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Interferon-Gamma-Activated Gene Expression

Molecular Mechanisms of Stat1-mediated Transcription

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ACADEMIC DISSERTATION

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1. ABSTRACT

Interferons are a group of secreted proteins belonging to the large family of cytokines. They have a critical role in the immune system in providing an early line of defence against viruses. Interferon-γ (IFN-γ) is also an important activator of monocytes and macrophages. One of the target genes for IFN-γ in macrophages is the high affinity receptor for immunoglobulin G, FcγRI, which mediates phagocytosis, antibody-dependent cytotoxicity and respiratory burst. IFN-γ binds to its specific receptor on the cell surface, and the signal is transduced into the cell via the JAK/Stat pathway. The receptor-associated JAK tyrosine kinases are activated upon IFN-γ binding and activate cytoplasmic latent Stat1 transcription factors by tyrosine phosphorylation. Stat1 molecules form dimers and translocate into the nucleus where they recognize their specific DNA binding elements and regulate transcription of IFN-γ-responsive genes. The molecular mechanisms of Stat-mediated transcription are not yet fully understood. The aim of this study was to establish a model system for analysing the functions and molecular mechanisms of Stat1 and other transcription factors in the tissue-specific expression of cytokine-responsive genes. Myeloid cell-specific IFN-γ-stimulated activation of the FcγRI gene was strictly dependent on binding of two factors, Stat1 and PU.1, to the promoter. The DNA binding and transactivation functions of both proteins were required, but no physical interaction between the factors was involved. PU.1 was found to mediate contacts with basal transcription machinery components TATA-binding protein and RNA polymerase II, whereas Stat1 recruited the coactivator protein CBP/p300 to the promoter. The mechanism by which glucocorticoids augment IFN-γ-induced activation of FcγRI in macrophages was investigated in the same model system. Glucocorticoid receptor (GR), a sequence-specific transcription factor belonging to the family of nuclear hormone receptors, was found to cooperate with Stat1 and PU.1 by an indirect mechanism involving the DNA binding and transactivation functions of GR and Stat1. No physical association between Stat1 and GR was demonstrated, and the requirement of glucocorticoid-induced protein synthesis suggests the involvement of newly produced transcriptional coregulators. The roles of PIAS (protein inhibitor of activated Stat) proteins, which have been described as negative regulators for Stats, were analysed in the modulation of cytokine-responsive genes. In the experimental conditions used in this study, none of the PIAS proteins was found to significantly affect transcriptional activation mediated by any of the Stats analysed in the study. In contrast, all PIAS proteins were able to modulate steroid receptor-dependent transcription positively or negatively, depending on the promoter and cell type.

2. LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I Aittomäki S, Yang J, Scott EW, Simon MC, Silvennoinen O. Distinct functions for signal transducer and activator of transcription 1 and PU.1 in transcriptional activation of Fc gamma receptor I promoter. Blood 100:1078-80, 2002

II Aittomäki S, Yang J, Scott EW, Simon MC, Silvennoinen O. Molecular basis of Stat1 and PU.1 cooperation in cytokine-induced Fc γ receptor I promoter activation. In press

III Aittomäki S, Pesu M, Groner B, Jänne OA, Palvimo JJ, Silvennoinen O. Cooperation among Stat1, glucocorticoid receptor, and PU.1 in transcriptional activation of the high-affinity Fc gamma receptor I in monocytes. J Immunol 164:5689-97, 2000

IV Kotaja N, Aittomäki S, Silvennoinen O, Palvimo JJ, Jänne OA. ARIP3 (androgen receptor-interacting protein 3) and other PIAS (protein inhibitor of activated STAT) proteins differ in their ability to modulate steroid receptor-dependent transcriptional activation. Mol Endocrinol 14:1986-2000, 2000

3. ABBREVIATIONS

AF	Activation function
AP-1	Activator protein-1
ARIP3	Androgen receptor interacting protein-3
ATP	Adenosine triphosphate
bp	Base pair,-s
BSA	Bovine serum albumin
C	Carboxy
CIITA	MHC class II transactivator
CBP	CREB binding protein
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer binding protein
CREB	Cyclic AMP response element binding protein
Dex	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
EMSA	Electrophoretic mobility shift assay
ES	Embryonic stem
Ets	E-twenty-six
FACS	Fluorescence activated cell sorter
FcγR	Fcγ receptor
FCS	Foetal calf serum
FERM	Band four point one, ezrin, radixin and moesin
FITC	Fluorescein isothiocyanate
GAS	Gamma activated sequence
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRR	Gamma response region
GRE	Glucocorticoid response element
GRIP1	Gluococorticoid receptor interacting protein-1
GST	Glutathione-S-transferase
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMG	High mobility group protein
Hsp	Heat shock protein
ICSBP	Interferon consensus binding protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK	Janus kinase
JNK1	C-Jun N-terminal kinase
KDa	Kilodalton

LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocombatibility complex
MIF	Migration inhibitory factor
mRNA	Messenger RNA
N	Amino
N-CoR	Nuclear corepressor
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
PBS	Phosphate-buffered saline
PCAF	p300/CBP-associated factor
PEST	Proline, glutamic acid, serine and threonine
PI-3K	Phosphatidyl-inositol 3-kinase
PIAS	Protein inhibitor of signal transducer and activator of transcription
PMSF	Phenylmethylsulfonyl fluoride
RNAPII	RNA polymerase II
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology-2
SOCS	Suppressor of cytokine signalling
Spi-1	SFFV provirus integration site-1
SRC	Steroid receptor coactivator
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
Stat	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TAD	Transactivation domain
TBP	TATA-binding protein
TFII	Transcription factor for RNA polymerase II
Th	T helper
TK	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor

4. INTRODUCTION

The human body has developed a number of defence mechanisms against pathogenic organisms involving a variety of cells, primarily leukocytes of the immune system. A multitude of soluble signalling molecules participate in modulating immune responses. Interferons (IFNs) are secreted proteins that are important in combating microbial infections. IFNs produced by infected cells act both in autocrine and paracrine fashion, inducing an antiviral state in the infected cell and in the uninfected neighbouring cells. IFNs fall into two categories, and the type II IFN, IFN-γ, performs a range of immunomodulatory functions, one of the most important being the activation of macrophages.

IFNs were discovered as early as in the 1950s, but it took several decades before the mechanism of the signalling pathway was elucidated. Subsequently the same pathway was found also to mediate the signalling by other cytokines. IFN-γ exerts its functions by inducing immediate transcriptional responses, regulating the expression of a large number of genes. The signal is transduced into the nucleus through the JAK/Stat pathway, which is activated when IFN-γ binds to its specific receptor on the cell surface. The receptor is expressed on most cells, and the components of the signalling pathway are also present in the majority of cells of the body. However, some of the IFN-γ-responsive genes are activated only in certain cell types, indicating that there must be other factors that determine the cell type specificity of transcription.

The effects of IFN-γ on transcription are mediated largely by the binding of Stat1 transcription factor to its response elements in the regulatory regions of genes. To study the molecular mechanisms that underlie the cell type-specific expression of IFN-γ-regulated transcription, we set up a model system with a previously characterised gene promoter. The high affinity IgG receptor FcγRI is expressed on cells of myeloid origin, i.e. on monocyte/macrophages and neutrophils, but by ectopic expression of a haematopoietic cell restricted transcription factor PU.1, the FcγRI promoter can also be activated in non-myeloid cells. This model allowed us to study in detail the functions of Stat1 and PU.1 factors and to ascertain the mechanism by which they cooperate with other components of transcription machinery to activate the expression of FcγRI.

5. REVIEW OF THE LITERATURE

5.1. Haematopoiesis

The production of blood cells, haematopoiesis, is a continuous process that in adults occurs mainly in the bone marrow. A limited population of pluripotent self-renewing stem cells gives rise to distinct lineage-committed common lymphoid and common myeloid precursor cells. These cells then differentiate further, lymphoid precursors into B and T lymphocytes, and myeloid precursors into monocytes, granulocytes, megakaryocytes and erythrocytes (Ogawa 1993). Haematopoietic stem cell commitment is regulated by the bone marrow microenvironment that includes cell-cell interactions, cell-extracellular matrix interactions and exposure to soluble factors including cytokines (Krause 2002). Both the commitment and maturation of haematopoietic cells arise from the gradual expression of lineage-specific genes, determined by the action of critical transcription factors regulated by the external differentiating signals (Valledor et al. 1998a). The differentiation of a particular lineage is dependent on the correct combination of transcription factors acting at the correct times and in the correct doses (Cheng et al. 1996, Rothenberg and Anderson 2002).

Numerous cytokines have been identified to be essential in haematopoiesis. Some, like stem cell factor, interleukin 3 (IL-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF), contribute to the progeny of several cell types, whereas others, such as erythropoietin, granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) exert their principal action on precursors of a single lineage (Ogawa 1993, Metcalf 2001). Two different hypotheses have been proposed to explain the requirement for cytokines in haematopoietic differentiation. The stochastic hypothesis suggests that the commitment of a progenitor cell to a particular lineage is a stochastic event, subsequent to which differentiation proceeds according a pre-determined programme, and cytokines are required to ensure the survival and proliferation of already committed precursors. According to the instructive hypothesis, cell fate will be determined by the type of cytokine acting on the cell (Socolovsky et al. 1998). Several studies support the former hypothesis, suggesting that the specificity of lineage-restricted cytokines is a result of a unique expression pattern of each cytokine receptor and that this expression is the result, rather than the cause, of lineage commitment (Socolovsky et al. 1997, Goldsmith et al. 1998, Stoffel et al. 1999, Kovanen et al. 2003). The expression of the receptors is induced by a set of critical transcription factors that are initially stochastically expressed in the stem cells and early multipotential progenitors and subsequently upregulated by undefined mechanisms (Tenen et al. 1997, Skalnik 2002).

5.2. Macrophages

Macrophages are the mature forms of monocytes and their primary function is phagocytosis. Macrophages are derived from bipotent granulocyte-macrophage progenitors that can differentiate into either granulocyte- or macrophage-forming

cells. After further divisions and differentiation steps the immature macrophage, called monoblast, leaves the bone marrow and enters the bloodstream, where it undergoes two more cell divisions, becoming a monocyte. Final differentiation into macrophages occurs when the monocyte exits the bloodstream and enters the site where it is required for host defence (Shepard and Zon 2000).

Macrophages are widely distributed throughout the body, either free or resident in different tissues and organs (Shepard and Zon 2000). As a result of their widespread tissue distribution and responsiveness to many endogenous and exogenous stimuli, macrophages are phenotypically heterogeneous (Gordon 2003). Liver Kupffer cells, bone osteoclasts, alveolar macrophages of the lungs, the antigen presenting dendritic and Langerhans cells, and microglia in the central nervous system are all derived from the macrophage lineage. The signals responsible for the tissue-specific phenotypes include secreted products and surface molecules of neighbouring cells and extracellular matrix. Macrophage migration is controlled by many types of adhesion molecules, and cytokines, chemokines and growth factors affect gene expression of the macrophage (Gordon 2003). Macrophages are activated in the tissues where they are already resident or recruited to as monocytes. Depending on the types of stimuli macrophages are exposed to, they can be activated to perform a variety of functions (Gordon 2003, Mosser 2003). They defend the host against microorganisms and tumour cells, regulate inflammatory and immune responses by secreting different mediators, presenting antigens and activating T cells, and contribute to wound healing (Shepard and Zon 2000).

Macrophages function as important effector cells in both innate and adaptive immunity, and they can be activated by several ways (Figure 1). In innate activation the macrophage recognizes microbial components that are absent in the host, e.g. lipopolysaccharide (LPS), peptidoglycans, and bacterial CpG DNA, by its pattern-recognition receptors. Macrophages express a number of different pattern-recognition receptors on their surfaces, such as those belonging to the scavenger receptor type A and Toll-like receptor families, CD14 and β-glucan receptor. Ligation of these receptors enhances phagocytosis and endocytosis, and induces the cell to produce pro-inflammatory cytokines, reactive oxygen species and nitric oxide. Some complement and Fc receptors mediate the humoral activation of the macrophage, resulting in cytolytic activity and cytokine secretion. In so-called classical activation the macrophage is exposed to two signals, cytokine interferon-γ (IFN-γ) and a microbial trigger, such as LPS. The ability of the cell to kill and degrade microbes is enhanced through the production of nitric oxide and reactive oxygen species, and it secretes a range of pro-inflammatory cytokines, like interleukins 1 and 6 (IL-1 and IL-6) and tumour necrosis factors (TNFs) (Gordon 2003). Stimulation with IL-4 or IL-13 results in the “alternatively activated macrophage” that is involved in humoral immunity, allergic and anti-parasite responses and tissue repair rather than microbicidal activities (Gordon 2003, Mosser 2003). Recent data indicate that there may be still another type of activated macrophage, type II activated macrophage, the induction of which requires two signals: ligation of its Fc receptors and another stimulatory signal. These cells produce the same pro-inflammatory cytokines as the classically activated macrophage, with the

exception of large amounts of IL-10 and absence of IL-12, suggesting that this type of cell may have anti-inflammatory effects. IL-10 secretion results in IL-4 production by T cells and preferential induction of T helper type 2 (Th2) immune responses (Mosser 2003).

