The Role of β_c Subunit Phosphorylation in the Functioning of the GM-CSF/IL-3/IL-5 Receptors.

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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 another person, except where due reference has been made in the text.

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Wendy Winnall

Acknowledgement of Contributions

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The assays used in Figures 3.11 and 3.12 were performed by Ms E.Barry and Dr M. Guthridge, constituting approximately 10% of the data in Chapter 3. Creation of the pRUFneoIRES construct and subcloning in the huGMRα and huβ$_c$ mutant constructs, and creation of the Ψ2 cell lines expressing these constructs, was performed by Dr H. Ramshaw (Sections 2.2.1.17, 4.3.1, Figure 2.1).

Abstract

The cytokines GM-CSF, IL-3 and IL-5 are central regulators of haemopoietic cell functions and are pivotal in the regulation of haemopoiesis and inflammatory responses of myeloid cells. In particular, these cytokines have been shown to perform essential functions in host defence against foreign pathogens through their ability to regulate innate immune responses in myeloid cells. As key regulators of such important processes, these cytokines play an important role in human inflammatory pathologies such as rheumatoid arthritis, asthma, multiple sclerosis and psoriasis as well as a number of leukemias such as JML and CMML.

GM-CSF, IL-3 and IL-5 signal through receptors containing α subunits specific to each cytokine and a common β subunit (β_c). Cytokine stimulation leads to tyrosine phosphorylation of the β_c and promotes specific responses such as proliferation, survival and activation of haemopoietic cells. Mouse knockout studies identified a key function of these cytokines in the activation of effector functions of myeloid cells, including production of reactive oxygen species (ROS) and phagocytosis. These earlier studies provide a link between cytokine signalling and inflammation, but the molecular mechanisms by which β_c activation regulates effector cell functions, and the receptor motifs involved, are unknown.

The aim of this thesis was to address two broad questions with regard to β_c signalling: (1) Does β_c regulate specific cellular responses by phosphotyrosine-independent mechanisms?

(2) What are the molecular mechanisms by which β_c initiates signalling to promote specific biological responses such as activation of effector cell functions?

To address the first question, we have focussed on Serine 585, a potential 14-3-3 binding site which lies in the cytoplasmic potion of huβ_c. Out results show that the mutation huβ_c^{S585G} disrupted the interaction of 14-3-3ζ with β_c, whilst not affecting receptor tyrosine phosphorylation. Both mouse and human β_c were shown to interact with 14-3-3 proteins, indicating that this interaction is conserved between these species. Significantly, a huβ_c^{S585G} mutant was unable to promote haemopoietic cell survival in response to IL-3. These results identify a new mechanism by which cytokine receptors are able to couple to downstream signalling pathways that regulate cell survival.

An approach was developed and optimised to analyse specific GM-CSF-mediated responses in monocytes/macrophages expressing wildtype or mutant huβ_c, (including huβ_c^{S585G} that was defective in regulating survival). Bone marrow-derived mu$\beta_c^{-/-}$;mu$\beta_{IL\text{-}3}^{-/-}$ monocytes/macrophages were retrovirally transduced with constructs expressing wildtype or mutant huβ_c, along with huGMRα, then purified by FACS. Two assays were established to measure effector functions in the transduced monocyte/macrophages; (1) a flow cytometry assay for ROS production, and (2) an assay for phagocytosis. The capacity for GM-CSF to prime (i.e. enhance effector functions) ROS production and phagocytosis was investigated in huGMRα-transduced monocytes/macrophages. Our results have identified two key residues in the cytoplasmic domain of β_c subunit: Tyrosine 577 (required for huβ_c interaction with the adaptor protein Shc) and serine 585 (required for 14-3-3 association), that are essential for the ability of GM-CSF to regulate key effector functions in monocytes/macrophages.

These novel findings are significant in that they establish a molecular link between the GM-CSF/IL-3/IL-5 receptor and the regulation of both haemopoietic cell survival and inflammatory responses, and therefore have important implications in our understanding of inflammatory diseases such as rheumatoid arthritis and asthma.

