The role and regulation of the p84 adaptor subunit in phosphatidylinositol 3-kinase γ lipid-kinase signalling and the control of PI3Kγ-dependent cell migration

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Declaration

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Abbreviations

A2AR	A2A adenosine receptor
A3AR	A3 adenosine receptor
Ab	Antibody
ABD	Adaptor-binding domain
ACN	Acetonitrile
AnxA1/A2	Annexin A1/A2
Arg	Arginine
ATP	Adenosine triphosphate
β-AR	Beta adrenergic receptor
BAD	BCL-2 antagonist of cell death
BCR	B cell receptor
BH	Breakpoint-cluster-region homology
BMMC	Bone marrow-derived mast cell
bp	Base pair
BSA	Bovine serum albumin
C2	Protein-kinase C homology-2 domain
cAMP	Cyclic adenosine monophosphate
CBB	Coomassie Brilliant Blue stain
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CNA	Copy number alteration
CNS	Central nervous system
ConA	Concanavalin A
CRISPR	Clustered regularly interspaced short palindromic repeat
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAG	Diacyl-glycerol
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DEPC	Diethylpyrocarbonate
DN	Double negative (thymocytes)
DP	Double positive (thymocytes)
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-related kinases 1/2
ES cell	Embryonic stem cell
EUCOMM	European Conditional Mouse Mutagenesis Program
FA	Formic acid
FcεRI	Fc epsilon receptor 1
fMLP	Formyl-methionyl-leucyl-phenylalanine
FOXO	Forkhead box O
FRET	Fluorescent resonance energy transfer
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor

gRNA	Guide RNA
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HA	Haemagglutinin
HBSS	Hank's Balanced Salt Solution
HDX-MS	Hydrogen-deuterium exchange mass spectrometry
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
ICPL	Isotope-coded protein labelling
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IMVS	Institute of Medical and Veterinary Science
INPP5	Inositol polyphosphate-5-phosphatase
InsP3	Inositol triphosphate
IP	Immunoprecipitation
iSH2	Inter-SH2
KD	Knockdown
kDa	Kilodalton
LCMV	Lymphocytic choriomeningitis virus
LDS-PAGE	Lithium dodecyl sulphate polyacrylamide gel electrophoresis
LPA	Lysophosphatidic acid
Lys	Lysine
mAb	Monoclonal antibody
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionisation time-of-flight MS
MSCV	Murine stem cell virus
MS/MS	Tandem mass spectrometry
Met	Methionine
MOG	Myelin oligodendrocyte glycoprotein
miRNA	Micro RNA
MRCRB	Mouse red cell removal buffer
mRNA	Messenger RNA
MS	Multiple Sclerosis
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NIP-OVA	Nitroiodophenylacetic acid conjugated to ovalbumin
NLS	Nuclear localisation sequence
NMR	Nuclear magnetic resonance
p-Akt	Phosphorylated Akt
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE3B	Phosphodiesterase 3B
PDK-1	Phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
PIP	PtdIns(4)P; PtdIns-4-phosphate
PIP_2	PtdIns(4,5)P_2; PtdIns-4,5-bisphosphate
PIP_3	PtdIns(3,4,5)P_3; PtdIns-3,4,5-triphosphate

PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PLP	Myelin proteolipid protein
PMS	N-methyl dibenzopyrazine methyl sulphate
PMSG	Pregnant mare's serum gonadotropin
PP2A	Protein phosphatase 2
PPMT-1	PP2A methyltransferase-1
PRM	Parallel Reaction Monitoring
PtdIns	Phosphatidyl inositol
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene fluoride (membrane)
PX	Phox homology
qPCR	quantitative PCR
RA	Rheumatoid arthritis
RBD	Ras-binding domain
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RVD	Repeat-variable diresidue
SAGE	South Australian Genome-Editing facility
SCID	Severe Combined Immunodeficient
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	Serine
SH2	Src homology 2 domain
SH3	Src homology 3 domain
Shh	Sonic hedgehog
SHIP	Src homology 2 domain-containing inositol phosphatase
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SP	Single positive (thymocytes)
S/T	Serine/Threonine
STRING	Search Tool for the Retrieval of Interacting Genes
TALEN	Transactivator-like effector nuclease
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T cell receptor
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
Th	T helper lymphocyte
Thr	Threonine
TNF	Tumour necrosis factor
Tyr	Tyrosine
VEGF-A	Vascular endothelial growth factor-A
VEGFR1	Vascular endothelial growth factor receptor 1
WB	Western blot
WEHI	Walter and Eliza Hall Institute
WT	Wildtype
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide

Publications arising from this work

Manuscripts

Turvey ME, Klingler-Hoffmann M, Hoffmann P, McColl SR. p84 forms a negative regulatory complex with p110γ to control PI3Kγ signalling during cell migration. *Immunol Cell Biol*. 2015 Mar. doi; 10.1038/icb.2015.35. (Epub ahead of print).
Refer to **Appendix A1**.

Turvey ME, Koudelka T, Comerford I, Greer JM, Carroll W, Bernard CC, Hoffmann P, McColl SR. Quantitative proteome profiling of CNS-infiltrating autoreactive $CD4^+$ cells reveals selective changes during experimental autoimmune encephalomyelitis. *J Proteome Res*. 2014 Aug 1;13(8):3655-70.
Refer to **Appendix A2**.