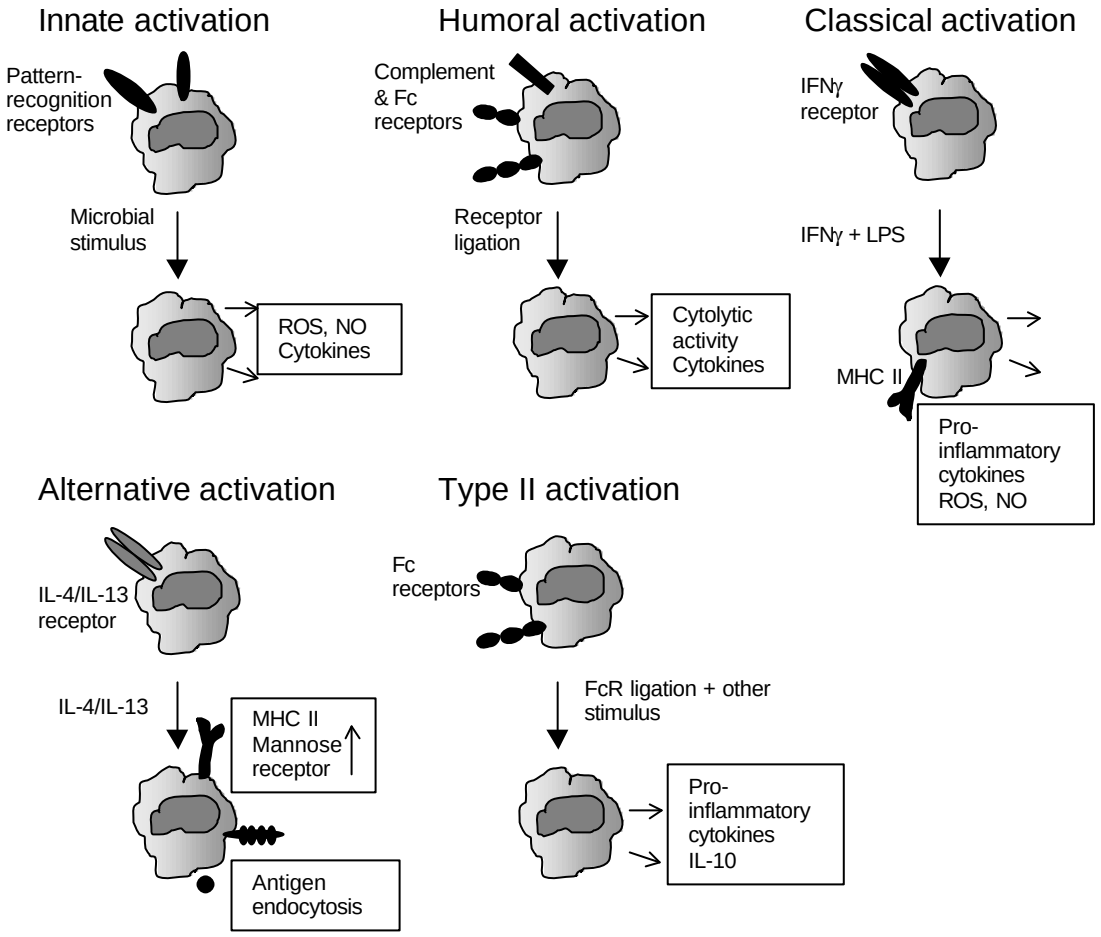

Figure 1. Activation of macrophages. Adapted from Gordon 2003.

5.3. Transcription factors in the differentiation of macrophages

Many transcription factors important in myeloid differentiation have been discovered through their involvement in leukaemias, particularly as parts of fusion proteins that are generated as a result of the chromosomal translocations common in leukaemias. Various previously known oncogenes have also been shown to play a role in myeloid differentiation. Additionally, factors have been identified by analysing the regulatory regions of myeloid-specific genes (Tenen et al. 1997). It appears that a relatively small number of factors are truly critical for regulating macrophage differentiation as well as the gene expression of mature cells. These factors are not myeloid-specific; the cell-type specific gene expression depends on combinatorial interactions of these factors with each other and with additional transcription factors. The activities of the transcription

factors can be controlled at multiple stages, e.g. at the level of transcription, via protein-protein interactions and through posttranslational modifications such as phosphorylation (Skalnik 2002).

Some factors are expressed in haematopoietic progenitors and downregulated with differentiation. Such factors include GATA-1, GATA-2, PLZF and SCL, which control the survival of stem cells, and the proto-oncogenes c-myb and c-myc that regulate early differentiation. Other factors like PU.1 and AML1 are expressed throughout differentiation, whereas others, e.g. C/EBPβ, Egr-1 and c-Jun, are involved in terminal differentiation regulating genes that determine the mature macrophage phenotype (Valledor et al. 1998b). The promoters of myeloid-specific genes generally contain binding sites for multiple transcription factors. In the chromosomal context the proximal promoter is, however, often not sufficient to drive proper expression, but distant regulatory elements, sometimes dozens of kilobases upstream and downstream of the coding region, are required. Many genes contain a number of cell-type restricted DNase I hypersensitive sites that can be located kilobases upstream of the flanking sequence. This suggests that *in vivo* chromatin structure remodelling is crucial in regulating cell-type specific gene expression (Skalnik 2002).

5.4. PU.1

PU.1 was originally isolated as a product of *spi-1* gene from mouse erythroid leukaemia cells. The gene locus is an integration site for the spleen focus-forming virus that induces leukaemia, hence the name *spi-1* (SFFV provirus integration site-1), and the integration into the 5' flanking region of *spi-1* results in upregulation of PU.1 expression (Moreau-Gachelin et al. 1988, Moreau-Gachelin et al. 1989). In an independent study, PU.1 was cloned from a mouse macrophage expression library using an oligonucleotide probe containing so-called PU box (GGAA) that binds PU.1 (Klemsz et al. 1990). PU.1 belongs to the large family of Ets-domain transcription factors, being its most divergent member (Lloberas et al. 1999). All family members (almost 50 identified in various metazoan organisms (Lloberas et al. 1999, Sharrocks 2001)) have a common 85-amino acid DNA binding domain, the Ets domain, that recognizes a purine-rich core sequence 5'-GGAA/T-3' (Janknecht and Nordheim 1993). The closest homologues to PU.1 in the family are Spi-B and Spi-C, which can bind to the same target sequences as PU.1 *in vitro* (Su et al. 1996, Bemark et al. 1999). The expression patterns of the three proteins are partially different, and gene-targeting experiments have shown that Spi-B and PU.1 are functionally redundant in myeloid but not in lymphoid development (Dahl et al. 2002). Spi-C is not so well characterized but appears to be expressed temporarily during B-cell differentiation and in macrophages (Bemark et al. 1999).

PU.1 is expressed solely in cells of haematopoietic origin, i.e. in B cells, early erythroblasts, neutrophils, macrophages, and mast cells, and also in multipotent haematopoietic progenitor cells (Lloberas et al. 1999). The promoter of PU.1 gene has been characterized in human and mouse (Chen et al. 1995, Chen et al. 1996). The proximal 120 bp of the promoter direct cell type-specific reporter

gene expression in myeloid and B cell lines, and three functional transcription binding sites have been identified in the region; an Octamer site, an Sp1 binding site, and a site for PU.1 itself. Ectopic PU.1 or Spi-B specifically transactivated the minimal promoter in PU.1-deficient cells, indicating that PU.1 can activate its own promoter elements in an autoregulatory loop (Chen et al. 1995, Chen et al. 1996). A more recent study indicates that in the chromosomal context, in stably transfected cell lines and in transgenic animals, other regulatory regions are also required. A 3.5-kb fragment containing a DNase I hypersensitive site, located -14 kb 5' of the transcriptional start site, conferred myeloid cell-type specific expression of PU.1 (Li et al. 2001). Altering chromatin structure by histone acetylation is crucial for the regulation of gene expression. In one report PU.1 expression was shown to be down-regulated following blocking the activity of histone deacetylases (Laribee and Klemsz 2001).

5.4.1. Structure of PU.1

PU.1 gene encodes a protein of 272 amino acids, the DNA binding Ets domain located at the C-terminal end (Lloberas et al. 1999). Different approaches have been used to identify functionally important domains of the protein: conventional biochemical and transactivation assays in established cell lines and *in vitro* differentiation system of PU.1-deficient embryonic stem (ES) cells transduced with retroviral PU.1 constructs. Different regions have been found to be important in different systems. The N-terminal part of the protein contains multiple transactivation domains, flanked by a central PEST region (proline, glutamic acid, serine and threonine rich domain) (Fisher and Scott 1998, Simon 1998).

PU.1 binds to its target sequence as a monomer (Kodandapani et al. 1996). The crystal structure of the PU.1 Ets domain bound to DNA has been solved (Kodandapani et al. 1996). The domain is similar to $\alpha + \beta$ (winged) helix-turn-helix proteins, consisting of three α helices and four β-sheets, and interacts with a ten-base-pair region of DNA, resulting in DNA bending. Four of the amino acids interacting with the DNA are highly conserved in Ets family members: two arginines from the recognition helix lying in the major groove, one lysine from the "wing" that binds upstream of the core GGAA sequence, and another lysine from the turn of the helix-turn-helix motif, which binds downstream on the opposite strand (Kodandapani et al. 1996).

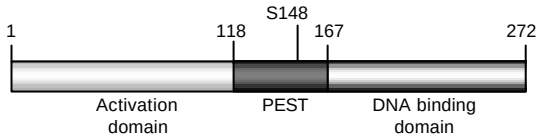

Figure 2. Schematic representation of the structure of PU.1. The N-terminal part contains multiple transactivation domains, the DNA binding domain is located in the C-terminal part. The PEST domain is important in protein-protein interactions and in the differentiation of macrophages.

The four transactivation domains identified reside between amino acids 7 to 100; three are rich in acidic amino acids (residues 7 to 74) and the fourth is glutamine-rich (residues 75 to 100). In different assay systems distinct domains appear to be critical. When various PU.1 deletion constructs were co-expressed with reporter plasmids containing multiple PU.1 binding sites in non-haematopoietic HeLa cells, the glutamine-rich domain was the weakest (Klemsz and Maki 1996). In the ES cell model this region was the only transactivation domain absolutely required for myeloid differentiation (Fisher et al. 1998). In a fibroblast transfection system using multimers of PU.1 binding site from immunoglobulin κ light chain 3' enhancer it was demonstrated that neither the regions 7-30 nor 33-100 were necessary for the formation of an active complex with other transcription factors (Pongubala and Atchison 1997). Two serine phosphorylation sites, Ser41 and Ser45, were necessary for the M-CSF-dependent proliferation of bone marrow macrophages (Celada et al. 1996). PU.1 has been shown to be induced by phosphatidyl-inositol 3-kinase-dependent externally regulated kinase Akt, phosphorylation of Ser41 being required (Rieske and Pongubala 2001).

The central PEST domain (amino acids 118 to 160) was not necessary for transcriptional activation by PU.1 in transfection assays in non-haematopoietic cell lines (Klemsz and Maki 1996, Wara-aswapati et al. 1999). However, a subregion of the PEST domain (amino acids 129 to 141) was critical for the differentiation of ES cells to macrophages (Fisher et al. 1998). The PEST domain is important in protein-protein interactions in some regulatory elements and has been shown to be phosphorylated on serine residues. Bacterial lipopolysaccharide (LPS) treatment was demonstrated to induce phosphorylation of PU.1 at Ser148 by casein kinase II, resulting in its enhanced transcriptional activity (Lodie et al. 1997). Phosphorylation of Ser148 augmented binding of interferon regulatory factor-4 (IRF-4) and IRF-8 (interferon consensus sequence binding protein ICSBP) to PU.1, which were required for optimal transactivation of immunoglobulin light chain 3' enhancer elements and IL-1β promoter, respectively (Pongubala et al. 1993, Eisenbeis et al. 1995, Marecki et al. 1999). IL-3 stimulation of mcl-1 gene transcription in a pro-B cell line involved phosphorylation of PU.1 at Ser142 by a p38 mitogen-activated protein kinase (MAPK)-dependent pathway and markedly increased the transactivation activity of PU.1 (Wang et al. 2003). In one study, the stress activated protein kinase JNK1 (c-Jun N-terminal kinase) was also shown to phosphorylate PU.1 (Mao et al. 1996).

	Reference
Genes positively regulated by PU.1	
Composite PU.1/IRF elements	
immunoglobulin κ light chain	(Pongubala et al. 1992)
immunoglobulin λ light chain	(Eisenbeis et al. 1993)
IL-1β 5' enhancer	(Marecki et al. 2001)
CD20	(Himmelmann et al. 1997)
gp91hox	(Eklund et al. 1998)
Toll-like receptor 4	(Rehli et al. 2000)
IFN-stimulated gene 15	(Meraro et al. 2002)
B cell-restricted genes	
immunoglobulin μ heavy chain	(Nelsen et al. 1993)
immunoglobulin J-chain	(Shin and Koshland 1993)
IgE germline promoter	(Stutz and Woisetschlager 1999)
Btk	(Himmelmann et al. 1996)
CD72	(Ying et al. 1998)
LSP1/pp52	(Omori et al. 1997)
mb-1	(Hagman and Grosschedl 1992)
mcl-1	(Wang et al. 2003)
IL-7Rα	(DeKoter et al. 2002)
P2Y10	(Rao et al. 1999)
c-rel	(Viswanathan et al. 1996)
Myeloid genes	
high affinity Ig receptor FcγRI	(Eichbaum et al. 1994, Perez et al. 1994)
low affinity Ig receptor FcγRIIIa	(Feinman et al. 1994)
scavenger receptor	(Moulton et al. 1994)
integrin CD18	(Bottinger et al. 1994, Rosmarin et al. 1995)
integrin CD11b	(Pahl et al. 1993)
mannose receptor	(Eichbaum et al. 1997)
GM-CSFR	(Hohaus et al. 1995)
M-CSFR	(Reddy et al. 1994, Zhang et al. 1994)
G-CSFR	(Smith et al. 1996)
lysozyme	(Ahne and Stratling 1994)
proteinase-3	(Sturrock et al. 1996)
elastase	(Oelgeschlager et al. 1996)
$gp91^{hox}$	(Eklund et al. 1998, Suzuki et al. 1998)
$gp67^{hox}$	(Li et al. 2001)
$gp47^{hox}$	(Li et al. 1997)
$gp40^{hox}$	(Li et al. 2002)
IL-12 (p40 subunit)	(Byrnes et al. 2001)
IL-1b	(Marecki et al. 2001)
IL-1 receptor antagonist	(Smith et al. 1998)
c-fes	(Heydemann et al. 1996)
allograft inflammatory factor-1	(Sibinga et al. 2002)
Genes negatively regulated by PU.1	
CD11c integrin	(Lopez-Rodriguez and Corbi 1997)
murine MHC II I-Ab	(Borras et al. 1995)
c-myb	(Bellon et al. 1997)

Table 1. Genes regulated by PU.1.

