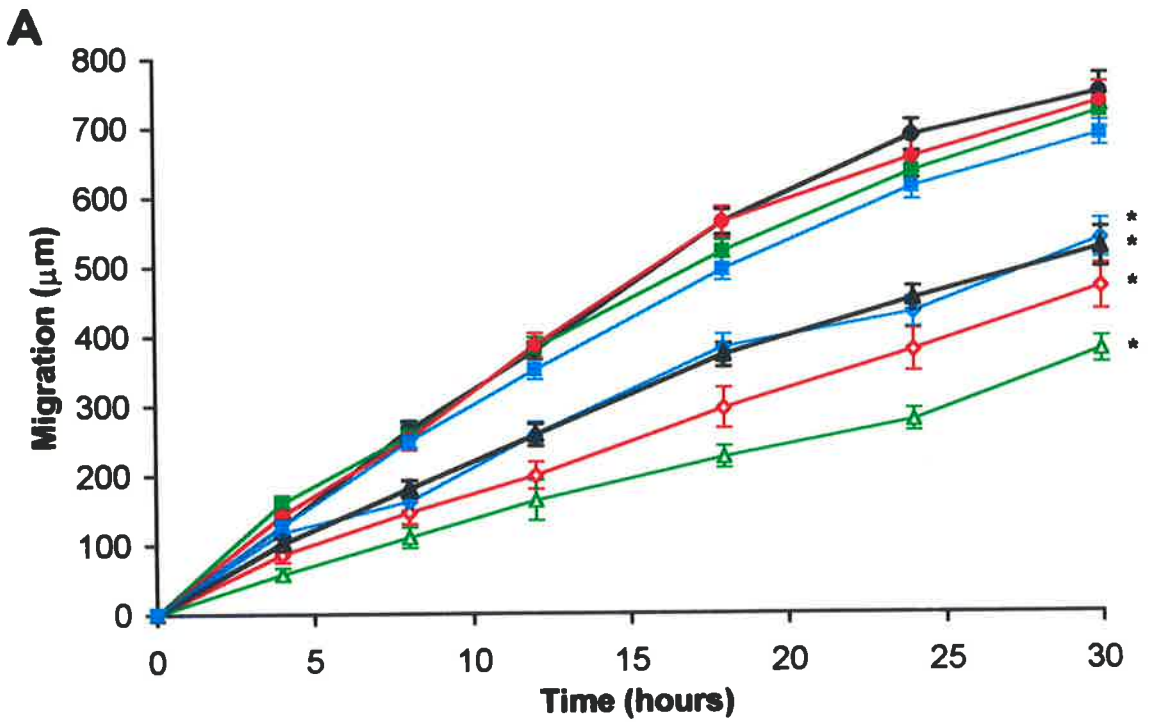
Figure 5.12 Anti-PETA-3 mAbs Inhibit EC Motility

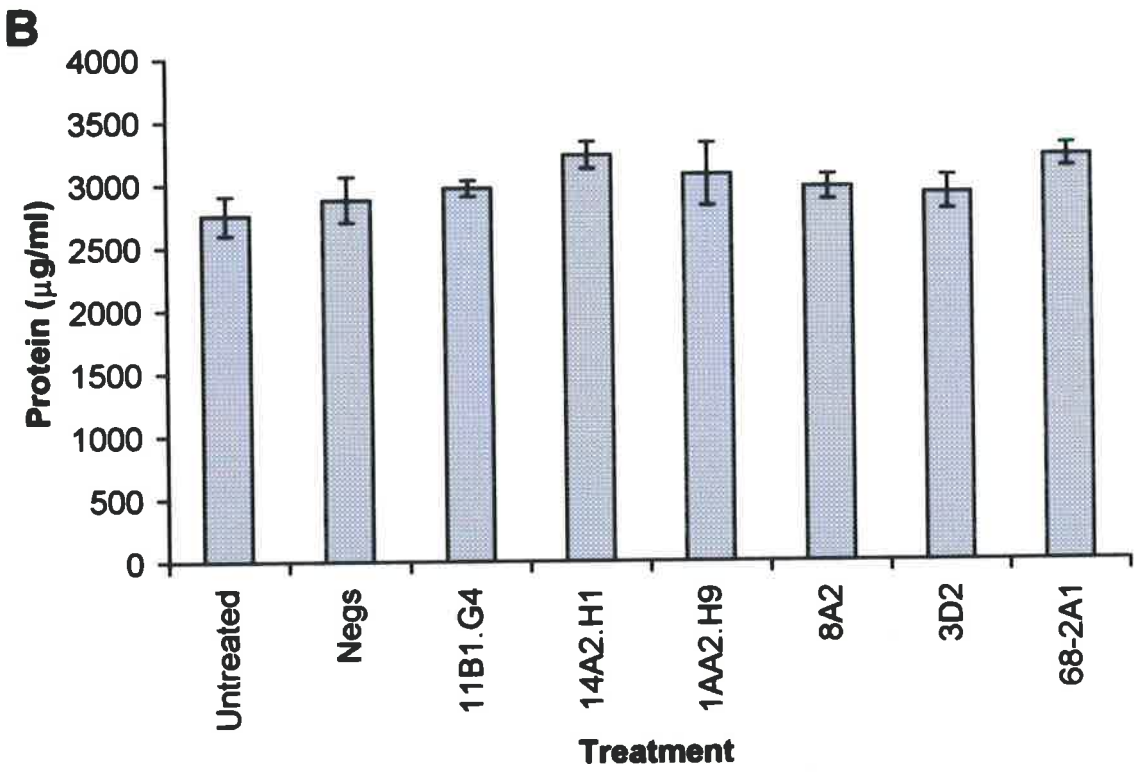
(A) Fourth passage EC monolayers were wounded, three times per well, medium aspirated and replaced with medium supplemented with purified mAbs (Section 2.10.1). Horizontal distance of cells from the initial wound was measured at five random points along each wound in duplicate wells at the times indicated as described (Section 2.10.1). Data presented represent the mean ± S.E.M. of duplicate wells (n=30) from one of two separate experiments performed. Statistical analysis using a two way ANOVA, demonstrated a significant difference between the migration rate of 1AA2.H9, 14A2.H1, 11B1.G4 and 8A2 treated EC when compared to untreated, isotype matched binding or non-binding controls, ($P<0.01$).