Conference proceedings

Australian Society for Immunology Annual Scientific Meeting (2013): Poster entitled 'Quantitative proteome profiling of CNS-infiltrating autoreactive $CD4^+$ cells reveals selective changes during experimental autoimmune encephalomyelitis'

Australian Society for Immunology (SA / NT Branch) 9th Adelaide Immunology Retreat (2013): Oral Presentation entitled 'The role and regulation of the adaptor subunit p84 in phosphatidylinositol 3-kinase γ signalling and implications for cancer metastasis'

Abstract

The Class IB phosphatidylinositol 3-kinase (PI3K) enzyme, PI3Kγ, is activated and recruited to the plasma membrane in response to G protein-coupled receptor stimulation. Upon activation, the lipid-kinase activity and downstream signalling cascades initiated by PI3Kγ lead to cytoskeletal rearrangements and the formation of a leading edge for the induction of directed cell migration. PI3Kγ consists of the catalytic subunit p110γ, which forms a mutually exclusive heterodimer with one of two regulatory adaptor subunits, p84 or p101. Although expressed by most cells in the organism, PI3Kγ subunits are expressed at highest levels in motile haematopoietic cells, where the regulation of PI3Kγ signalling is critical to controlling and maintaining coordinated cell migration during immune responses. Consistent with a central role in leukocyte chemotaxis, innate and adaptive immune cell subsets from p110γ-deficient mice have been shown to exhibit migration defects *in vitro* and *in vivo*. Furthermore, the aberrant expression of PI3Kγ subunits and dysregulation of PI3Kγ signalling pathways has been shown to contribute to pathologies such as cancer and autoimmunity where enhanced cell migration promotes disease progression. Despite this, the mechanistic basis for PI3Kγ signal regulation is not well understood, particularly with respect to the distinct contributions of the individual regulatory adaptor subunits, p84 and p101. Many PI3Kγ-dependent cell functions have been elucidated experimentally using p110γ- and p101-deficient genetically-modified mouse strains and the PI3Kγ-selective inhibitor, AS605240. However, detailed functional data regarding p84 is lacking due to the absence of a p84-deficient mouse strain and limited availability of high quality p84-specific reagents. Three major research goals were addressed in the present study to improve our understanding of the role of p84 in PI3Kγ lipid-kinase signalling and its implication in PI3Kγ-dependent cell migration.

The first goal was to examine the phosphorylation status of p84 during PI3Kγ signalling and assess the role of identified regulatory phosphorylation sites for p84 function using the mammary epithelial carcinoma model cell line, MDA.MB.231. Data presented in this thesis demonstrate that in contrast to the p110γ and p101 subunits that promote the migration and metastasis of carcinoma cells, the p84 adaptor protein has tumour suppressor function *in vitro* and *in vivo*, which was determined to be dependent on a potential phosphorylation site within p84, Thr607. It was found that Thr607 was required for p84 to form an inducible heterodimer with p110γ (after initial PI3Kγ signal activation) in a

complex sequestered from active signalling at the membrane. This Thr607-dependent p84/p110γ dimerisation may therefore represent a novel mechanism of negative PI3Kγ signal regulation that limits the migration and metastasis of cancer cells.

Next, the contribution of p84 to PI3Kγ-dependent immune cell function was determined through the generation and characterisation of a novel p84-deficient mouse ($Pik3r6^{-/-}$) using CRISPR gene-editing technology. $Pik3r6^{-/-}$ mice were characterised in the context of immune cell development, activation and migration in a variety of haematopoietic cell subsets. It was shown that $Pik3r6^{-/-}$ mice develop normally with respect to lymphoid organ and circulating leukocyte populations at homeostasis. However upon stimulation, neutrophils from $Pik3r6^{-/-}$ mice display reduced migration in response to GPCR agonists *in vitro* and in a murine model of inflammatory autoimmunity (experimental autoimmune encephalomyelitis; EAE), it was found that activated Th lymphocytes display impaired trafficking and reduced infiltration to inflammatory sites.

The final goal was to develop and optimise a proteomic platform to investigate and compare the proteomes of migratory $CD4^+$ lymphocytes isolated from tissues at different stages of inflammatory disease progression using experimental autoimmune encephalomyelitis as a model. An isotope-coded protein-labelling (ICPL) approach was developed and optimised to assess the proteomes of CNS-infiltrating $CD4^+$ lymphocytes during disease progression in two models of EAE; chronic MOG_{35-55}-induced EAE and relapsing-remitting $PLP_{139-151}$-induced EAE. This study identified differentially regulated proteins related to immune cell function and represented a initial feasibility study to verify the validity of ICPL as an approach to examine the differential proteomes of wildtype and p84-deficient migratory $CD4^+$ lymphocytes during inflammatory disease.

Collectively, the data presented in this thesis represent the identification and characterisation of novel roles for p84 within PI3Kγ lipid-kinase signalling during both the regulation of cell migration in carcinoma cells and in haematopoietic cells during immune responses. In addition to furthering the understanding of the unique roles for p84 within PI3Kγ signal regulation, the generation of a p84-deficient mouse strain constitutes an important tool to further experimental research in this area.