5.4.2. Genes regulated by PU.1

Functional PU.1 binding sites have been identified in most myeloid-specific promoters and in a number of B-cell-restricted enhancers and promoters (Table 1). PU.1 can bind to DNA either alone, recognizing the core element GGAA, or in a complex together with IRF-4 or IRF-8, when it binds to a composite PU.1/IRF motif containing adjacent PU.1 and IRF binding elements. Several genes, both myeloid and B-lymphoid, contain these composite elements (Marecki et al. 2001). In myeloid cells PU.1 activates a number of receptors involved in phagocytosis, such as immunoglobulin binding Fc receptors, scavenger receptor, complement receptor-forming integrins and mannose receptor (Pahl et al. 1993, Bottinger et al. 1994, Eichbaum et al. 1994, Feinman et al. 1994, Moulton et al. 1994, Perez et al. 1994, Rosmarin et al. 1995, Eichbaum et al. 1997). PU.1 also regulates the expression of colony-stimulating factor receptors (Reddy et al. 1994, Zhang et al. 1994, Hohaus et al. 1995, Smith et al. 1996). In addition, PU.1 binds to the promoters of several enzymes involved in bacterial killing and of NADPH oxidase components (Ahne and Stratling 1994, Oelgeschlager et al. 1996, Sturrock et al. 1996, Li et al. 1997, Eklund et al. 1998, Suzuki et al. 1998, Li et al. 2001, Li et al. 2002). In most cases PU.1 appears to have an activating effect on transcription, but it can also function as a repressor. It has been demonstrated to repress the genes for CD11c integrin, murine MHC II I-Aβ and c-myb through binding to PU boxes in their promoters (Borras et al. 1995, Bellon et al. 1997, Lopez-Rodriguez and Corbi 1997). In macrophages but not in osteoblasts it acts as a silencer of integrin β_5 expression, and in glomerular mesangial cells it silences gelatinase A gene (Feng et al. 2000, Harendza et al. 2000). PU.1 has also been shown to inhibit the activities of several promoters, e.g. that of c-myc, without binding to DNA. The suggested mechanism is recruitment of a complex containing corepressor protein mSin3A and histone deacetylase HDAC1 by PU.1 (Kihara-Negishi et al. 2001).

5.4.3. Biological role of PU.1

The pivotal role of PU.1 for myeloid and B cell development has been demonstrated through the generation of PU.1 deficient mice by two independent research groups (Scott et al. 1994, McKercher et al. 1996). The two mouse strains have some differences in their phenotypes, but both lack mature B cells and macrophages and have abnormal T cell and neutrophil development. The gene targeting strategies of the two studies were different in some respects and it has been suggested that in the mice strain of McKercher et al. there could be a truncated PU.1 protein still expressed, possessing some limited functional ability that contributes to the different phenotype (Fisher and Scott 1998). In the study by Scott et al. amino acids 200 to 272 of PU.1 were replaced with a neomycin resistance cassette, and by RT-PCR and Western blotting it was verified that no mRNA or protein was produced in PU.1 null animals (Scott et al. 1994, Scott et al. 1997). This targeting strategy resulted in embryonic lethality, null foetuses dying at a late gestational stage (between days 17 and 18 of gestation). The foetuses produced normal numbers of megakaryocytes and proerythroblasts, but completely lacked progenitor cells for T and B lymphocytes, granulocytes and

monocytes (Scott et al. 1994). Further studies performed using these PU.1-/- mice showed that the majority of PU.1-/- thymocytes were blocked in differentiation prior to T cell commitment, but almost 40% of PU.1-/- thymi placed in foetal thymic organ culture developed into mature thymocytes, demonstrating that PU.1 is not absolutely required for T cell development, but does play a role in the efficient commitment and/or early differentiation of T progenitors (Spain et al. 1999). On the other hand, overexpression of PU.1 blocked T cell differentiation at pro-T stage showing that a correct level of PU.1 is critical at different stages and lineages in haematopoiesis (Anderson et al. 2002). By retroviral transduction of PU.1 cDNA into PU.1-/- progenitor cells, it has been demonstrated that a low concentration of PU.1 promotes B cell differentiation, whereas a high concentration induces macrophage development and blocks B cell generation (DeKoter and Singh 2000). The PU.1 null mice by McKercher et al. were generated by inserting a neomycin resistance cassette at amino acid 232 of the Ets domain. These mice were born alive but died of septicaemia within 48 hours. They lacked mature macrophages, neutrophils, B and T lymphocytes. However, when the mice were maintained on antibiotics, they survived up to 17 days and developed a reduced number of T cells and a few abnormal neutrophils (McKercher et al. 1996). Due to absence of osteoclasts, the mice also suffered from osteopetrosis (Tondravi et al. 1997). Early progenitors generated from neonate liver of these mice were able to commit to the myeloid lineage, as cells possessing early myeloid characteristics could be grown from foetal liver cultures (Henkel et al. 1999).

By replacing PU.1 coding sequence with cDNA of Spi-B in the PU.1 locus of mouse ES cells, it was shown that PU.1 protein was absolutely necessary for lymphoid development, whereas Spi-B could rescue myeloid differentiation of the ES cells (Dahl et al. 2002). The results suggest that genes involved in lymphoid lineage commitment and/or maturation are strictly dependent on PU.1. Spi-B is expressed at high levels in B-cells, being co-expressed with PU.1 throughout most of B-cell development, and also in lymphoid-derived plasmacytoid dendritic cells and at low levels in T cells (Su et al. 1996, Schotte et al. 2003). Mice with homozygous mutation of Spi-B and with one functional allele of PU.1 (Spi-B-/- PU.1+/-) exhibited reduced numbers of B cells, their poor survival and a more severe B-cell receptor signalling defect than either the Spi-B-/- or PU.1+/- mutations alone (Garrett-Sinha et al. 1999). Hu et al. showed that this was largely due to misexpression of the Rel/NF-κB family member c-Rel, which is directly regulated by PU.1/Spi-B (Hu et al. 2001).

PU.1 was originally characterized as putative oncogene in murine erythroleukaemia cells (Moreau-Gachelin et al. 1988, Moreau-Gachelin et al. 1989). Recent evidence suggests that it may also be involved in human neoplasias. Several mutated PU.1 alleles were found in patients with acute myeloid leukaemia: deletions affecting the DNA binding domain, and point mutations in the DNA binding domain, PEST domain and transactivation domain. Thus disruption of PU.1 function may contribute to the block in differentiation found in acute myeloid leukaemia patients (Mueller et al. 2002).

5.4.4. Mechanism of action of PU.1

Most myeloid-specific genes regulated by PU.1 have PU boxes close to the transcriptional initiation sites, whereas the PU.1 binding sites in the immunoglobulin genes are mostly found in enhancer regions. Thus PU.1 may activate transcription by at least two different mechanisms. The close proximity of its binding sites and transcription start sites and its ability to interact with TATA-binding protein TBP through its transactivation domain (Hagemeier et al. 1993) suggest that its role might be the recruitment of the TFIID complex to the promoter, the other adjoining transcription factors working to stabilize the TFIID complex (Fisher and Scott 1998). On the other hand, in the enhancers binding of PU.1 appears to promote the binding of other transcription factors to neighbouring binding elements, and bending the DNA by PU.1 repositions the transactivation domains of one or more of the enhancer complex proteins to facilitate transcription (Nikolajczyk et al. 1996, Pongubala and Atchison 1997, Fisher and Scott 1998). In the Ig κ 3' and Ig μ heavy chain enhancers the PU.1 transactivation domains are not required for activation but PU.1 rather seems to play an architectural role, bending DNA (Erman and Sen 1996, Pongubala and Atchison 1997).

PU.1-regulated genes typically contain binding sites for other transcription factors, both cell type-specific and ubiquitously expressed. PU.1 has been shown to associate with various proteins in addition to the afore-mentioned IRF-4 and IRF-8, such as C/EBPδ, HMG I(Y) (high mobility group protein), SSRP, Retinoblastoma protein, TATA-binding protein (TBP), C/EBPβ and CREB binding protein (CBP) (Hagemeier et al. 1993, Nagulapalli et al. 1995, Yamamoto et al. 1999, Yang et al. 2000). PU.1 cooperates with Sp1 in the activation of c-fes, CD11b, CD18 and Tec promoters, with C/EBPα, AML1 and c-myb in elastase promoter activation, with AML1 and C/EBPα in M-CSFR promoter activation, with C/EBPε in eosinophil granule genes for major basic protein and eosinophil peroxidase, with NF-κB (p50/c-Rel) in immunoglobulin heavy chain (IgH) enhancer HS 1,2 activation and with Microphtalmia transcription factor the tartrate-resistant acid phosphatase promoter (Chen et al. 1993, Rosmarin et al. 1995, Heydemann et al. 1996, Oelgeschlager et al. 1996, Honda et al. 1997, Petrovick et al. 1998, Linderson et al. 2001, Luchin et al. 2001, Gombart et al. 2002). c-Jun has been shown to act as a co-activator for PU.1 in the induction of M-CSFR promoter (Behre et al. 1999).

Some interactions between PU.1 and other proteins have a repressive effect on transcription. A master regulator of erythroid development, GATA-1, inhibits myeloid differentiation by binding to PU.1 and blocks its interaction with c-Jun (Zhang et al. 1999). Conversely, PU.1 binds to GATA-1 and inhibits its activity through blocking its CBP-mediated acetylation (Hong et al. 2002). C/EBPα possibly plays a role in directing haematopoietic differentiation to the granulocytic lineage as it interacts with the DNA binding domain of PU.1 and displaces c-Jun (Hong et al. 2002). C/EBPα additionally downregulates c-Jun expression in myeloid cells (Rangatia et al. 2002). Similarly, in t(8;21) myeloid leukaemia, AML1-ETO fusion protein down-regulates the transcriptional

activity of PU.1 by also binding to PU.1 Ets domain and disrupting its interaction with c-Jun (Vangala et al. 2003).

PU.1 associates with the transcriptional coactivator CBP, which is a widely expressed transcriptional regulator possessing acetyltransferase activity and interacting with several other transcription factors (Yamamoto et al. 1999, Blobel 2002). CBP has been suggested to function by physically connecting transcriptional activators with components of the basal transcription machinery, and it has been shown that PU.1 cooperates with IRF-1 and IRF-8 (ICSBP) to recruit CBP to the promoters of $gp91^{hox}$ and $gp67^{hox}$ genes and that CBP is necessary for the activation of these genes (Eklund and Kakar 1999). CBP may also be required for the transcription of other PU.1-dependent promoters. HMG-I(Y) (high mobility group protein) has been identified as a coactivator for PU.1 in the Ig μ heavy chain enhancer, where it interacts physically with PU.1 and induces a structural change in PU.1 that results in enhanced DNA binding and transcriptional activation of PU.1 (Lewis et al. 2001).

The mechanisms of PU.1 regulation are poorly understood. In a recent study G-CSF treatment of myeloid 32D cells stimulated the expression and activation of PU.1 in a Stat3-dependent manner (Panopoulos et al. 2002). PU.1 was also upregulated by conditionally active C/EBPα in myeloid cells (Wang et al. 1999). PU.1 protein levels seem to be unchanged when macrophage activation or proliferation is induced, suggesting that the activity of PU.1 in mature macrophages is largely controlled by post-translational modifications, such as phosphorylation, as discussed in (Lloberas et al. 1999). LPS treatment has been shown to increase the level of phosphorylation of PU.1 and its activity (Lodie et al. 1997). The effect of LPS may be mediated through a mechanism involving intracellular expression of macrophage migration inhibitory factor (MIF), since stable transfection of RAW 264.7 mouse macrophages with a plasmid encoding an antisense MIF messenger RNA impaired basal PU.1 DNA-binding activity and transcriptional activity (Roger et al. 2001). It was shown recently that the activation of Notch1 receptor signalling increased PU.1 mRNA and protein levels in multipotent haematopoietic progenitor cells, thus inducing myeloid differentiation (Schroeder et al. 2003).

5.5. Glucocorticoid receptor

Glucocorticoid hormones, synthesized by the adrenal cortex, have a plethora of functions in the body. They regulate glucose homeostasis e.g. by stimulating gluconeogenesis and decreasing glucose uptake in peripheral tissues, they are involved in maintaining normal cardiovascular function and in bone turnover, and they accelerate many developmental processes, such as maturation of the lungs. Glucocorticoids are critical in stress resistance. They are also very potent immunosuppressive and anti-inflammatory agents, functioning by inhibiting the expression of cytokines, chemokines and many cell surface receptors and adhesion molecules involved in the activation and migration of lymphocytes (Karin 1998). Monocyte/macrophages are particularly sensitive for the effects of glucocorticoids, and glucocorticoids suppress the release of most known

macrophage inflammatory mediators, including TNF-α. In human monocytic cell lines, glucocorticoids primarily suppress transcriptional activation through adjacent promoter binding sites for NF-κB transcription factor complexes and for complexes of c-Jun with activating transcription factor-2 (Joyce et al. 2001). Glucocorticoids also induce apoptosis of human monocytes, possibly partially through inhibiting IL-1β production by the monocytes (Schmidt et al. 1999). By contrast, glucocorticoids have been reported to enhance M-CSF and GM-CSF-mediated proliferation of murine macrophages (Lloberas et al. 1998), and they markedly increase the phagocytic potential of macrophages by inducing changes in the capacity for reorganization of cytoskeletal elements (Giles et al. 2001).

Glucocorticoids exert their functions through binding to their specific receptor, GR, which is a sequence-specific transcription factor belonging to the superfamily of nuclear hormone receptors. In their unliganded form GR-like steroid hormone receptors reside in the cytoplasm associated with a multi-protein complex including heat shock proteins Hsp90, Hsp70, Hsp56 and p23. Upon hormone binding GR undergoes conformational changes, dissociates from the heat shock protein complex, and translocates to the nucleus where it regulates the expression of glucocorticoid-responsive genes (Beato et al. 1996). The same molecular chaperones that associate with GR in the cytoplasm, namely Hsp90 and especially p23, have been implicated to be involved in the rapid cessation of GR-mediated transcriptional activation by promoting disassembly of transcriptional regulatory complexes in the nucleus (Freeman and Yamamoto 2002). GR has a modular structure with the N-terminal domain that exhibits ligand-independent activation functions (AF-1), a zinc-finger-based DNA binding domain, a hinge region and a C-terminal ligand binding domain that also harbours regions mediating dimerization and ligand-dependent activation (AF-2) (Beato et al. 1996). Glucocorticoid response and sensitivity differ among species, individuals, tissues and cell types, and it has been hypothesized that at least some of this variety is due to multiple isoforms of GR (Yudt and Cidlowski 2002). The single gene encoding GR has multiple promoters that may contribute to cell type-specific regulation of GR expression, and GR mRNA can be alternatively spliced. Additional isoforms are produced by alternative translation start sites, and GR is also posttranslationally modified by phosphorylation. Mutation of multiple phosphorylation sites influences receptor stability and signalling in a promoter-specific context (Yudt and Cidlowski 2002).