Black Closed Circles	Untreated
Red Closed Circles	Isotype matched non-binding control mAbs (10μg/ml)
Green Closed Squares	IgG1 binding control, 3D2 (10μg/ml)
Blue Closed Squares	IgG2a binding control, 68-2A1 (10μg/ml)
Black Closed Triangles	CD9, 1AA2.H9 (10μg/ml)
Blue Open Diamonds	PETA-3, 14A2.H1 (10μg/ml)
Red Open Diamonds	PETA-3, 11B1.G4 (10μg/ml)
Green Open Triangles	β1 Integrin, 8A2 (2μg/ml)

* $P<0.01$ when compared to untreated, isotype matched binding or non-binding controls.

(B) Bradford total protein determination performed at the conclusion of the migration assay is shown. Statistical analysis using the unpaired T-test, demonstrated no significant difference in protein content between isotype non-binding control mAb and any of the other mAb treatments examined ($P>0.05$).


A
800
700
600
500
400
300
200
100
0
Migration (µm)
0
5
10
15
20
25
30
Time (hours)
*
*
*
*


B
4000
3500
3000
2500
2000
1500
1000
500
0
Protein (µg/ml)
Untreated
Negs
11B1.G4
14A2.H1
1AA2.H9
8A2
3D2
68-2A1
Treatment

11B1.G4, 14A2.H1, 1AA2.H9 or 8A2 when compared to untreated, isotype matched binding or non-binding controls ($P<0.01$). These findings were reproduced in two separate experiments performed and for concentrations of 11B1.G4 ranging from 1-10μg/ml. Lower doses of 14A2.H1 (1μg/ml) were not as effective as higher doses (10μg/ml) (data not shown).

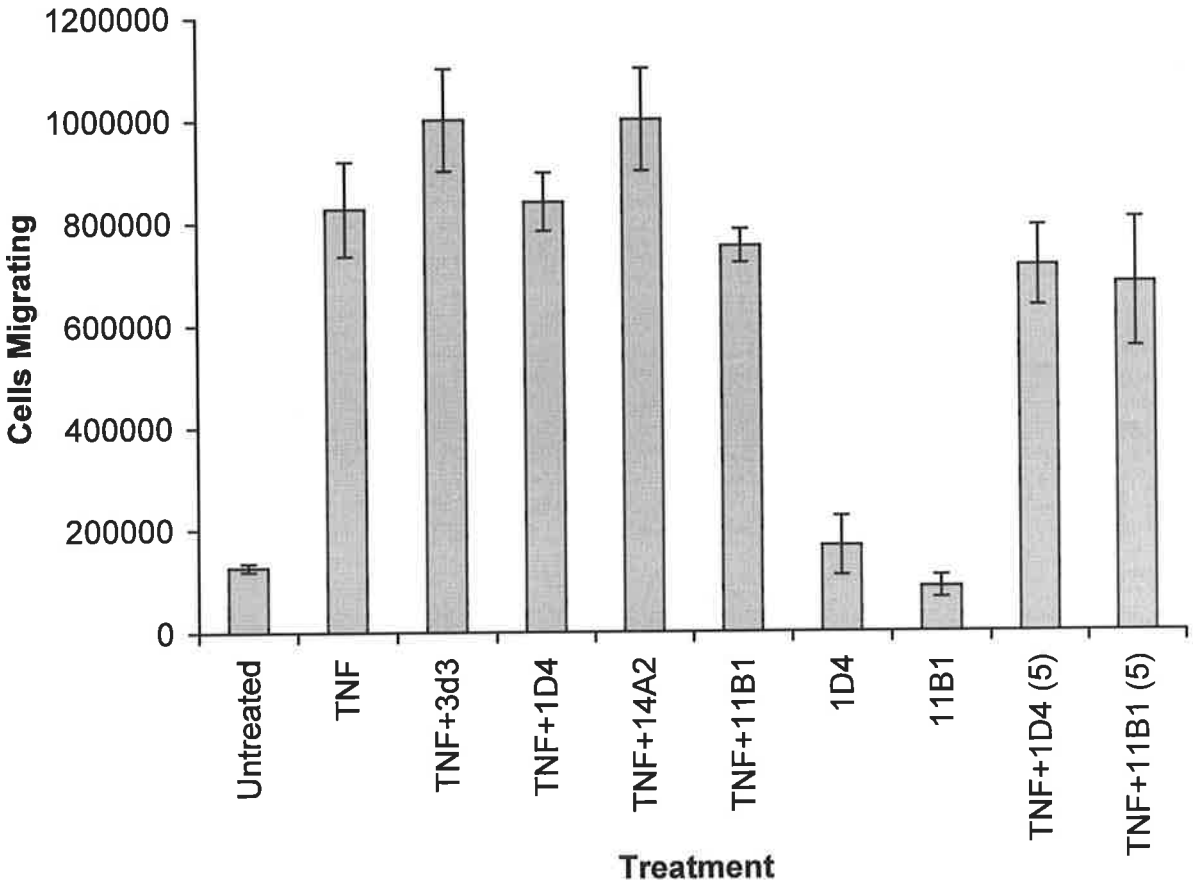
To determine whether mAb induced proliferation may have contributed to the movement of cells into the wound, total protein content within each well was determined at the end of the experiment. No significant change in total protein was observed ($P>0.05$), for any of the treatments, as determined by T-tests (Figure 5.12.B).