Publications arising from this thesis

Important results from Chapter 3 of this thesis are published in these papers:

Stomski,F.C., Dottore,M., Winnall,W., Guthridge,M.A., Woodcock,J., Bagley,C.J., Thomas,D.T., Andrew,R.K., Berndt,M.C., and Lopez,A.F. (1999). **Identification of a 14-3-3 binding sequence in the common β chain of the GM-CSF, IL-3 and IL-5 receptor that is serine-phosphorylated by GM-CSF.** Blood *94*, 1933-1942.

Guthridge,M.A., Stomski,F.C., Barry,E.F., Winnall,W., Woodcock,J.M., McClure,B.J., Dottore,M., Berndt,M.C., and Lopez,A.F. (2000). **Site-specific serine phosphorylation of the IL-3 receptor is required for hemopoietic cell survival.** Mol. Cell *6*, 99-108.

Abbreviations

Ab: Antibody
AML: acute myeloid leukemia
APS: ammonium persulphate
ATP: adenosine triphosphate
β_c: common β-subunit of the GM-CSF, IL-3, IL-5 receptor
β_{IL3}: β-subunit of the mouse IL-3 receptor
bisacrylamide: N,N'-methylene-bisacrylamide
βME: beta-mercaptoethanol
bp: base pairs
BSA: Bovine serum albumin
°C: degrees Celcius
cAMP: cyclic adenosine monophosphate
cDNA: complementary DNA
CFU: colony forming unit
CIP: calf intestinal phosphatase
CMML: chronic myelomonocytic leukemia
CRM: cytokine receptor module
CsCl: caesium chloride
DMEM: Dulbecco's modified Eagle's medium
DTT: dithiothrietol
EDTA: ethylenediaminetetra acetic acid
ERK: extra-cellular signal-related kinase
EtBr: ethidium bromide
ETOH: ethanol
FACS: fluorescene activated cell sorting
FcR: "fragment, crystallisable" region of antibody receptor
FCS: fetal calf serum
FITC: fluorescein
fMLP: *N*-formylmethionyl-leucyl-phenylalanine
GM-CSF: granulocyte macrophage colony-stimulating factor
GMR: GM-CSF receptor
GMRα: GMR alpha subunit
GRB2: growth factor receptor bound G-CSF
HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HRP: horseradish peroxidase
hu: human
IFNg: interferon gamma
Ig: immunoglobulin
IL: interleukin
IL-3: interleukin-3
IL-3R: IL-3 receptor
IL-3Rα: IL-3R alhpa subunit
IL-5: interleukin-5
IL-5R: IL-5 receptor
IL-5Rα: IL-5R alpha subunit
IMDM: Isocove's modified Dulbecco's medium
JAK: Janus Kinase
JNK: c-jun N-terminal kinase
KAc: potassium acetate
kb: kilobase pairs
kD: kiloDaltons
krpm: kilorevolutions per minute
L: litre(s)

LB: Luria broth
M: molar
mu: murine/mouse
MAP kinase: mitogen-activated protein kinase
MQ: Milli Q purified water
mu: murine
NaAc: sodium Acetate
NADPH: reduced form of nicotinamide adenine dinucleotide phosphate
NO: nitric oxide
OD: optical density
p: plasmid
PAG: polyacrylamide gel
PAP: pulmonary alveolar proteinosis
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PE: phycoerythrin
***Pfu*:** *Pyrococcus furiosis* bacterium
PI 3-kinase: phosphoinositide 3-kinase
PKA: protein kinase A
PKC: protein kinase C
PMSF: phenylmethylsulfonyl fluoride protein-2
PTB domain: phospho-tyrosine binding domain
R: receptor
RNA: ribonucleic acid
ROS: reactive oxygen species
rpm: revolutions per minute
SCF: Stem cell factor
SDS: sodium dodecyl sulphate
SH2: shc homology 2
SH3: shc homology 3
STAT: signal transducer and activator of transcription
TEMED: N,N,N',N-tetramethylenediamine
TNF: tumour necrosis factor
tris:tris(hydroxymethyl) aminomethane
µl: microlitres
UV: ultraviolet light
v/v: volume per volume
w/w: weight per weight

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