GR controls transcription by several different ways. The one first discovered involves binding of GR homodimers to palindromic elements with a consensus sequence 5'-GGTACAnnnTGTTCT-3', called glucocorticoid response elements (GREs), in regulatory sequences of GR target genes (Beato et al. 1989). GREs are found in varying copy numbers and at varying distances from the transcription initiation sites of glucocorticoid responsive genes. Many GRE-containing genes are positively regulated by glucocorticoids, but GR can also inhibit gene expression through binding to so-called negative GREs. A more common mode of suppressing gene expression is, however, independent of direct DNA binding by GR. GR antagonises the activity of other transcription factors, such as AP-1, NF-AT and NF-κB (Karin 1998). GR has been shown to interact with NF-κB and AP-1 *in vitro,* and it may directly interfere with transactivation

by these factors (Jonat et al. 1990, Ray and Prefontaine 1994, Scheinman et al. 1995b, De Bosscher et al. 1997). Other mechanisms have also been suggested, such as inducing expression of IκB-α, an inhibitory molecule for NF-κB, and competition for nuclear coactivator proteins CBP or SRC-1 (Auphan et al. 1995, Scheinman et al. 1995a, Kamei et al. 1996, Lee et al. 1998). GR can also positively regulate transcription without binding to GREs, by interacting with other factors. It forms a complex with Stat5, acting as a transcriptional coactivator in the mammary gland-specific β-casein gene (Stocklin et al. 1996). GR cooperates with Stat5 and C/EBPβ on the β-casein promoter and with Stat5 and nuclear factor I B isoform on the whey acidic protein promoter (Mukhopadhyay et al. 2001, Wyszomierski and Rosen 2001). The complex formation between Stat5 and GR, on the other hand, diminishes the glucocorticoid response of a GRE-containing promoter (Stocklin et al. 1996). Interleukin-2-induced resistance of T cells to glucocorticoids may be mediated via association of activated Stat5 with GR and subsequent retardation of GR nuclear translocation (Goleva et al. 2002).

Nuclear hormone receptors are now known to promote gene activation or repression by recruiting a number of coregulator proteins to the promoter regions of hormone-responsive genes. These coregulators are often components of large multiprotein complexes containing chromatin remodelling activities. Many of the coregulators interact ligand-dependently with the C-terminal ligand binding and transcriptional activation domain of nuclear hormone receptors. GR interacts with human Brg-1-containing complexes that act to locally disrupt nucleosomal structures in an ATP-dependent manner and enhance transcription from organised chromatin templates (Fryer and Archer 1998). The coactivators CBP/p300 have been shown to interact with GR and mediate glucocorticoid-responsive promoter activation (Chakravarti et al. 1996, Kamei et al. 1996). Members of the so-called p160 family that include SRC-1, SRC-2/TIF2/GRIP1 and SRC-3/pCIP/AIB1, stimulate the transactivation of all steroid receptors (Onate et al. 1995, Hong et al. 1996, Voegel et al. 1996, Anzick et al. 1997, Torchia et al. 1997). SRC-3 possesses acetyltransferase activity, but p160 family coactivators have also been shown to associate with other acetyltransferase activity-containing coactivators such as CBP/p300 and PCAF suggesting that steroid hormone-dependent transcription is regulated by acetylation of nearby histones (Yao et al. 1996, Chen et al. 1997, Torchia et al. 1997, Blanco et al. 1998). Additional enzymatic activities may be important for the function of coactivators. For example, GRIP1 can associate with arginine methyltransferase 1, which enhances transcription by several nuclear hormone receptors (Chen et al. 1999). Other coactivators have also been identified for GR, for example PPARγ coactivator 1 (PGC-1) up-regulates gluconeogenesis in the liver by acting as a coactivator for GR and HNF-4 (Yoon et al. 2001).

Unlike GR, which in its unliganded form resides in the cytoplasm, some other nuclear hormone receptors, such as thyroid hormone and retinoid acid receptors, are predominantly nuclear and can bind to DNA even in the absence of ligand and either activate or repress transcription. Repression involves the recruitment of corepressor proteins N-CoR (nuclear corepressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptors), which in turn recruit

histone deacetylases via interaction with Sin3 (McKenna and O'Malley 2002). The glucocorticoid antagonist RU486 binds to GR and interferes with glucocorticoid-mediated transcriptional activation, and RU486-bound GR directly associates with NCoR and SMRT (Schulz et al. 2002). RIP140 protein was initially defined as a coactivator for nuclear hormone receptors, but in glucocorticoid signalling it inhibits GR function both in transactivation and transrepression, indicating that it is a general corepressor of glucocorticoid-dependent gene regulation (Subramaniam et al. 1999). Glucocorticoid signalling is also repressed by an atypical orphan receptor and corepressor SHP (short heterodimer partner), which interacts with various nuclear hormone receptors and inhibits their DNA binding and transcriptional activity (Borgius et al. 2002).

Even though GR was initially characterized as a sequence-specific transcription factor, gene-targeting studies indicate that DNA binding of GR is not essential for many of the physiological functions of glucocorticoids. Mice with a null mutation of GR die shortly after birth due to a maturation defect of the lungs and display a number of other defects, such as impaired feedback regulation via the hypothalamic-pituitary-adrenal axis, resulting in elevated levels of glucocorticoids in plasma, and reduced expression of gluconeogenic enzymes (Cole et al. 1995). A mouse strain with a “knock-in” mutation of dimerization-defective GR (amino acid alanine 458 located in the DNA binding domain changed into threonine) also lack expression of gluconeogenic enzymes and other GRE-dependent genes, but are relatively healthy under standard laboratory conditions (Reichardt et al. 1998). Repression of inflammatory responses in these mice is comparable to that of wild-type mice, indicating that the glucocorticoid-dependent inhibition of pro-inflammatory protein expression by GR occurs independently of DNA binding of GR through negative interference with NF-κB and possibly other pro-inflammatory transcription factors (Reichardt et al. 2001).

5.6. Overview of cytokine signalling

Cytokines are a large family of secreted proteins that mediate cell-to-cell communication. They are involved in a variety of biological processes, including cell growth and activation, inflammation, immunity, differentiation and development, and they stimulate biological responses in basically all cell types. One important group of cytokines is the haematopoietic cytokines, whose main role is to regulate haematopoietic cell differentiation and activity. In mammals there are at least 50 members in the haematopoietic cytokine subfamily, consisting of a large number of interleukins (ILs) and related molecules such as colony-stimulating factors. They exert their effects through binding to and activating a family of structurally and functionally conserved cell surface receptors, designated as type I cytokine receptors. The receptors are classified into several subfamilies according to the number and type of chains they consist of. The signal is transduced into the cell and ultimately into the nucleus to affect gene transcription. Signal transduction occurs via a common pathway, the JAK/Stat pathway (Ihle 1995, Schindler 2002).

In mammals there are four Janus kinases or JAKs, and seven signal transducers and activators of transcription or Stats that are used in different combinations depending on the cytokine and its receptor. Haematopoietic cytokine receptors have no intrinsic catalytic activity but instead associate with JAKs that are activated upon ligand binding and subsequent receptor chain aggregation and mediate signalling by phosphorylating tyrosine residues of other signalling molecules of the pathway, including Stats. Phosphorylated Stats form homo- or heterodimers and translocate to the nucleus where they recognize specific target DNA sequences, mostly with a consensus TT(N_{4-6})AA, and regulate the expression of genes containing these elements (Darnell et al. 1994, Ihle 1995, Leonard and O'Shea 1998). The JAK-Stat pathway was first discovered in the context of IFN signalling, and is described in more detail below, in the context of the interferon-γ signal transduction pathway.

5.6.1. JAKs

There are four JAK family kinases in mammals: Jak1, Jak2, Jak3 and Tyk2, each selectively associating with specific cytokine receptor chains. For example, Jak2 binds to erythropoietin and prolactin receptors, which are single-chain cytokine receptors. Jak2 also binds to the common β_c chain associating with the α chains of IL-3, IL-5 and GM-CSF receptors (Ihle 1995). The crucial role of JAKs in cytokine signalling was first revealed in genetic complementation experiments using mutant cell lines defective in type I and II IFN signalling pathway components, and the requirements of individual JAKs for signalling via particular receptors *in vivo* have been verified by the analysis of mice deficient of different JAKs. Jak3 expression is restricted to cells of haematopoietic origin, but the other three members of the family are widely expressed (Taniguchi 1995, O'Shea et al. 2002).

JAKs are large kinases, being over a thousand amino acids in length and between 110-140 kDa in molecular mass. They all have a similar modular structure consisting of a C-terminal kinase domain responsible for the catalytic activity, an adjacent pseudokinase domain that possesses regulatory functions, and the N-terminal half, originally divided into five additional domains based on sequence similarities between the family members, but recently suggested to have homology to two domain structures, namely FERM (“band four point one, ezrin, radixin and moesin”) and SH2-like domains (Girault et al. 1999, Chen et al. 2000, Kampa and Burnside 2000, Saharinen et al. 2000, Saharinen and Silvennoinen 2002). The FERM domain has been suggested to have a role in receptor association and maintenance of kinase integrity, whereas the function of the SH2-like domain is not known (Hilkens et al. 2001, Zhou et al. 2001).

All the individual JAK family genes have been knocked out from the mouse genome. Jak1 deficient mice die shortly after birth, probably due to a failure of cytokine signalling in neurogenesis, and have defective lymphopoiesis. They fail to respond to IFNs and to cytokines using receptors that utilize the common γ subunit and the gp130 subunit (Rodig et al. 1998). Jak2 deficiency is embryonically lethal, probably caused by a failure in definitive erythropoiesis. Jak2 plays an essential role in signalling through erythropoietin, IL-3, GM-CSF,

IL-5, thrombopoietin and IFN-γ receptors (Neubauer et al. 1998, Parganas et al. 1998). Jak3-deficient mice are viable but have severe defects in lymphopoiesis, similar to the mice deficient in the common γ chain of cytokine receptors to which Jak3 associates. The mice fail to respond to IL-2, IL-4 and IL-7, and exhibit a phenotype resembling SCID, severe combined immunodeficiency (Nosaka et al. 1995, Park et al. 1995, Thomis et al. 1995). Tyk2-deficient mice develop normally but lack responsiveness to low doses of IFN-α/β and are resistant to endotoxin shock induced by high doses of LPS. T cell functions induced by IL-12 are also defective. IFN-γ signalling is also impaired in Tyk2-/- mice, possibly due to reduced levels of Stat1 expression (Karaghiosoff et al. 2000, Shimoda et al. 2000, Karaghiosoff et al. 2003).

5.6.2. Stats

Stats are well conserved during eukaryotic evolution, and Stat-like proteins have been identified in *Dictyostelium*, *Caenorhabditis elegans*, *Drosophila*, and zebrafish. In *Drosophila* the JAK/Stat pathway plays an important role in many aspects of development, including larval haematopoiesis, and the pathway consists of one cytokine receptor, a ligand, a single JAK and a single Stat. Several regulatory proteins, such as SOCS and PIAS, are also found in *Drosophila* (Kisseleva et al. 2002). The seven mammalian Stat genes are segregated into three clusters formed by tandem duplications. The mouse Stat1 and Stat4 map to chromosome 1, Stat2 and Stat6 to chromosome 10 and Stat3 and Stat5 to chromosome 11. The Stat5 gene has undergone an additional duplication to Stat5a and Stat5b more recently during evolution (Copeland et al. 1995). In addition to cytokine receptor signalling complexes, many receptors with intrinsic tyrosine kinase activity can activate Stats in mammals. For example, the receptors for platelet-derived growth factor and epidermal growth factor can activate Stat1 and Stat3 (Leaman et al. 1996, Vignais et al. 1996).

Although Stats can be activated by partially overlapping sets of cytokines and growth factors, generation of null mice has demonstrated non-redundant biological roles for the individual Stat family members. Stat1 and Stat2 are largely responsible for IFN signalling, Stat4 mediates responses for IL-12 and thus controls T cell differentiation to the Th1 type cells, and Stat6 functions in the IL-4 and IL-13 signalling, being critical for Th2 type differentiation and allergic responses (Durbin et al. 1996, Kaplan et al. 1996a, Kaplan et al. 1996b, Meraz et al. 1996, Shimoda et al. 1996, Takeda et al. 1996, Thierfelder et al. 1996, Park et al. 2000). Mice null for all the other Stats are born viable, but knocking out Stat3 results in embryonic lethality (Takeda et al. 1997). Reflecting the role of Stat3 in mediating responses for multiple cytokines, mice strains with tissue-specific targeting of Stat3 exhibit poor responses to IL-6 in the liver and T cells, enhanced proliferation and production of cytokines in myeloid cells, defective apoptosis in mammary epithelium and impaired wound repair, keratinocyte migration and second hair cycles (Takeda et al. 1998, Chapman et al. 1999, Sano et al. 1999, Takeda et al. 1999, Alonzi et al. 2001, Lee et al. 2002). For Stat5, both single mutations for Stat5a and Stat5b and a double mutant mouse have been generated. Stat5a and Stat5b single null mice have distinct phenotypes, and the Stat5a/b double knockout mouse exhibits a

combined phenotype. Stat5a-/- mice have impaired prolactin-dependent mammary gland development, and Stat5b-/- mice are unresponsive to growth hormone and thus have sexually dimorphic growth retardation (Liu et al. 1997, Udy et al. 1997, Teglund et al. 1998). Stat5a/b double knockout mice have infertility in the females, are small in size, and despite grossly normal blood cell counts have multiple defects in the development of myeloid and lymphoid lineages resulting in lymphopenia, thrombocytopenia, neutrophilia and bone marrow hypocellularity (Teglund et al. 1998, Bunting et al. 2002, Snow et al. 2002).

5.7. Interferons

Interferons (IFNs) are related to type I cytokines, and belong to the type II cytokine family that also includes IL-10 and many other, recently identified proteins (IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29) (Kotenko 2002, O'Shea et al. 2002, Sheppard et al. 2003). IFNs were discovered as early as in the 1950's as agents capable of impeding replication of viruses (Samuel 2001). IFNs have a vital role in higher vertebrates, providing an early line of defence against viral infections. They inhibit cell growth and control apoptosis, and are potent immunomodulatory cytokines, affecting almost all phases of innate and adaptive immune responses (Stark et al. 1998). IFNs constitute a multigene family that has been divided into two classes, type I (IFN-αs, IFN-β, IFN-ε, IFN-κ, IFN-λ1-3, IFN-ω, IFN-τ and limitin) and type II (IFN-γ) (Kotenko and Pestka 2000, LaFleur et al. 2001, Schindler 2002, Kotenko et al. 2003). IFN-α is a family of 17 closely related proteins encoded by a gene cluster. IFN-β is a single protein encoded by a distinct, more distantly related gene (Levy 2002). IFN-α and IFN-β are found across many species, whereas IFN-ω expression is restricted to humans and IFN-τ has been identified in ruminants, acting as an early signal in pregnancy (Chesler and Reiss 2002). IFN-κ is selectively expressed in epidermal keratinocytes, and IFN-λs mediate antiviral protection in a wide variety of cells (LaFleur et al. 2001, Kotenko et al. 2003). Limitin has been described as type I IFN selectively inhibiting B-lineage growth (Oritani et al. 2000). IFN-γ bears no structural resemblance to type I IFNs at the protein level, and the gene resides in a different chromosome from that of the type I IFN locus (Bach et al. 1997). Type I and type II IFNs are also produced by a distinct set of cells. Type I IFNs can be synthesized by most cells, whereas IFN-γ is produced by cells of the immune system, predominantly by natural killer cells, T helper type 1 cells (Th1) and cytotoxic T cells. In the presence of IL-12 and IL-18 IFN-γ is also produced by activated dendritic cells and macrophages (Shtrichman and Samuel 2001, Decker et al. 2002, Levy 2002). In T cells the main inducer of IFN-γ production is cross-linking of the T cell receptor complex, whereas in natural killer cells, IFN-γ expression is stimulated by macrophage-derived cytokines, especially TNF-α and IL-12 and by IFN-γ itself (Boehm et al. 1997).