5.4.2 PETA-3 MAbs do Not Regulate Neutrophil Transendothelial Migration

MAbs against the β3 integrin associated protein, IAP (CD47), have been demonstrated to modulate neutrophil transendothelial migration (Cooper *et al.*, 1995). Since PETA-3 was also found to associate with β3 integrin, the anti-PETA-3 mAbs 11B1.G4 and 14A2.H1 were assessed for their ability to modulate neutrophil transendothelial migration. For these experiments, $F(ab')_2$ fragments of anti-PETA-3 and control mAbs were used to avoid effects mediated by Fc binding to neutrophils. Second passage EC were plated onto transwells with 2μm pore size, cultured until confluent, then treated with various $F(ab')_2$ fragments or cytokines as described (Section 2.10.2). 100% migration was defined as the number of neutrophils migrating through TNF-α (100U/ml) treated HUVEC monolayers during the 1 hour incubation. Preincubation with 11B1.G4 $F(ab')_2$ fragments at 50 or 5μg/ml for 10 minutes did not significantly alter the number of neutrophils migrating through TNF-α stimulated or untreated EC monolayers (Figure 5.13), as determined by three separate experiments ($P>0.05$).

Figure 5.13 Anti-PETA-3 mAbs Do Not Regulate Neutrophil Transendothelial Migration

Neutrophil transendothelial migration assays were performed as described (Section 2.10.2). 10^6 neutrophils were placed into transwells, 3μm pore size, containing EC monolayers either untreated, pretreated with 100U/ml TNF-α for 4 hours and/or $F(ab')_2$ fragments at 50μg/ml (or 5μg/ml as indicated by numbers in parenthesis) for 10 minutes as indicated. After 1 hour incubation at 37°C, migrating neutrophils in the lower chamber were counted. Data presented are the mean ± S.E.M. of triplicate wells of one representative experiment from three. Statistical analysis using unpaired T-tests, demonstrated no significant difference between untreated EC and mAb treatments or TNF-α treated EC and TNF-α+mAb treated EC, ($P>0.05$).


1200000
1000000
800000
600000
400000
200000
0
Cells Migrating
Untreated
TNF
TNF+3d3
TNF+1D4
TNF+14A2
TNF+11B1
1D4
11B1
TNF+1D4 (5)
TNF+11B1 (5)
Treatment

5.4.3 Anti-PETA-3 mAbs Modulate *In Vitro* Capillary Formation

The process of *in vitro* tube formation involves EC movement, realignment to form sprouts and subsequent fusion and extension to form mature capillary tubes (Gamble *et al.*, 1993; Meyer *et at.*, 1997). Treatment of EC with the activating β1 mAb 8A2 has been previously shown to inhibit *in vitro* tube formation (J. Gamble, unpublished). To investigate whether the association of PETA-3 with β1 (or other integrins) may modulate this process, the effect of anti-PETA-3 mAb on *in vitro* tube formation was investigated as described (Section 2.10.3). Fourth passage EC cultured on Matrigel in the presence of 1-10μg/ml anti-PETA-3 mAbs showed retarded tube formation (Figure 5.14) in three separate experiments performed. Although not as pronounced as the modulation observed with 2μg/ml 8A2, tube formation in the presence of anti-PETA-3 mAbs was significantly altered when compared to untreated, binding or non-binding negative control mAbs (Table 5.2). At the 10 hour time point, treatment with 8A2 showed a statistically significant decrease ($P<0.05$) in tube length and increase in tube number when compared to controls. Although the anti-PETA-3 mAbs 11B1.G4 and 14A2.H1 (1-10μg/ml) showed similar results at 10 hours, only the increase in the number of tubes of 11B1.G4 treated EC was statistically significant ($P<0.05$) when compared to controls. However, at 20 hours both the decrease in tube length and the number of tubes formed were significantly altered by both anti-PETA-3 and 8A2 mAbs when compared to controls ($P<0.05$). The anti-CD9 mAb 1AA2.H9 failed to modulate tube formation ($P>0.05$).

Figure 5.14 Anti-PETA-3 mAbs Modulate the Formation of Capillaries *In Vitro*

EC were plated onto Matrigel at $2.5x10^4$/well in the presence or absence of various mAbs as indicated (Section 2.10.3). Treatment with anti-PETA-3 mAb 11B1.G4 (10μg/ml) or β1 mAb 8A2 (2μg/ml) resulted in more tubes of shorter length (see Table 5.3 for summary of all treatments). Data shown are representative photographs of treatments carried out in duplicate from one of three experiments performed. Bar; 800μm.