5.8. Interferon-g

The human IFN-γ molecule is a noncovalent homodimer that consists of two 17-kDa-polypeptide chains, which are variably N-glycosylated (Bach et al. 1997). The crystal structure of IFN-γ indicates that the polypeptides associate in an anti-parallel fashion to form a symmetrical dimer that expresses two identical receptor-binding sites. Each subunit contains six α helices, and the helices from the subunits of the dimer are intimately intertwined, forming a compact and globular overall structure (Ealick et al. 1991).

IFN-γ induces the expression of major histocompatibility complex (MHC) class I and class II proteins that play an important role in antigen recognition (Park and Schindler 1998, Shtrichman and Samuel 2001). IFN-γ is a potent activator of macrophages. It promotes the differentiation of naïve T cells into Th1 cells and induces Ig isotype switching in B cells into certain types (IgG2a and IgG3 in the mouse) (Boehm et al. 1997). IFN-γ enhances the synthesis of cytokines involved in antimicrobial immunity, e.g. the p40 subunit of IL-12, TNF-α and IL-1 (Decker et al. 2002). IFN-γ promotes anti-tumour responses, protecting the host against tumour development (Ikeda et al. 2002). IFN-γ is also involved in the regulation of leukocyte-endothelium interactions and in the induction of components of the complement cascade (Boehm et al. 1997).

It is estimated that IFN-γ regulates the expression of hundreds of genes in different types of cells (Ramana et al. 2002). DNA microarray technique has been a powerful tool to analyse the transcription profiles of IFN-γ treated cells (Der et al. 1998, Ehrt et al. 2001). IFN-γ mediates the induction of genes encoding e.g. iNOS (inducible nitric oxide synthase) involved in bacterial killing by phagocytes, transcription factors IRF-1, (interferon regulatory factor-1) IRF-9, IRF-8/ICSBP and CIITA (MHC class II transactivator), SOCS-1 (suppressor of cytokine signalling 1), MHC class I pathway proteins such as TAP1 and TAP2 (transporters associated with antigen presentation), and several chemokines (Shtrichman and Samuel 2001, Decker et al. 2002). A large number of genes are also suppressed by IFN-γ, including cell cycle genes such as cyclins D and A and CDC25A, many matrix metalloproteinases and several thyroid-specific genes (Ramana et al. 2000). In a recent study using primary mouse macrophages and microarrays containing ~10,000 probe sets, 1300 genes were responsive to IFN-γ, surprisingly many, i.e. roughly half, being suppressed (Ehrt et al. 2001).

5.8.1. Fcγ receptor I as an example of an IFN-γ regulated gene

Important targets for IFN-γ upregulation in macrophages are Fcγ receptors (FcγRs) (Guyre et al. 1983). They are a group of membrane glycoproteins, belonging to the Ig superfamily, and bind to the Fc portion of IgG. There are three classes of FcγRs, FcγRI, FcγRII and FcγRIII, and they may initiate either activating (FcγRI, FcγRIII) or inhibitory (FcγRII) signals in the cell (Gerber and Mosser 2001). FcγRI is the high affinity receptor for IgG and expressed in cells of myelomonocytic origin, being constitutively present only on monocytes and

macrophages. IFN-γ induces its expression several folds in monocyte/macrophages and also in neutrophils (Pan et al. 1990a, Pearse et al. 1991). FcγRI differs from the other FcγRs in having three Ig-like extracellular domains instead of two, and this third domain contributes to its high affinity for IgG (Gerber and Mosser 2001). FcγRI plays an important role in increased phagocytosis, enhanced antibody-dependent cellular cytotoxicity, respiratory burst, immune complex-dependent antigen presentation, and regulation of antibody responses, as recently demonstrated by studying FcγRI deficient mice (Barnes et al. 2002). Three genes encoding the human FcγRI protein have been identified, but only one appears to encode the functional form of the receptor. In mice there is only a single gene for FcγRI (Gerber and Mosser 2001). The 72-kDa FcγRI polypeptide chain associates with a homodimeric γ chain that is also used by many other receptor complexes, e.g. the high affinity receptors for IgE and IgA, the T cell receptor, and FcγRIII. The γ chain is the signal transducing chain of the receptor complex, while the FcγRI α chain binds the ligand (Gerber and Mosser 2001).

The proximal region of the human FcγRI gene promoter has been characterized and two important *cis* elements have been identified; one that is responsible for the IFN-γ induction, designated as GRR (gamma response region), and the other which is involved in the myeloid cell-restricted expression of the gene. The transcription factors binding to these elements have also been identified: Stat1 binds to GRR and PU.1 to the myeloid cell activating element. The promoter lacks a TATA element, but contains a motif similar to initiator consensus sequence that is able to direct transcription initiation (Pearse et al. 1993, Eichbaum et al. 1994, Perez et al. 1994). Monocyte FcγRI expression is upregulated with IFN-γ and IL-10, and downregulated with IL-4 and IFN-β (Guyre et al. 1983, te Velde et al. 1992, Van Weyenbergh et al. 1998). IFN-β downregulated IFN-γ-induced FcγRI protein levels but not mRNA (Van Weyenbergh et al. 1998). In human neutrophils, which do not constitutively express FcγRI, the expression is induced by IFN-γ or granulocyte colony-stimulating factor (G-CSF) but not by IL-10, whereas murine neutrophils do not express FcγRI even after G-CSF treatment (Bovolenta et al. 1996, Bovolenta et al. 1998, Ioan-Facsinay et al. 2002). IFN-γ-induced FcγRI mRNA and protein levels are also up-regulated by glucocorticoids in monocyte/macrophages but not in immature myeloid cells. However, the mechanism of this up-regulation is not known (Warren and Vogel 1985, Pan et al. 1990b). There are sequences showing homology to glucocorticoid-responsive elements within introns 4 and 5 of the FcγRI gene, and IFN-γ treatment of primary mouse macrophages increases GR expression (Salkowski and Vogel 1992, Sivo et al. 1996).

5.9. Interferon-g signal transduction pathway

IFN-γ receptor is expressed on the surface of virtually all cell types (Bach et al. 1997). It belongs to the family of class II cytokine receptors and consists of two different polypeptide chains, IFN-γRI and IFN-γR2, both of which are composed of an extracellular domain, a single membrane spanning domain and an

intracellular domain. The homodimeric IFN-γ binds to two chains of IFN-γR1, which also function as signal transducing units. Two IFN-γR2 chains are additionally required to assemble a functional receptor complex (Kotenko and Pestka 2000). Ligand binding induces receptor component oligomerization and subsequent activation of the signalling pathway. Both receptor chains constitutively associate with members of the JAK family tyrosine kinases, R1 with Jak1 and R2 with Jak2, which are activated upon receptor aggregation and cross-phosphorylate each other. The R1 intracellular domains are also phosphorylated on tyrosine residues. One of the phosphorylated tyrosine residues of R1, Tyr 440, serves as a docking site for the Src homology 2 (SH2) domain of the latent cytoplasmic transcription factor Stat1. Stat1 is recruited to the receptor complex where it becomes phosphorylated on a single tyrosine residue (Tyr701) that mediates its dimerization by reciprocal interactions between the phosphotyrosine of one Stat1 molecule and SH2 domain of another Stat1. These homodimers then translocate into the nucleus, where they bind to their target sequences called GAS (gamma activated sequence) elements and regulate the transcription of IFN-γ responsive genes (Darnell et al. 1994). A schematic representation of the signalling pathway is shown in Figure 3.

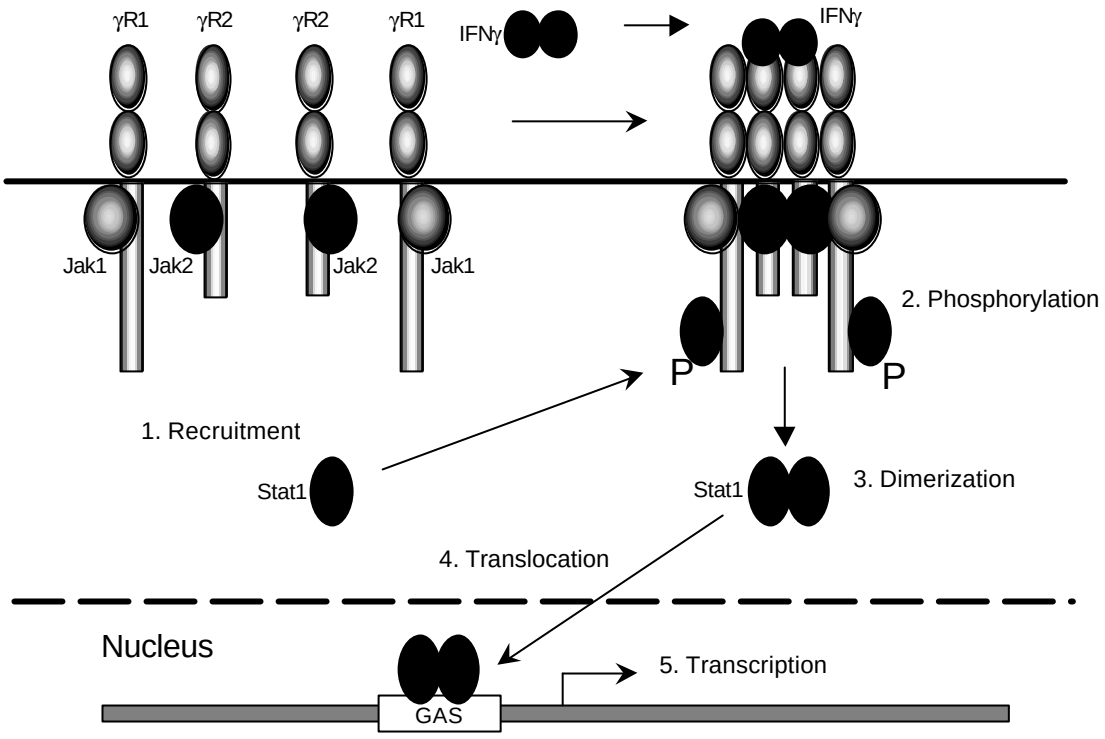

Figure 3. Schematic representation of the IFN-γ signalling pathway. The binding of IFN-γ to the receptor chains leads to activation of Jak1 and Jak2 and subsequent tyrosine phosphorylation of Stat1. Activated Stat1 molecules form dimers and translocate into the nucleus, where they regulate transcription by binding to their target sequences.

Signalling by most cytokine receptors is mediated via activating Stat homodimers, or sometimes in the case of Stat1 and Stat3, heterodimers. In IFN-α/β signalling, a distinct heterotrimeric complex called ISGF3 (interferon-stimulated gene factor 3) is formed that binds to completely different DNA sequences from GAS, designated as ISRE (interferon-stimulated response elements). ISGF3 consists of Stat1-Stat2 heterodimer and IRF-9 (p48) that is required for the DNA binding of the complex (Darnell et al. 1994). IFN-α/β signal is also transduced via a different receptor complex composed of IFNAR1 chain, which is associated with Tyk2, and IFNAR2 chain associating with Jak1 (Kotenko and Pestka 2000).

In addition to the JAK/Stat pathway, several other signal transduction pathways have been reported to be activated by IFN-γ but their roles in IFN-γ regulated gene expression are less well studied. Activated proteins include mitogen-activated protein kinases (MAPK) Pyk2 and ERK1/2 (extracellular-signal-regulated kinase), Src-family tyrosine kinase Fyn, adaptor molecules c-Cbl, CrkL, CrkII and Vav, and tyrosine phosphatases SHP-1 (SH2-containing phosphatase 1) and SHP-2 (Ramana et al. 2002). IFN-β and IFN-γ induce the phosphorylation of phosphatidyl-inositol 3-kinase (PI-3K) pathway component serine/threonine kinase Akt independently of Stat1, and inhibition of PI-3K signalling prevents IFN-induced monocyte adhesion (Navarro et al. 2003).

5.10. Structure of Stat1

All Stat proteins share a similar overall structure that consists of several domains, and different human Stats vary in length, being between 750 and 850 amino acids (Figure 4) (Kisseleva et al. 2002). The crystal structure of Stat1 residues 132-713 bound to DNA has been solved (Chen et al. 1998). Residues 136-317 comprise a four-stranded helical coiled-coil domain whose exposed surfaces present many sites for protein-protein interactions either on target promoters or in solution. The DNA-binding domain (residues 318–488) follows next and contains an immunoglobulin-type fold similar to DNA binding domains of NF-κB and p53, formed from several β-sheets. Each Stat polypeptide of the dimer recognizes bases in the most proximal half GAS element, with only few direct contact sites between amino acid residues and DNA. Thus, effective transactivation likely requires cooperativity in DNA binding. Residues 488–576 constitute a mostly α-helical linker domain that plays a role in transcriptional responses, affecting the stability of DNA binding of Stat1 (Yang et al. 1999, Yang et al. 2002). The SH2 domain (residues 577–683), which is critical for Stat to be recruited to the cytokine receptor, to associate with the activating JAK and to dimerize with another Stat, consists of an anti-parallel β-sheet flanked by two α-helices, which form a pocket. An absolutely conserved arginine residue 602 lies at the base of the pocket and mediates the interaction with Tyr701 that is phosphorylated upon Stat activation. The SH2 domain is followed by a segment (residues 700–708) including Tyr701. The carboxy-terminal transactivation domain (TAD) is missing from the crystal. The amino-terminus also missing

from the Stat1 crystal has been crystallized from another Stat, namely Stat4 (Vinkemeier et al. 1998). The N-domain is composed of eight short α-helices assembled in a hook-like configuration and is involved in tetramer formation on promoters containing tandem low affinity Stat binding sites (Chen et al. 1998, Vinkemeier et al. 1998, Levy and Darnell 2002). The N-domain also influences Stat receptor recognition, phosphorylation, dephosphorylation and nuclear translocation (Shuai et al. 1996, Strehlow and Schindler 1998, Murphy et al. 2000).