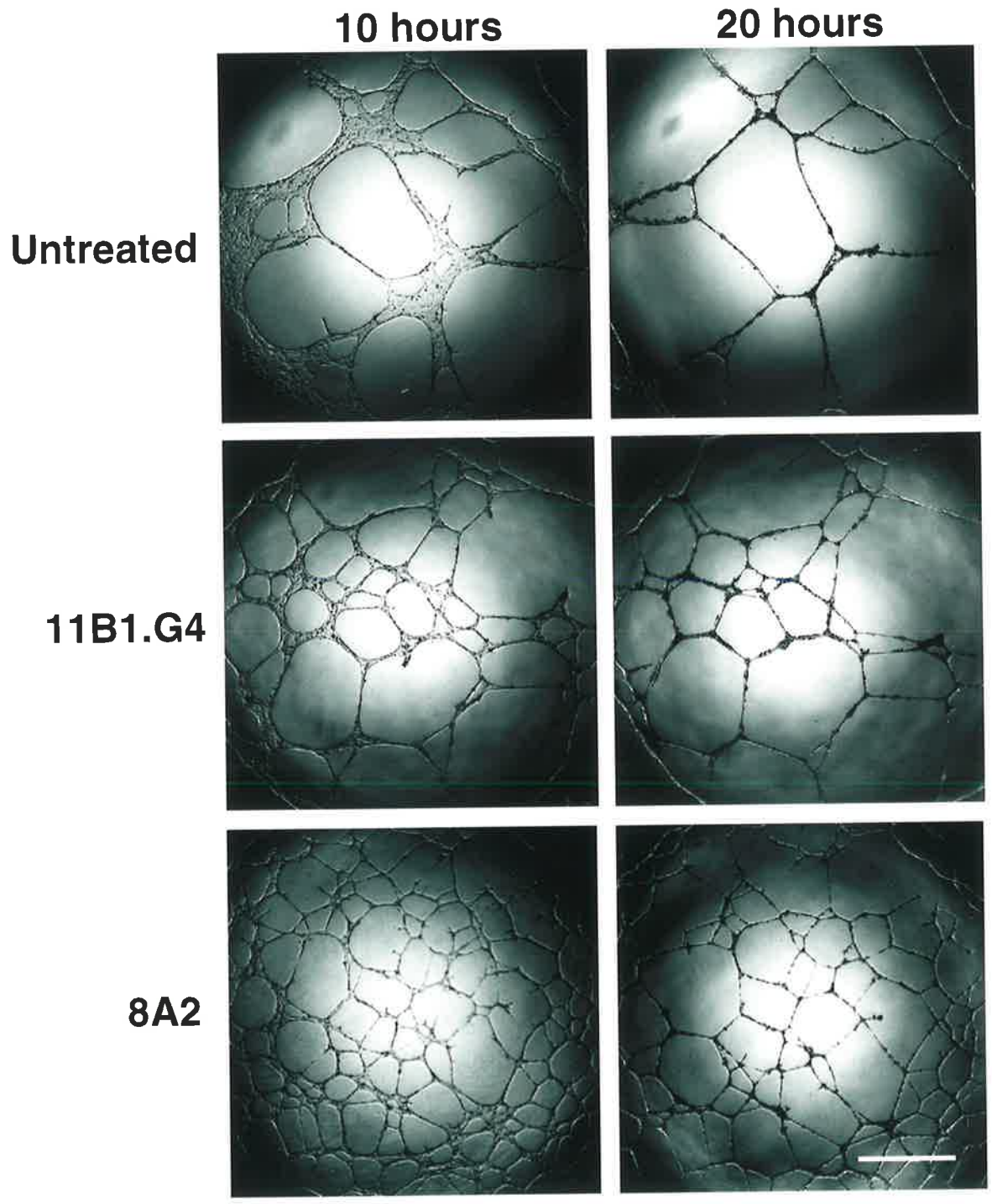

10 hours
20 hours
Untreated
11B1.G4
8A2

Table 5.2 Anti-PETA-3 mAbs Modulate *In Vitro* Capillary Formation

	10 hours		20 hours	
Treatment	**Length (μm)**	**Number**	**Length (μm)**	**Number**
Untreated	408 ± 46	11.0 ± 0.5	537 ± 68	22.5 ± 1.4
3D3.3 + 1D4.5 (Control)	374 ± 16	12.6 ± 2.7	473 ± 46	26.0 ± 1.6
3D2 (PECAM-1)	441 ± 57	12.1 ± 3.1	560 ± 45	22.4 ± 2.4
68-2A1 (E-Selectin)	430 ± 63	9.3 ± 1.5	561 ± 56	21.8 ± 2.4
1AA2.H9 (CD9)	423 ± 23	9.7 ± 1.6	466 ± 65	24.6 ± 4.7
11B1.G4 (PETA-3)	344 ± 33	24.3 ± 1.3*	364 ± 24*	33.9 ± 3.0*
14A2.H1 (PETA-3)	374 ± 14	10.7 ± 1.6	386 ± 29*	31.3 ± 2.2*
8A2 (β1)	221 ± 7*	175.8 ± 40*	260 ± 23*	126.1 ± 17*

Table shows the length and number of tubes formed at 10 and 20 hours. Results presented represent the mean ± S.E.M. of pooled data from three assays performed in duplicate (Section 2.10.3). T-tests were performed to compare each mAb treatment with untreated and isotype matched non-binding controls, * $P<0.05$.

5.5 Discussion

Co-immunoprecipitation studies in EC have demonstrated the association of PETA-3 with a range of integrins and the two TM4SF members CD9 and CD63. Precipitation from lysates, prepared with the mild detergent CHAPS, using mAbs to the various α chains and Western blotting for PETA-3, showed association of PETA-3 with β1, β3, β4, α3 and α6 integrin chains. In addition, very weak co-precipitation with α2 and α5 was observed. Under these conditions, both intracellular and surface associations were able to be detected. However, when surface-biotinylated cells were subjected to mild detergent lysis, immunoprecipitation with PETA-3 mAb, followed by solubilisation of the precipitates in stronger detergent (1% NP 40 supplemented with 0.2% SDS), and re-precipitation with mAbs to integrin chains, PETA-3/integrin complexes at the cell surface could be specifically examined. Using this protocol, the weak association of α2 and α5, as detected by immunoprecipitation and Western blotting for PETA-3, was able to be verified. The association of PETA-3 with integrins was supported by their colocalisation in EC by 2-colour confocal microscopy. The colocalisation of PETA-3 with integrins in cytoplasmic vesicles, especially the β1, β3, α5 and α6 chains, suggests that integrin complexes containing PETA-3 may also occur intracellularly. Based on the immunofluorescence and electron microscopic studies described in the Chapter 4, such complexes might exist within the endocytic pathway.