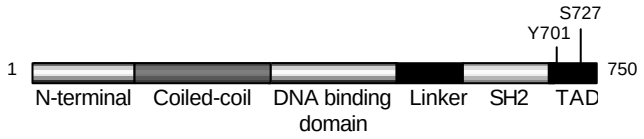

Figure 4. Schematic representation of Stat1 structure. The coiled-coil domain near the N-terminal end mediates interactions with various proteins. The DNA binding domain is located in the middle of the molecule, followed by linker and SH2 domains. The transactivation domain is located at the C-terminus and contains the single serine phosphorylation site of Stat1.

In addition to tyrosine phosphorylation, Stat1 undergoes serine phosphorylation in its transactivation domain on Ser727, which is a potential mitogen-activated protein kinase (MAPK) phosphorylation site. In cell lines, mutation of Ser727 results in only 20% of wild-type activity of Stat1 in reporter gene assays (Wen et al. 1995). The phosphorylation of Ser727 has a particularly strong enhancing effect on the expression of selected genes and mediates the interaction of Stat1 with certain coactivator proteins (see below) (Zhang et al. 1998, Ouchi et al. 2000, Kovarik et al. 2001). Tyrosine and serine phosphorylation events of Stat1 can occur independently of each other, suggested by studies showing that LPS, ultraviolet radiation or TNF-α stimulus can cause serine but not concomitant tyrosine phosphorylation (Kovarik et al. 1998, Kovarik et al. 1999). The mechanisms through which Stat1 serine phosphorylation occurs are not well understood. Several kinases have been implicated, and serine phosphorylation seems to occur via different signalling pathways (Decker and Kovarik 2000). IFN-γ-responsive serine phosphorylation of Stat1 is abrogated and IFN-γ-induced gene expression is reduced by inhibition of PI-3K activity, and constitutively active Akt serine protein kinase activates Stat-dependent transcription. Serine phosphorylation by the PI-3K/Akt pathway also requires Jak1 and Jak2 and the tyrosine 440 docking site of Stat1 on IFN-γR1 (Nguyen et al. 2001). Stress-induced phosphorylation of Stat1 Ser727 requires p38α MAPK activity, whereas IFN-γ-stimulated Ser727 phosphorylation occurs independently of p38α, and stress-induced activation of the MAPK pathway increases Stat1 transcriptional activity independently of Ser727 phosphorylation (Ramsauer et

al. 2002). IFN-γ mobilizes a Ca^{2+} flux in cells and activates Ca^{2+}/calmodulin-dependent kinase (CaMK) II that can interact directly with Stat1 and phosphorylate Stat1 on Ser727. Inhibition of Ca^{2+} flux or CaMKII results in a lack of Ser727 phosphorylation and reduction of Stat1-dependent gene activation (Nair et al. 2002). Inhibition of protein kinase C δ activation diminishes the type I IFN-dependent serine phosphorylation of Stat1 (Uddin et al. 2002). Engagement of Toll-like receptors (TLR) 2 and 4 induce Ser727 phosphorylation by different mechanisms, both involving p38 MAPK activity (Rhee et al. 2003).

Two Stat1 isoforms are produced by alternative splicing of a single mRNA: the full-length Stat1α and the shorter Stat1β that lacks an exon encoding the C-terminal 38 amino acids (Fu et al. 1992, Schindler et al. 1992). Stat1α has a size of 91 kDa, Stat1β 84 kDa (Fu et al. 1992). The two isoforms are in most cases interchangeable for ISGF3 activity in response to type I IFN stimulation, but only the full-length Stat1α completely restores transcriptional responses to IFN-γ treatment (John et al. 1991, Muller et al. 1993, Shuai et al. 1993). Thus, Stat2 TAD is critical in the transcriptional activation of IFN-α/β-regulated genes.

5.11. Transcriptional regulation by Stat1

The expression of most genes is activated only when several different transcription factors bind to their regulatory regions, and this also holds true for IFN-γ responsive genes. The transcription factors associate with coactivators that facilitate contact with the basal transcriptional machinery. Stat1 has been shown to cooperate with several transcription factors bound to neighbouring DNA sequences to activate transcription. Stat1 physically associates with Sp1 and these factors synergistically activate the intercellular adhesion molecule-1 (ICAM-1) gene (Look et al. 1995), and IFN-γ-induced Stat1 and TNF-α-induced NF-κB cooperatively regulate the IRF-1 and the chemokine CXC ligand 9 (MIG) genes (Ohmori et al. 1997, Hiroi and Ohmori 2003). On the IFN-inducible promoter of the MHC class II transactivator gene, CIITA, Stat1 binds to its GAS element only in the presence of USF-1, a ubiquitous member of the helix–loop–helix/leucine zipper family (Muhlethaler-Mottet et al. 1998). Stat1 and heat shock factor-1 (HSF-1) interact directly and bind to neighbouring elements on both heat shock protein 70 (Hsp70) and Hsp90β promoters, having an additive effect on transcription (Stephanou et al. 1999). In macrophages Stat1 interacts with vitamin D receptor (VDR). This prevents VDR association with retinoid acid receptor X and thus 1,25-dihydroxyvitamin D (1,25D) inducible gene activation, but enhances IFN-γ-induced transcription by preventing Stat1 deactivation by tyrosine dephosphorylation (Vidal et al. 2002).

Like a large number of transcription factors, Stats bind to the important coactivators CBP/p300 (Levy and Darnell 2002). Both N-terminal and C-terminal regions of Stat1 interact directly with CBP/p300 and this interaction has been shown to promote IFN-γ-dependent transcription (Zhang et al. 1996, Horvai et al. 1997, Hiroi and Ohmori 2003). In the activation of the CXC ligand 9 gene, Stat1 and NF-κB simultaneously interacted with CBP, which in turn recruited RNA polymerase II complex to the promoter. Acetyltransferase activity of CBP

was dispensable for the transcriptional synergy (Hiroi and Ohmori 2003). Adenoviral protein E1A binds to both CBP and p300 and has been widely used to demonstrate the requirement of CBP/p300 in the transcriptional activation of various genes (Eckner et al. 1994, Bannister and Kouzarides 1995). E1A has also been shown to directly inhibit Stat1 independently of CBP (Look et al. 1998). Stat1, like several other Stats, has been reported to associate with Nmi (N-Myc interactor). Nmi increased the interaction between Stat1 and CBP and augmented IFN-γ-dependent transcription (Zhu et al. 1999). Serine phosphorylation of Stat1 on Ser727 mediates its interaction with at least two proteins implicated to function as coactivators, MCM5 (mini-chromosome maintenance) and BRCA1 (Zhang et al. 1998, Ouchi et al. 2000). BRCA1 coactivator function was dependent on IFN-γ target genes, i.e. it was required for the activation of the $p21^{waf}$ but not for the IRF-1 gene (Ouchi et al. 2000). A large multi-subunit coactivator complex called Mediator appears to be involved in type I IFN activated gene regulation. IFN-α-induced transcription was dependent on Stat2 interactions with DRIP150, a subunit of the Mediator complex. DRIP150 both enhanced IFN-α-dependent transcription and coimmunoprecipitated with Stat2 (Lau et al. 2003). The Mediator may well also be used as a coactivator for other Stat-responsive genes.

5.12. Biological role of Stat1

The importance of Stat1 in IFN signalling was initially found by studying chemically mutagenized human fibrosarcoma cells lacking Stat1 (Muller et al. 1993, Darnell et al. 1994). In cell culture, Stat1 has been shown to be activated by multiple cytokines and growth factors in addition to IFNs, but through the generation of Stat1 deficient mice the specific role of Stat1 was confirmed to be restricted to IFN signalling (Durbin et al. 1996, Meraz et al. 1996). The mice strains generated by two different laboratories showed similar phenotypes, being born viable with no obvious developmental defects, but were completely unresponsive to IFNs and highly sensitive to infections by viruses and intracellular bacteria, such as *Listeria monocytogenes*. The growth inhibitory and antitumour effects of IFNs were also demonstrated by using Stat1 deficient mice. Lymphocytes from Stat1-/- mice exhibited increased proliferation and reduced apoptosis *in vitro* and entered S phase of the cell cycle more efficiently than wild type cells (Lee et al. 2000). Stat1-/- mice developed tumours more rapidly and more frequently than wild-type mice when chemical carcinogenesis was induced, and when the Stat1 deficiency was placed on a p53 null background there were increased rates of tumour formation and an increase in non-lymphoid tumours. These increases were suggested to be due to a loss of tumour surveillance, possibly through the loss of the ability of IFNs to promote antigen processing and presentation by MHC class I (Kaplan et al. 1998). By using fibrosarcoma cells of a Stat1 null mouse, Stat1 was shown to be required for the inhibitory effect of IFN-β on the expression of angiogenic factor basic fibroblast growth factor, demonstrating that Stat1 expressed by tumour cells is a negative regulator of tumour angiogenesis (Huang et al. 2002). Stat1 appears to be involved in several genetic forms of human dwarfism caused by mutated constitutively active fibroblast growth factor (FGF) receptor. Persistent activation of Stat1 is

associated with reduced proliferation of chondrocytes, as well as their excessive apoptosis (Sahni et al. 1999). When transgenic mice overexpressing human FGF2 and exhibiting phenotypes characterized by chondrodysplasia were crossed with Stat1-/- mice, their chondrodysplasic phenotype was significantly corrected (Sahni et al. 2001).

Two recent reports describe human Stat1 mutations. Three patients heterozygous for a point mutation resulting in amino acid substitution serine 706 to leucine, were not susceptible to viral infections but developed mycobacterial infections (Dupuis et al. 2001). The mutant Stat1 protein could not be phosphorylated on Tyr701 and was dominant negative in IFN-γ signalling and recessive for ISGF3 activation by type I IFNs. In the second study, two patients were reported homozygous for mutant Stat1 genes, one with a small deletion generating a premature stop codon at position 603, and the other with a point mutation resulting in the substitution of a proline for a leucine at amino acid position 600 (Dupuis et al. 2003). Both of these patients were completely deficient of Stat1 protein, suffered from mycobacterial infections and died of viral disease at 12 and 16 months of age. Cell lines (Epstein-Barr virus-transformed B cells) derived from the two patients did not restrict the growth of viruses in response to type I IFNs. Thus IFN-α/β is crucial for protective immunity to viruses in natural conditions of infection in humans, whereas protection from mycobacterial infections requires intact IFN-γ signalling pathway.

In addition to having a critical role in IFN signalling, Stat1 is involved in the constitutive expression of a number of genes (Chatterjee-Kishore et al. 2000). Unphosphorylated Stat1 mediates constitutive expression of caspases 1-3, and binds together with IRF-1 to the partially overlapping interferon consensus sequence 2 and GAS sites in the low molecular mass polypeptide 2 (LMP2) promoter to activate transcription (Kumar et al. 1997, Chatterjee-Kishore et al. 2000). Stat1 was demonstrated to be able to bind to the LMP2 promoter either as a monomer or a dimer, and residues 135–200 within the coiled-coil domain were required for dimer formation (Chatterjee-Kishore et al. 2000).

However, not all effects of IFN-γ are mediated via Stat1. By performing microarray analyses with macrophages derived from wild-type and Stat1-/- mice, a number of genes were shown to be either induced or suppressed independently of Stat1 (Gil et al. 2001). In Stat1-/- cells the majority of IFN-γ-regulated genes were suppressed, whereas in wild-type cells IFN-γ regulated predominantly the activation of genes. Genes such as pim-1, SOCS3, MCP-1 and fibronectin were upregulated in both wild-type and Stat1-/- macrophages. Stat1-/- mice were significantly less susceptible to certain viral infections than mice lacking both IFN-α/β receptor and IFN-γ receptor, showing that a Stat1-independent IFN signalling pathway plays a physiologically relevant role in protection against at least some pathogens (Gil et al. 2001).

5.13. Regulation of Stat1-dependent signalling

5.13.1. Regulation of Stat1 protein levels

The functions of transcription factors can be regulated in multiple ways, for example by controlling their abundance at the level of transcription or translation, by introducing post-translational modifications, by regulating their cellular localization or by interaction with other molecules.

Stat1 protein is ubiquitously expressed and relatively long-lived (having a half-life of <24 hours in Daudi B cells (Lee et al. 1997)), and its activity is regulated primarily by phosphorylation and dephosphorylation events. However, Stat1 expression has been shown to be stimulated in human blood monocytes and macrophages by IFN-α and IFN-γ, and in myeloid leukaemia cells by retinoic acid, and upon the isolation of the human Stat1 gene, an intronic enhancer element responsive to type I and II IFNs was identified (Lehtonen et al. 1997, Matikainen et al. 1997, Wong et al. 2002). The element bound an IFN-induced complex containing IRF-1 and CBP, and as Stat1 reciprocally regulates the IRF-1 gene promoter, the authors proposed that Stat1 and IRF-1 could form an intracellular amplifier circuit that would regulate cellular responsiveness to IFNs (Wong et al. 2002). In addition, Stat1 protein levels have been shown to be down-regulated by the dsRNA-dependent protein kinase PKR, a well-known IFN-inducible gene product (Tam et al. 1999).

5.13.2. Regulation of Stat1 signalling by the ubiquitin-proteasome pathway

Protein degradation is a complex tightly regulated process, which plays important roles in a great variety of basic cellular functions. A multistep process involving different enzymes generates polyubiquitinated proteins, which are degraded by the 26S proteasome. Among numerous substrates of the ubiquitin-proteasome pathway are components of signal transduction pathways (Ciechanover 1998). Proteasome inhibitors have been shown to increase the duration of signalling by multiple cytokines, IFN-γ among them (Kim and Maniatis 1996). The turnover of certain cytokine receptors is controlled by proteasomes, and the ubiquitin-proteasome pathway directly regulates activated Jak2 (Verdier et al. 2000, Yu and Malek 2001, Ungureanu et al. 2002). IL-3 and IFN-γ stimulation increases the accumulation of polyubiquitinated forms of Jak2, which are then degraded through proteasomes (Ungureanu et al. 2002). Stat1 molecules have been shown to cycle into the nucleus as tyrosine phosphorylated molecules and return quantitatively to the cytoplasm in non-phosphorylated form, suggesting that the removal of activated Stat1 molecules from the nucleus is not proteolytic but depends on protein tyrosine phosphatase activity (Haspel et al. 1996, Haspel and Darnell 1999).