Co-immunoprecipitation of PETA-3 with the β3 chain provides the first description of TM4SF/β3 complexes in nucleated cells, as studies to date have investigated CD9 associations with αIIbβ3 in platelets only (Slupsky *et al.*, 1989; Indig *et al.*, 1997). As αvβ3 is the only integrin of the β3 subfamily to be expressed by HUVEC, PETA-3 was assumed to associate with this integrin complex. This study also provides the first report of TM4SF associations with the β4 integrin. PETA-3 coimmunoprecipitated by the α6 and β4 mAbs was predominantly of the higher molecular weight species. Therefore the complexes of

PETA-3 with α6β4 and perhaps α6β1 would be expected to be localised to the plasma membrane. Since the α6 chain heterodimerises with both β1 and β4, the specific association of PETA-3 with α6β1 in EC remains to be verified, however parallel studies in haemopoietic cells, which lack the β4 chain, have demonstrated the association of PETA-3 with the α6 chain (Fitter *et al.*, submitted). No expression of α4 by cultured HUVEC was detected in this study, thus the possibility of α4β1/PETA-3 interactions could not be examined. However, the association of α4β1 with PETA-3 was detected in haemopoietic cell lines (Fitter *et al.*, submitted).

Apart from the complexes comprising TM4SF members and α3β1, α4β1 or α6β1 (reviewed in Hemler *et al.*, 1996; Maecker *et al.*, 1997), associations with the β3, β4, α2 and α5 integrin chains observed in this study have been less widely reported. Nonetheless, other TM4SF proteins have also been shown to associate with non-β1 integrin sub-families, including CD9 with αIIbβ3 in platelets and α4β7 and CD63 with αLβ2 (Slupsky *et al.*, 1989; Mannion *et al.*, 1996; Skubitz *et al.*, 1996). Despite the promiscuous association of PETA-3 with integrins observed in this study, the lack of co-immunoprecipitation with other EC adhesion molecules and transferrin receptor, with which it colocalised (Section 4.2.2), demonstrates the specificity of the reported interactions. Although some of the controls were expressed at lower levels (Figure 5.1) PECAM-1 was abundant on the plasma membrane of EC. Furthermore many of the PETA-3/integrin complexes were also detectable using 1% NP 40. Based on their association in CHAPS versus NP 40, PETA-3/α3β1 appears to be the most stable, followed by β4 and α6 while α2 and α5 associations appear to be the weakest. From this study, it was concluded that PETA-3 associates with a wide range of integrins including members of the β1, β3 and β4 sub-families. These findings extend the TM4SF/integrin associations reported. The PETA-3/integrin and PETA-3/CD9/CD63 associations observed in this study add to the recent report of PETA-3 associations with CD81 and α3β1 in EC

(Yanez-Mo *et al.*, 1998). TM4SF proteins have been observed to complex with other TM4SF members in several studies (Sections 1.2.3, 1.2.4 and 1.2.5), and the association of PETA-3 with CD9 and CD63 in EC was demonstrated in this study. In contrast to the results obtained with integrins, the association of PETA-3 with CD9 and CD63 was not detected when more stringent lysis conditions were used. Others have also reported loss of TM4SF/TM4SF and TM4SF/integrin associations in NP 40 (Mannion *et al.*, 1996), which might be due to the lower affinity of certain complexes or the disturbance of hydrophobic interactions in the presence of different detergents.

The ability of anti-PETA-3 mAbs to induce aggregation of human platelets has previously been examined (Ashman *et al.*, 1991; Roberts *et al.*, 1995). Both of these previous studies had used the IgG1 mAb 14A2.H1 and demonstrated the requirement of FcγRII ligation. The 11B1.G4 mAb displays different binding properties to 14A2.H1 (Sections 3.2.19 and 3.3) and is of the IgG2a subtype. Since mAbs of the IgG2a subtype interact poorly with the platelet FcγRII, the ability of 11B1.G4 to cause platelet activation was examined. The sub-optimal response of PRP treated with high concentrations of 11B1.G4 indicates a possible prozone effect, such that di-valent binding, or crosslinking, of PETA-3 contributes to the stimulatory signal. This finding differs from the dose-dependent response of 14A2.H1 and suggests that FcγRII ligation does not alone account for the activation induced by mAb 11B1.G4. This was further supported by the ability of $F(ab')_2$ fragments of 11B1.G4 but not 14A2.H1, to cause aggregation which appeared to require PETA-3 crosslinking. The possibility of intrinsic signalling through PETA-3 crosslinking differs from the mechanism of FcγRII ligation reported for most other platelet activating mAbs (Section 1.5.4). However, studies using the FcγRII blocking mAb IV.3 suggest that FcγRII ligation may still contribute to 11B1.G4 Ig induced aggregation. This may also be responsible for the shorter LT_{50} and lower minimum responding concentrations observed with 11B1.G4 whole Ig, as compared to

$F(ab')_2$ fragments. In addition complement fixation by the IgG2a mAb may be involved (see below).

Not all donors responded to 11B1.G4 and the response was slow and labile. During the 14A2.H1 study (Roberts *et al.*, 1995), preliminary evidence indicated that PETA-3 copy number was not dramatically different on platelets from responders or non-responders, suggesting that the level of PETA-3 expression was not a major contributing factor in mAb responses (Ashman *et al.*, unpublished). These results suggest that PETA-3 may require costimulatory signals for platelet aggregation and that these may be provided by release of endogenous mediators by platelets from some donors. Other studies have utilised weak platelet agonists, such as ADP, to provide co-stimulatory signals and potentiate the effects of platelet activating mAbs (Varon *et al.*, 1998). Such co-stimulatory signals might assist in detecting weak 11B1.G4 responders and maintaining the reactivity of stored PRP. However, attempts to demonstrate synergy between 11B1.G4 and ADP were not successful. Additional studies investigating synergy with other platelet agonists may provide further insight as to the signalling pathway initiated by PETA-3 crosslinking. The association of CD63 with PI4-K implicates a role for TM4SF members in phosphatidylinositide signalling pathways (Berditchevski *et al.*, 1997a). Since ADP did not synergise with 11B1.G4, synergy with collagen appears unlikely, as aggregation induced by this agonist is primarily dependent on released ADP (Cowan *et al.*, 1981). Therefore pathways stimulating arachidonic acid metabolism and subsequent eicosanoid production may be required, as suggested by the inhibitory effect of aspirin on 11B1.G4 induced aggregation. Also, platelet activating factor, which amplifies platelet activation independently of ADP release or arachidonic acid metabolism (Chignard *et al.*, 1980), may provide an alternative co-stimulatory signal.