In addition to ubiquitination, proteins can be modified by covalent attachment of other small polypeptides that are structurally related to ubiquitin, such as SUMO-1, -2 and -3 and ISG15 (interferon stimulated gene 15) (Yeh et al. 2000). The function of these modifications, however, is at present poorly understood, but recently both Jak1 and Stat1 have been shown to be ISGylated in human

thymic cells, and Stat1 ISGylation may regulate its transcriptional activity (Malakhov et al. 2003, Malakhova et al. 2003).

5.13.3. Dephosphorylation

A general feature of cytokine signalling is a rapid activation and subsequent inactivation of the pathway. After activation by phosphorylation, the components of the pathway are inactivated by dephosphorylation. Several tyrosine phosphatases have been identified to negatively regulate the JAK/Stat pathway: cytoplasmic SHP-1 (SH2-containing phosphatase 1), SHP-2, PTP1B (protein-tyrosine-phosphatase 1B) and CD45 dephosphorylate JAKs, and TC45 appears to dephosphorylate Stat1 in the nucleus but also Jak1 and Jak3 in the cytoplasm (David et al. 1995, You et al. 1999, Irie-Sasaki et al. 2001, Myers et al. 2001, Simoncic et al. 2002, ten Hoeve et al. 2002). SHP-2 was demonstrated to dephosphorylate nuclear Stat1 at both tyrosine and serine residues (Wu et al. 2002). During a full response to cytokine stimulation, an individual Stat1 molecule undergoes several cycles of phosphorylation, nuclear import, dephosphorylation and export from the nucleus (Haspel and Darnell 1999).

5.13.4. Nucleocytoplasmic transport

Eukaryotic cells have distinct nuclear and cytoplasmic compartments, separated by the nuclear envelope that is penetrated by nuclear pore complexes, which allow exchange of macromolecules between the two compartments. Passive diffusion through the nuclear pore complexes is reasonably fast only for proteins of <20–30 kDa, and nucleocytoplasmic transport of larger molecules is a tightly regulated process (Gorlich and Kutay 1999). Accurate localization is essential for the function Stats. They reside in a latent state mainly in the cytoplasm and upon stimulation by cytokines or growth factors they rapidly accumulate in the nucleus. Stat1 has been suggested to move from the plasma membrane to the nuclear pore by diffusion, independently of the actin cytoskeleton or microtubules (the random walk model) (Lillemeier et al. 2001). No classical nuclear localization signal (NLS) motifs have been identified in Stat molecules, and a shuttling transport receptor superfamily member importin-α5 has been shown to bind Stat1 and to be necessary for the nuclear import of activated Stat1 (Sekimoto et al. 1997, Melen et al. 2003). Several residues within the Stat1 DNA binding domain have been shown to be crucial in the transport of activated Stat1: lysines 410 and 413, which also affected the DNA binding of Stat1, and leucines 407 and 409, the mutation of which only blocked the nuclear import (Melen et al. 2001, McBride et al. 2002, Meyer et al. 2002). The ability of Leu407Ala mutant Stat1 to translocate into the nucleus as a dimer with either wild-type Stat1 or Stat2 molecule implies that the NLS resides in a Stat monomer (McBride et al. 2002), whereas in another study, no binding of monomeric Stat1 to importin-α5 was seen *in vitro*, and mutant Stat1 with lysines 410 and 413 changed into alanines was unable to bind to importin-α5 as a heterodimer with wild-type Stat2 (Fagerlund et al. 2002). The constitutive functions of unphosphorylated Stat1 in regulating certain genes indicates its presence in the nucleus (Kumar et al. 1997, Chatterjee-Kishore et al. 2000), and nuclear import of unphosphorylated Stat1 has been demonstrated to occur through a pathway distinct from that of activated

Stat1, independently of the transport receptor importin-α5 (Meyer et al. 2002). The export of Stat1 from the nucleus occurs after dephosphorylation via interaction with the export receptor CRM1 (chromosome region maintenance 1) (McBride et al. 2000). Two different leucine-rich nuclear export signals have been mapped in Stat1: one residing in the coiled-coil domain (amino acids 302-314) and the other in the DNA binding domain (amino acids 400-409) (Begitt et al. 2000, McBride et al. 2000).

5.13.5. Regulation by interacting proteins: the suppressors of cytokine signalling (SOCS)

In addition to phosphatases, two families of negative regulators of the JAK/Stat pathway have been discovered, the suppressors of cytokine signalling (SOCS) and protein inhibitors of signal transducer and activator of transcription (PIAS) protein families (Greenhalgh and Hilton 2001). The SOCS family includes at least eight members (SOCS-1–7 and cytokine-induced SH2 protein CIS), characterized by a divergent N-terminus, a central SH2 domain and a C-terminal region containing a conserved SOCS box motif. SOCS proteins are induced by a variety of cytokines, and appear to inhibit cytokine signalling via a number of different mechanisms (Greenhalgh and Hilton 2001). SOCS-1 directly binds to JAKs and inhibits their activities, whereas SOCS-3 interacts with multiple activated receptors and also JAKs, but without affecting JAK activity (Endo et al. 1997, Nicholson et al. 1999). CIS interacts with receptors to block the recruitment and activation of Stats (Matsumoto et al. 1999). SOCS box containing proteins can bind to ubiquitin-proteasome pathway components elongin B and elongin C, and it is possible that the SOCS-bound signalling complex is targeted to proteasomal degradation (Greenhalgh and Hilton 2001). Generation of SOCS-1-/- mice has demonstrated this protein to be an essential regulator of IFN-γ signalling (Alexander et al. 1999, Marine et al. 1999). The mice died perinatally with a complex disease characterized by anomalously activated T cells, lymphopaenia, macrophage infiltration of tissues, and liver necrosis, and they exhibited excessive responses similar to those induced by elevated levels of IFN-γ. The phenotype could be temporarily attenuated by the lack of IFN-γ or Stat1, but the long-term consequence of combined deficiency of SOCS1 and IFN-γ was the development of chronic disease states including polycystic kidneys, pneumonia and granulomas in various organs (Metcalf et al. 2002).

5.13.6. The family of protein inhibitors of signal transducer and activator of transcription (PIAS)

PIAS1 was isolated as a Stat1β interacting protein in a yeast two-hybrid screen, and additional PIAS family members PIAS3, PIASy, PIASxα/ARIP3 and PIASxβ/Miz1 were identified by database search and cDNA library screen. PIAS1, PIAS3 and PIASy are encoded by separate genes, and PIASxα and PIASxβ are probably splice variants of the same gene, differing in their C-terminal regions (Liu et al. 1998). PIAS1 and PIAS3 were characterized as specific inhibitors of Stat1 and Stat3, respectively (Chung et al. 1997, Liu et al. 1998), and PIASy has been suggested to act as a transcriptional co-repressor of

Stat1 (Liu et al. 2001). PIAS1 binds to activated Stat1 dimers and inhibits their DNA-binding activity, whereas IFN treatment triggers interaction of Stat1 with PIASy, which represses Stat1-mediated gene activation without blocking the DNA binding activity of Stat1 (Liu et al. 1998, Liao et al. 2000, Liu et al. 2001). Recently methylation of Stat1 on arginine 31 has been shown to decrease PIAS1 association with Stat1 and thus regulate IFN-induced transcription (Mowen et al. 2001). One mechanism by which methylation regulates Stat1-dependent transcription has been revealed in a subsequent study by the same authors. In the absence of methylation the increase in binding of PIAS1 to Stat1 concomitantly decreased the association between TC45 phosphatase and Stat1, resulting in a reduced rate of Stat1 dephosphorylation (Zhu et al. 2002).

In addition to Stats, members of the PIAS family have been reported to interact with a multitude of proteins and perform a variety of functions. For example, PIAS1 functions as a proapoptotic molecule, activating c-Jun N-terminal kinase and increasing the transcriptional activity of p53 proapoptotic protein (Liu and Shuai 2001, Megidish et al. 2002). PIASx isoforms interact with steroid receptors glucocorticoid receptor, progesterone receptor and oestrogen receptor, and androgen receptor has been shown to interact with all family members (Junicho et al. 2000, Kotaja et al. 2000, Tan et al. 2000, Gross et al. 2001). PIAS proteins can modulate nuclear hormone receptor-dependent transcription, acting as corepressors or coactivators depending on the receptor, promoter context and cell type (Kotaja et al. 2000, Tan et al. 2002). Rat PIAS3 homologue (KChAP, for K+ channel-associated protein) functions as a chaperone protein to facilitate the cell surface expression of voltage-gated K+ channels (Wible et al. 1998).

Recently, the PIAS proteins have been implicated in possessing enzymatic activity, acting as SUMO E3 ligases. SUMO (small ubiquitin-related modifier) is a small protein structurally homologous to ubiquitin and is covalently attached to specific lysine residues of proteins via a mechanism related to ubiquitination. The function of SUMO modification of proteins is not well understood, but it may play a critical role in various processes, including nuclear transport, regulation of transcriptional processes, targeting of transcriptional regulators to subnuclear domains, and the regulation of mitosis (Jackson 2001). PIAS-like proteins Siz1 and Siz2 of *Saccharomyces cerevisiae* were characterized as E3-like factors and found to be required for most SUMO conjugation in yeast (Johnson and Gupta 2001), and mammalian PIAS family members have subsequently been shown to function as SUMO ligases for various proteins. PIAS1 and PIASxβ promote sumoylation of p53 and c-Jun and repress the transcriptional activity of p53 (Schmidt and Muller 2002). PIAS1 and PIASx interact with and function as SUMO ligases for the nuclear hormone receptor coactivator GRIP-1 (glucocorticoid receptor-interacting protein 1) and thus cooperate in steroid receptor-dependent transcriptional regulation (Kotaja et al. 2002a). PIASxα, xβ, 1, and 3 interact with SUMO-1 and its E2 conjugase Ubc9 (ubiquitin-conjugating E2 enzyme), are themselves sumoylated and enhance the sumoylation of androgen receptor (Kotaja et al. 2002b). Axin interacts with PIAS1, PIASxβ and PIASy and its sumoylation is required for its ability to activate c-Jun N-terminal kinase (JNK) pathway (Rui et al. 2002). PIAS1 acts as a SUMO-1 E3 ligase for Sp3, and the sumoylation of a single lysine residue of

Sp3 silences its transcriptional activity (Sapetschnig et al. 2002). Similarly, PIAS3 functions as a SUMO ligase for IRF-1 and decreases its ability to induce activation of an ISRE-containing reporter gene (Nakagawa and Yokosawa 2002). Stat1 has also demonstrated to be modified by SUMO ligation, but the role of this modification is not clear (Ungureanu et al. 2003).

6. AIMS OF THE STUDY

The pivotal role of Stat factors in cytokine receptor signalling has been recognized for more than a decade and the basic mechanism of the JAK/Stat pathway is fairly well characterized. However, there are limited data available on how the Stats exert their function in the nucleus and how the tissue-specific expression of cytokine-responsive genes takes place. The aim of this study has been to investigate the mechanism of Stat action in cell type-specific gene activation at the molecular level using the promoter of the FcγRI gene as a model.

The specific aims of the study were:

1) To study the roles of Stat1 and PU.1 in IFN-γ-mediated activation of the FcγRI promoter and the molecular mechanisms of the functional interaction of Stat1 and PU.1 in detail.

2) To characterize the mechanism of glucocorticoid receptor-mediated enhancement of cell type-specific IFN-γ-induced transcription of the FcγRI gene.

3) To compare different PIAS family proteins in the regulation of gene activation by different Stat family members.

7. MATERIALS AND METHODS

7.1. Plasmid constructs

The GAS-luc luciferase reporter construct contains a GAS site TTTCCCCGAAA from the IRF-1 gene promoter inserted upstream of the thymidine kinase (TK) promoter driving the firefly luciferase (luc) coding region, Mut-GAS-luc contains the same GAS site with an AA to CC substitution (underlined in the sequence) (Pine et al. 1994). GRE-luc contains two copies of the rat tyrosine aminotransferase gene glucocorticoid response elements inserted upstream of the TK promoter (Palvimo et al. 1996). Spi-luc has six copies of GAS-like elements from the serine protease inhibitor 2.1 promoter upstream of the TK promoter (Wood et al. 1997), and pfNεN4-luc contains four copies of IL-4-responsive elements from the human Igε heavy chain constant region promoter placed upstream of c-fos minimal promoter (Pesu et al. 2000). The reporters derived from the FcγRI promoter sequence (Eichbaum et al. 1994) were constructed by cloning fragments of FcγRI promoter by PCR using human genomic DNA as a template and inserting them into a promoterless luc plasmid (Saksela and Baltimore 1993) or a luc vector containing TATA element from IFN-β promoter (Fujita et al. 1992). 189/66-ΔGAS-luc was constructed by mutating CAG (from –142 to –140) to ACT to disrupt the Stat1 binding site. A TCC sequence from PU.1 binding site (from –94 to –92) was mutated to GAA to give 189/66-ΔPU.1-luc. 189/66-ΔGASΔPU.1-luc harbours both CAG to ACT (from –142 to –92) and TCC to GAA (from –94 to –92) mutations. Schematic diagrams of the FcγRI promoter constructs are presented in Figure 5.

The Stat1 expression plasmids were constructed by subcloning the Stat1 cDNAs (Horvath et al. 1995, Wen et al. 1995, Bromberg et al. 1996) into EF-BOS vector containing HA (hemagglutinin) epitope tag (Mizushima and Nagata 1990). PU.1 expression plasmids (Fisher et al. 1998) were made by subcloning the PU.1 cDNAs into pCI-neo vector (Promega). TBP-GST plasmid was kindly provided by Dr. L. Tora. Human GRwt (pRSVhGRα) (Giguere et al. 1986) and GRD4× harbouring mutations Asn454Asp/Ala458Thr/Arg460Asp/Asp462Cys (Heck et al. 1994) were kind gifts from Dr. A. Cato, and rat GR3-556 and GR407-795 from Dr. S. Rusconi (Wieland et al. 1991). Stat5a (MGF) (Wakao et al. 1994) was kindly donated by Dr. B. Groner, human Stat6 (Quelle et al. 1995) and murine erythropoietin receptor (D'Andrea et al. 1989) by Dr. J. Ihle. Wild-type E1A, ΔCR1 and ΔCR2 (Bannister and Kouzarides 1995), gifts from Dr. T. Kouzarides, were tagged with HA epitope and subcloned into pCI-neo vector. E1A R2G and E1A ΔN mutants were generated by PCR and also tagged with HA, and cloned into pCI-neo. PIAS1 and PIAS3 cDNAs obtained from Dr. K. Shuai (Chung et al. 1997, Liu et al. 1998), and ARIP3 (Moilanen et al. 1999) were subcloned into pFLAG-CMV2 vector (Kodak IBI).