Previous studies have shown that mAbs of the IgG2a subtype may induce platelet aggregation via activation of complement (De Reys *et al.*, 1993). This mechanism of platelet

activation could not be blocked by pretreatment with mAb IV.3. Interestingly, the results reported here suggest that this may not always be the case for platelet activating mAbs of the IgG2a isotype. Nonetheless, the role of complement in 11B1.G4 induced aggregation was not addressed in this study and warrants further investigation, either through the use of washed platelets, or the protease inhibitor leupeptin which blocks complement activation. Whether the activation of platelets by 11B1.G4 involves the crosslinking and possible signalling via integrin complexes has not yet been investigated.

In addition to the activation of platelets, the effects of anti-PETA-3 mAbs on EC function were also assessed. Since anti-integrin mAbs may inhibit certain biological responses induced by other anti-TM4SF mAbs (Masellis-Smith and Shaw, 1994; Shaw *et al.*, 1995; Behr and Schriever, 1995), it appears that the functional effects of anti-TM4SF mAbs are mediated indirectly. Since EC motility is inhibited by anti-β1 and β3 antibodies (Leavesley *et al.*, 1993), the association of PETA-3 with integrins may indirectly mediate the activity of anti-PETA-3 mAbs on EC migration. The effect of anti-PETA-3 mAbs on EC motility has also been observed by other investigators (Yanez-Mo *et al.*, 1998) and was attributed to the association of PETA-3 with α3β1 integrin which resulted in a small but statistically significant increase in adhesion to fibronectin, type I collagen and laminin. Although modulation of integrin affinity has been demonstrated by the direct binding of anti-β1 mAbs such as 8A2 and QE2.E5 (Kovach *et al.*, 1992; Faull *et al.*, 1996), other studies to date have failed to demonstrate increased adhesion of cells to ECM after stimulation by anti-TM4SF mAbs (Mannion *et al.*, 1996; Fitter *et al.*, submitted). In contrast such mAbs usually induce cell-cell adhesion.

Unlike migration, no detectable effect on neutrophil transendothelial migration was observed when monolayers were treated with anti-PETA-3 mAbs. Leukocyte transendothelial migration had been shown to be inhibited by antibodies against PECAM-1 (DeLisser *et al.*,

1994; Muller *et al.*, 1993) and the β3 integrin associated protein IAP (Cooper *et al.*, 1995), through distinct mechanisms. PECAM-1 is believed to be involved in assisting leukocyte migration/attachment to EC margins, whilst CD47 antibodies may block transient intracellular calcium fluxes in EC which are implicated in transendothelial migration (Huang *et al.*, 1993). Based on co-immunoprecipitation experiments, PETA-3 and PECAM-1 were not physically associated and PETA-3 did not appear to be involved in leukocyte recognition of EC or the transendothelial migratory process. In preliminary experiments, mAb to IAP failed to co-immunoprecipitate PETA-3, although co-immunoprecipitation of β3 was observed (Sincock and Ashman, unpublished). These data suggest that separate pools of β3 associate with these molecules.

We have also demonstrated modulation of *in vitro* capillary formation by anti-PETA-3 mAbs. MAbs against α6 and β1 integrins have been shown to block EC tube formation on Matrigel, thus demonstrating a major role for α6β1 in this process (Davis and Camarillo, 1995). Although the mechanism of altered tube formation by anti-PETA-3 mAbs remains undefined, it does not solely correlate with decreased cellular motility, as treatment of EC with anti-CD9 mAb which also retarded EC migration, had no detectable effect on tube formation.

In summary, this Chapter describes the association of PETA-3 with multiple integrins in EC. The observed associations differ from those reported for other TM4SF members, and extend the range of integrin chains demonstrated to complex with TM4SF proteins. The effect of mAbs to PETA-3 on platelets, EC migration and capillary formation imply the functional significance of integrin associations. The results obtained from platelet aggregometry suggest that crosslinking PETA-3 by mAbs might induce signalling events. It is possible that these signals may also be mediated by integrin pathways.

CHAPTER 6: DISCUSSION

6.1 Overview

Shortly before the commencement of this project in 1995, a review on the TM4SF was published in which the 15 members that had been identified at that time were described (Wright and Tomlinson, 1994). Although the associations with integrins had yet to be reported, it was becoming evident that members of this superfamily were components of multi-protein complexes. However in their closing remarks the authors stated, "The TM4SF molecules remain a superfamily in search of a function:". To date 22 members of the TM4SF have been described and their role in cell biology has continued to be studied by many investigators. The work described in this thesis, and reports of others, are slowly seeking out the function of this unique family of proteins.

The aims of this study were to characterise the expression of PETA-3, CD9, CD63 and β1 integrins in normal human tissues, to determine the subcellular localisation in EC and platelets, to investigate protein-protein interactions involving PETA-3 and to examine the effects of anti-PETA-3 mAbs on platelet and EC function. Immunohistochemical analysis demonstrated expression of PETA-3 in endothelial, epithelial, muscle and Schwann cells *in vivo*. Expression of PETA-3 in polarised epithelial cells was mostly basolateral suggesting a possible role in the anchoring of cells to the basement membrane and in cell-cell adhesion. Consistent with a role in cell-ECM and cell-cell adhesion, PETA-3 was found to localise to EC margins and to associate with a broad range of members of the integrin superfamily. Additionally, CD9 and CD63 were also associated with PETA-3 in EC, demonstrating that like some other members of the TM4SF, PETA-3 is a component of integrin/TM4SF complexes (reviewed in Maecker *et al.*, 1997; Hemler *et al.*, 1996). The partial colocalisation of PETA-3 with integrins, CD9 and CD63 in some tissues, and in cultured EC, further

supported the results obtained by biochemical methods. Studies examining the effects of anti-PETA-3 mAbs on platelet function provided evidence to suggest that crosslinking of PETA-3 by the IgG2a mAb 11B1.G4 may contribute to the stimulatory signal required for aggregation of PRP. Ligation of PETA-3 by mAb also retarded EC migration and modulated the formation of capillaries in an *in vitro* model of angiogenesis.

6.2 *In Vivo* Distribution of PETA-3

Much of the work examining the expression and possible function of CD9 and CD63 has been performed in haemopoietic cells, including platelets, cell lines and primary transformed tissues, as described in Chapter 1. Although several studies have examined CD9 and CD63 in particular tissues or cell types, the systematic characterisation of the *in vivo* distribution of these molecules in normal tissues was not readily available from the current literature. Therefore the data presented in Chapter 3 not only provided evidence demonstrating the colocalisation of CD9 and CD63 with PETA-3 in some cells/tissues, but also served to catalogue their *in vivo* distribution. The co-expression of these molecules provides evidence for the existence of TM4SF/TM4SF and TM4SF/integrin complexes *in vivo*. However it is important to note that there were several cases where these molecules were not co-expressed, or resided within different subcellular compartments (Section 3.3), suggesting they play distinct roles in certain cells. Furthermore, examining the co-distribution of PETA-3, CD9, CD63 and the β1 integrin chain by immunohistochemical methods does not provide direct evidence of the physical association of these proteins. Other biochemical studies in a range of cell types would be required to confirm this.

Of the molecules examined, PETA-3 specifically localised to the basolateral membranes of basal keratinocytes in skin and gut enterocytes. Biochemical studies in EC demonstrated the association of PETA-3 with α6β4 integrin, and provides the only report of a

TM4SF association with this integrin (Section 5.2.2). Preliminary studies have shown strong colocalisation of PETA-3 with the α6 integrin in keratinocytes (Sincock and Ashman, unpublished). Since α6β4 is a major component of hemidesmosomes (Section 1.3.2), PETA-3 might play a specific role within these structures. Future studies examining the localisation of PETA-3 in ultrathin frozen sections of human skin would address this. The basolateral distribution of PETA-3 in epithelial cells was consistent with a role in anchoring of cells to the basal lamina. Furthermore, PETA-3 was predominantly expressed on cells associated with the basement membrane, including basal epithelial, endothelial, muscle (smooth, skeletal and cardiac) and Schwann cells, further emphasising a potential role for PETA-3 in cell-ECM adhesion.

During the studies investigating the tissue distribution of PETA-3, it became apparent that several anti-PETA-3 mAbs displayed different staining patterns (Section 3.2.19). However, despite the different tissue specificities of these mAbs, all bind equally to cells transfected with PETA-3 cDNA, but not control cells (Figure 2.2.A). Glycosylation patterns do not appear to effect mAb binding as PETA-3 has a single N-linked glycosylation site and all mAb immunoprecipitate both the glycosylated and non-glycosylated forms. Finally, treatment of normal bone marrow cells with neuraminidase did not abolish the differences in binding of the anti-PETA-3 mAbs used in this study to these cells (Ashman *et al.*, unpublished). Therefore it appears that other molecules, perhaps integrins, mask the epitopes of some anti-PETA-3 mAbs in a cell/tissue specific manner as discussed (Sections 3.2.19 and 3.3). Experiments investigating the complexes involved in the possible epitope masking of PETA-3 would be useful both in verifying the proximity of these molecules, and further characterising the binding properties of these mAbs. By assessing mAb binding to PETA-3 transfectants co-infected with individual integrin chains, the molecular basis for epitope

masking may be identified. Alternatively, the anti-PETA-3 mAbs 14A2.H1 and 11G5 may be sensitive to a conformational change in PETA-3.

6.3 PETA-3 in Platelet Biology

Within haemopoietic cells, PETA-3 is mostly restricted to platelets and megakaryocytes, where it might be involved in recognition of the subendothelial matrix during thrombus formation and in thrombopoiesis respectively. Consistent with a role in cell-cell adhesion, anti-PETA-3 mAbs promote homotypic aggregation of human myeloid cell lines (Fitter, Sincock, Jolliffe and Ashman, submitted) and stain the lateral margins of EC in culture and epithelial cells *in vivo*. The importance of TM4SF molecules during haemopoiesis has been demonstrated by the inhibition of myeloid cell production by anti-CD9 mAb (Oritani *et al.*, 1996). Therefore PETA-3 may play a role in co-ordinating adhesive events required for the differentiation of myeloid cells, especially those involved in megakaryopoiesis, within the bone marrow environment.

Although the experiments described in Section 5.3 provide evidence of signalling via PETA-3 crosslinking in platelets, these studies clearly need to be furthered with emphasis on characterising the mechanism of platelet activation. Initial studies could focus on the role of complement, and verification of the role of the FcγRII. Due to time constraints, Western blotting for anti-phosphotyrosine on 11B1.G4 stimulated platelets was not carried out. Since tyrosine phosphorylation plays such a critical role in platelet activation (Section 1.5.3), such studies would provide insight as to the downstream events initiated by $F(ab')_2$ fragments of 11B1.G4. These findings might also be useful in determining other platelet agonists which may synergise with 11B1.G4, as discussed (Section 5.5). Additionally, increasing the donor pool would be beneficial in examining the frequency of responding donors. The kinetics of 11B1.G4 induced platelet activation (ie. the prozone effect), were different from those of other

platelet activating mAbs of both IgG1 and IgG2a isotypes (De Reys *et al.*, 1993), suggesting that the mechanism was dependent on di-valent binding. Therefore, the initiation of signalling events induced by PETA-3 crosslinking appears likely.

6.4 PETA-3, Integrins and Endocytosis: A Functional Link?

The data presented in Chapter 4 provide evidence suggesting the involvement of PETA-3 in cell adhesion, as it was strongly localised to regions of cell-cell contact. However, upon examination of permeabilised EC, the presence of a large intracellular pool of PETA-3 became apparent. Further experiments using confocal microscopy and immunoelectron microscopy localised intracellular PETA-3 to the endocytic pathway, as described in Chapter 4. Based on the demonstration of multiple integrin associations, and the intracellular colocalisation with integrins in Chapter 5, PETA-3 may play a role in the internalisation and recycling of integrins. Endocytosis and subsequent recycling of integrins to the leading edge promotes cellular migration (reviewed in Lauffenberger and Horwitz, 1996). Furthermore, recycling of $\alpha5\beta1$, $\alpha v\beta3$ and $\alpha6\beta4$, which all associate with PETA-3, has been described (Bretscher, 1992; Sczekan and Juliano, 1990; Panetti and McKeown-Longo, 1993; Bretscher, 1989; Raub and Kuentzel, 1989). Although several integrin chains contain NPXY coated pit-mediated internalisation motifs in their cytoplasmic tails (Chen *et al.*, 1990), these domains do not necessarily appear to be directly involved in integrin internalisation (Vignoud *et al.*, 1994; Filardo *et al.*, 1995). Therefore the mechanism by which integrins are internalised, sorted and recycled to the leading edge of migrating cells remains unclear. The intracellular colocalisation of integrins with PETA-3, suggests that PETA-3 may enter the endocytic pathway complexed with integrins. Also, the internalisation of PETA-3/integrin complexes appears to be selective, as CD9, which is also present in integrin complexes on the plasma membrane, was not detected within the endocytic pathway. Therefore PETA-3 may play a

specific role in the internalisation and recycling of integrin complexes via the endocytic pathway. Furthermore, the functional effects of anti-PETA-3 antibodies on EC migration, and perhaps *in vivo* capillary tube formation, may have been due to disruption of integrin turnover. Using pulse-chase methodology, it would be useful to determine the turnover of PETA-3. Also, such experiments would verify actual internalisation of PETA-3 from the plasma membrane and would provide an estimate of the rate at which this might occur.

Several recent studies have described the localisation of CD63 to the internal membranes of multivesicular bodies in megakaryocytes, platelets (Heijen *et al.*, 1998) and CD37, CD53, CD63, CD81 and CD82 in MHC class II-enriched multivesicular bodies in B cells (Escola *et al.*, 1998). Multivesicular bodies are derived from the endocytic pathway and are thought to be precursors of platelet granules and B cell class II MHC enriched vesicles. These findings are consistent with the localisation of PETA-3 to the endocytic pathway of platelets and EC. However, although PETA-3 might be associated with these structures, no evidence for the enrichment of PETA-3 in platelet granules was found. Therefore, PETA-3 may be predominantly recycled out of these structures as they mature, as suggested by the low levels of labelling in platelet granules and EC Weibel-Palade bodies detected by immunoelectron mircoscopy. Interestingly, the megakaryoblastic cell line M-07e displays abundant intracellular staining for PETA-3, approximately 80% of the total (Fitter *et al.*, submitted), which might be associated with such vesicles. In B cells, class II MHC enriched multivesicular bodies are ultimately exocytosed as exosomes, suggesting a possible role for in antigen presentation. The lack of PETA-3 expression in B lymphocytes suggests that this molecule is not required for the formation of class II MHC enriched multivesicular bodies, nor is it involved in antigen presentation. However PETA-3 might still be involved in immune responses due to its upregulated expression on activated T lymphocytes (Hasegawa *et al.*, 1996).

The localisation of PETA-3 to the endocytic pathway suggests that the modulation of EC function may have been due to perturbation integrin turnover. However, studies using platelets in suspension suggest that crosslinking of PETA-3 by mAb might also transduce signals (Section 5.3). Other studies using mAb against CD9 have demonstrated the requirement of FcγRII crosslinking to promote full aggregation (Section 1.5.5). Nonetheless, several studies have provided evidence to suggest that ligation of CD9, although not sufficient to induce full aggregation, may contribute to platelet activation (Section 1.5.5). With the reports of signalling events initiated by anti-TM4SF mAbs (Sections 1.2.3, 1.2.4 and 1.2.5) and the association of PI4-K with CD63 (Berditchevski *et al.*, 1997a), it appears that TM4SF members may link integrin complexes with signalling molecules.

6.5 Towards a PETA-3 Knockout

With the characterisation of the murine PETA-3 genomic structure (Fitter *et al.*, 1998), the generation of PETA-3 knockout, or null, mice may also provide valuable insight as to the function of this molecule. The only reported TM4SF member to be deleted is CD81, which lead to a subtle phenotype involving impaired B cell responses (Levy *et al.*, 1998). As to what the phenotype of PETA-3 knockout mice might be remains speculative. The tissue distribution and electron microscopy studies described in this project have provided evidence to suggest a specific role for PETA-3 in cell adhesion events and endocytosis. Perhaps PETA-3 null mice would show major defects in the anchoring of cells to the basal lamina, analogous to those of β4 (Dowling *et al.*, 1996; Van der Neut *et al.*, 1996), α6 (Georges-Labouesse *et al.*, 1996), or α3 (DiPersio *et al.*, 1997) integrin knockouts. Whatever the phenotype of PETA-3 null mice, such studies would provide direct evidence as to the role this TM4SF member plays in cell biology.

6.6 Closing Remarks

In closing, the work described in this thesis has characterised the tissue distribution of PETA-3, determined its subcellular distribution in EC and platelets, demonstrated its association with multiple integrins and examined the function of anti-PETA-3 mAbs on platelet and EC function. These findings demonstrate that PETA-3, like other TM4SF members, is a component of integrin complexes and suggest a major role for this molecule in cell-cell and cell-ECM adhesion.

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