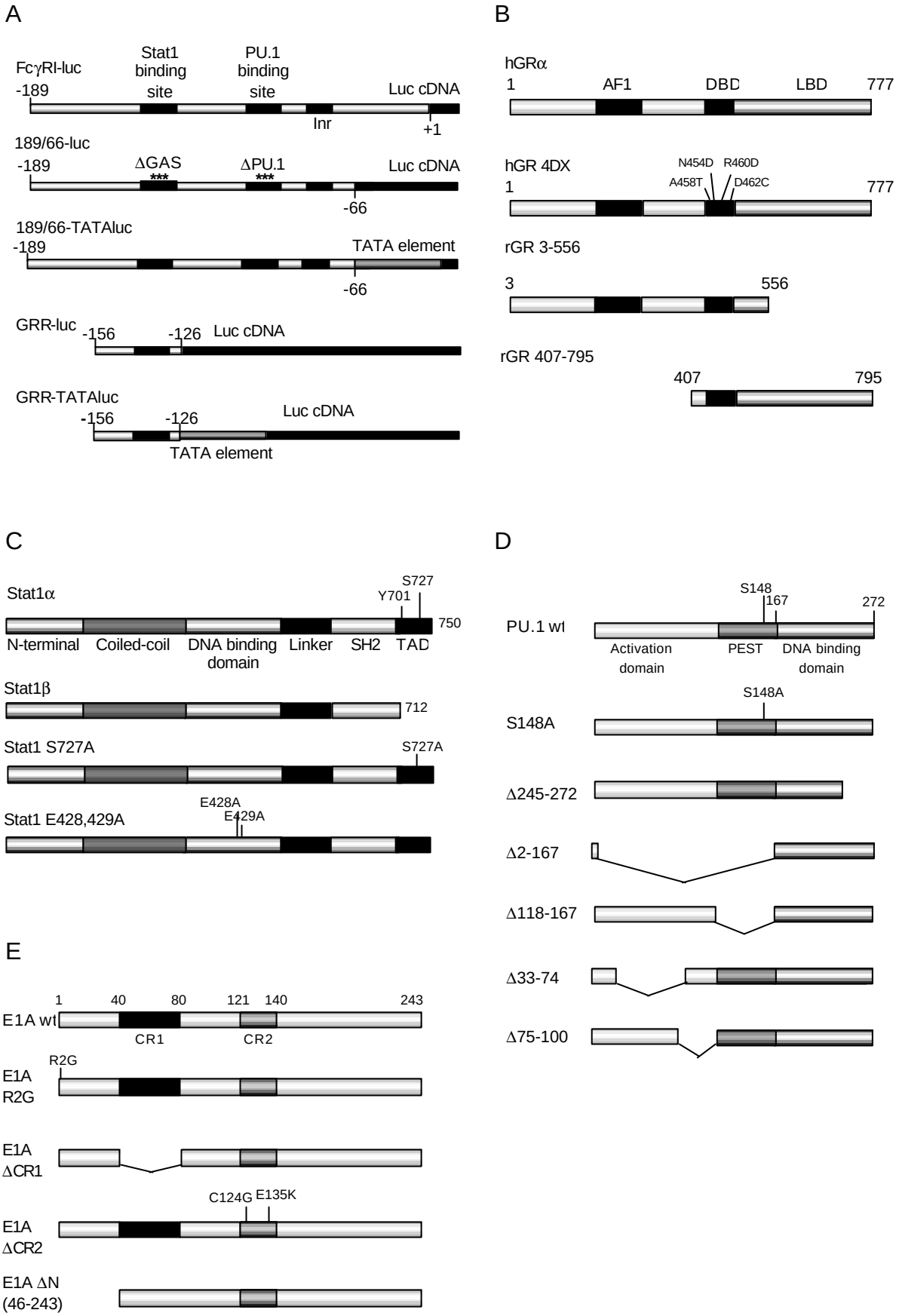

Figure 5. A) The FcγRI promoter constructs cloned for the study. Other plasmid constructs used in the thesis: B) Glucocorticoid receptor C) Stat1 D) PU.1 and E) E1A mutants.

7.2. Antibodies and cytokines

Monoclonal antibodies against the N-terminus and C-terminus of Stat1 were purchased from Transduction Laboratories, and monoclonal anti-Stat5a antibody was from Zymed. Polyclonal anti-GR antiserum was from Dr. Groner (Stocklin et al. 1996), and rabbit polyclonal anti-PU.1 and anti-RNAPII were purchased from Santa Cruz Biotechnology. Human recombinant IFN-γ was from Immugenex, and murine IFN-γ from Diaclone Research. Human IL-4 and erythropoietin were from PeproTech EC and Janssen-Cilag, respectively.

7.3. Cell lines and culture

Human HepG2 and HeLa cells, monkey COS-7 cells and murine RAW264.7 cells (from American Type Culture Collection, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and streptomycin, and human THP-1 and HL-60 cells (also from ATCC) in RPMI plus 10% FCS and antibiotics (all from Gibco/BRL). Human fibrosarcoma U3A cells (kindly provided by Dr. I. Kerr) (Muller et al. 1993) were cultured in DMEM containing 10% Cosmic calf serum (HyClone) plus antibiotics. Human peripheral blood monocytes were isolated from leukocyte-enriched buffy coats obtained from the Finnish Red Cross Blood Transfusion Service with density gradient centrifugation using Optiprep solution (Nycomed Pharma, Norway) according to the manufacturer's instructions. The purity of the monocyte preparation was analysed by FACS (fluorescence assorted cell sorter). After isolation the cells were maintained in RPMI medium with 10% FCS.

7.4. Transfections of cell lines

7.4.1. Calcium phosphate coprecipitation

Transfection of HepG2 and U3A cells was performed using the calcium phosphate precipitation method. Cells were seeded on 3.5 cm plates 24 hours before transfection. Plasmid DNA, 62 µl of 2 M $CaCl_2$ and water up to a total of 500 µl were mixed with 500 µl of 2 × HEPES solution (280 mM NaCl, 1.5 mM Na_2HPO_4, 55 mM N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid, pH 7.0) and the mixture was added to semiconfluent cells. 24 hours after transfection the cells received fresh medium with 1% untreated (**I,II**) or charcoal-stripped FCS (**III, IV**). Cells were either left untreated or were treated with appropriate cytokines and/or dexamethasone (Dex) for different time periods before harvesting.

7.4.2. FuGene 6

3×10^4 HeLa cells or 7×10^4 HepG2 cells were seeded onto 12 well plates, and 24 hours later transfected with FuGene 6 transfection reagent (Roche Molecular

Biochemicals) according to the manufacturer's instructions. For each transfection, 200 ng reporter plasmid, 20 ng β-galactosidase pCMV-βgal plasmid and 20 ng of Stat5 or Stat6 vectors plus different amounts of PIAS family expression plasmids were used. With Stat5, 30 ng of erythropoietin receptor expression vector was cotransfected. The DNA amounts were equalized by adding appropriated amounts of empty vector DNA. Twenty hours after transfection, the medium was changed into fresh one supplemented with 2% charcoal-stripped FCS with or without 10 ng/ml IFN-γ, 4 units/ml erythropoietin or 10 ng/ml IL-4. The cells were harvested 48 hours after transfection.

7.4.3. Electroporation

COS-7 cells and RAW264.7 cells were transfected by electroporation with Bio-Rad GenePulser apparatus, 260 V and 250 V, respectively, both 960 μF, with 0.5 cm cuvettes. Plasmid DNA was mixed with herring sperm DNA added up to 40 μg and mixed with the cells in 250 μl of DMEM (2-3 $\times$ 10^6 COS-7 cells or 6 $\times$ 10^6 RAW264.7 cells) prior to electroporation. After electroporation COS-7 cells were plated on 10 cm plates and RAW264.7 cells divided on four 3.5 cm plates per transfection.

7.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from human peripheral blood monocytes that had been cultured for 16 hours in medium containing 1% charcoal-stripped FCS and treated with IFN-γ (100 ng/ml) for 15 minutes or Dex (5μM) for 30 minutes. Cells were lysed in a buffer containing 50 mM HEPES pH 7.5, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium molybdate, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 0.2% NP-40, 3 μg/ml aprotinin, and 0.4 mM PMSF, the nuclei were collected by brief centrifugation and extracted with a buffer containing 0.3 M NaCl, 10% glycerol, 50 mM HEPES pH 7.5, 50 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium molybdate, 2 mM EDTA, 1 mM Na_3VO_4, 0.1% NP-40, 3 μg/ml aprotinin, and 0.4 mM PMSF. In (**II**), transfected COS-7 cells (by electroporation with 1 μg Stat1 or Stat1 and 0.5 μg PU.1 expression plasmid) and HL-60 and RAW264.7 cells were starved overnight with medium plus 1% FCS and treated with 100 ng/ml IFN-γ or left untreated, and nuclear extracts prepared as in (**I**). The GAS element from the murine IRF-1 gene (IRF-GAS, made by annealing the oligonucleotide 5`-CTAGAGCCTGATTTCCCCGAAATGATGAG-3` and its complement) and the GRR from the human FcγRI gene (5`-GATATGAGCATGGGAAAAGCATG-TTTCAAGGATTTGAGATGTATTTCCCAGAAAAGGAACATGATGAAAA TG-3`) were end-labelled with T4-polynucleotide kinase using [γ-^{32}P]ATP and used as probes. The 190-bp sequence from FcγRI-luc promoter was excised from the FcγRI-luc reporter plasmid and labelled like the other probes. Binding assays were performed with 10 μg of nuclear extracts from monocytes, HL-60 cells or RAW264.7 cells, or with 6 μg of COS-7 extracts. The extracts were incubated for 30 minutes on ice with ^{32}P-labelled oligonucleotide, 0.1 μg/μl herring sperm DNA and 1.5 μg/μl bovine serum albumin (BSA) in a total volume of 15 μl. For

supershift analysis, nuclear extracts were incubated with 0.5 μg of anti-Stat1 antibody (N-terminal) for 30 minutes on ice before adding the BSA, herring sperm DNA and probe. Reactions were resolved by 4.5% PAGE in 2.2 × TBE (175V +4°C). The gel was dried and autoradiographed.

7.6. Reporter gene assays

After transfection using pCMV-βgal as an internal transfection efficiency control, reporter plasmids and expression vectors, the cells were treated with appropriate cytokines for 6 h (**I,II**), 16 h (**III**) or 20 h (**IV**), and lysed in reporter lysis buffer (Promega) according to the manufacturer's instructions. Luciferase activity was measured with a luminometer (Luminova 1254, ThermoLabsystems) with Promega luciferase assay reagent and normalized against β-galactosidase activity of the lysates, determined by using 5 mM O-nitrophenyl-β-D-galactopyranoside as a substrate and measuring the absorbances at 420 nm.

7.7. Immunoprecipitation and Western blot analysis

COS-7 cells were transfected with 3 μg of Stat5a or Stat1, 2 μg of erythropoietin receptor, and 5 μg GR expression plasmids, and 48 hours after transfection the cells were treated with Dex (5μM) for 30 minutes and either erythropoietin (40 IU/ml) or IFN-γ (100 ng/ml) for 15 minutes. Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Na-deoxycholate, 20 mM NaF, 1% Nonidet P-40, 10% glycerol, 0.2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 20 μg/ml aprotinin). The lysates (1 mg protein) were precipitated with anti-Stat5a antibody (Zymed, 13-3600) or anti-Stat1 antibody (Transduction Laboratories, S21120) or anti-GR antiserum (Hoeck et al. 1989). Immunoprecipitates were separated on SDS-PAGE and transferred to nitrocellulose membrane for subsequent immunoblotting with specific antibodies followed by biotinylated secondary antibodies and streptavidin-biotin horseradish peroxidase conjugate. Proteins were visualized by enhanced chemiluminescence.

7.8. Flow cytometry

After treating human peripheral blood monocytes with IFN-γ and/or Dex for 20 hours, the cells were suspended in medium supplemented with 10% human AB+ serum and stained with FITC-conjugated mouse anti-human-CD64 antibody (Immunotech, Marseille, France) or with control antibody, isotype matched FITC-conjugated mouse anti-KLH antibody (Becton Dickinson, CA) for 30 minutes at 4°C and washed twice with PBS. The cells were analysed with FACSscan (Becton-Dickinson) and the expression was analysed from the gated monocyte population.

7.9. Glutathione S-transferase (GST) pull-down assay

GST and TBP-GST proteins were produced in BL21 bacteria and purified with Glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. 2×10^6 COS-7 cells were transfected with 2 μg of PU.1 expression plasmid and grown for 48 hours. Nuclear extracts were prepared as in the EMSA protocol, and 100 μg protein from COS-7 extracts or 200 μg protein from HL-60 nuclear extracts in 80 μl of lysis buffer was incubated overnight at 4°C with the GST fusion proteins bound to the Glutathione Sepharose beads in 240 μl of binding buffer (12.5 mM HEPES pH 7.4, 0.1 mM EDTA, 0.05% Nonidet P-40, 0.5% BSA, 1 mM DTT, 1 mM PMSF and 3 μg/ml aprotinin). The beads were washed five times with binding buffer containing 70 mM NaCl. The proteins were boiled in SDS-PAGE sample buffer, separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-PU.1 (Santa Cruz Biotechnology) or anti-Stat1 antibody (Transduction Laboratories).

7.10. In vitro coimmunoprecipitation

PU.1 and Stat1 proteins were translated *in vitro* and labelled with ^{35}S-methionine with TnT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. COS-7 whole cell extracts were prepared by lysing the cells in a buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM PMSF and 3 μg/ml aprotinin, and HeLa nuclear extracts were prepared as in EMSA protocol. COS-7 or HeLa extracts were immunoprecipitated with anti-RNA polymerase II antibody (Santa Cruz Biotechnology) in lysis buffer with NaCl concentration adjusted to 100 mM, and, after washing, incubated with *in vitro* translated PU.1 wild-type or deletion mutant Δ33-74 or Stat1. The bound proteins were subjected to SDS-PAGE and visualized by autoradiography.

8. RESULTS AND DISCUSSION

8.1. The molecular mechanism of Stat1 and PU.1 cooperation in IFN-g-induced transcription of the FcgRI promoter (I, II)

Since the proximal FcγRI promoter has been well characterized and shown to confer regulatory elements determining both IFN-γ responsiveness and myeloid cell specificity of the gene (Pearse et al. 1993, Eichbaum et al. 1994), the promoter could serve as a model for studying cell type-specific effects of IFN-γ. With reporter gene assays using transient transfections of different cell lines lacking Stat1 and PU.1, the functions of these proteins and of their functional domains could be analysed. The immediate 190-base-pair region of the FcγRI promoter (-189 to +1) was cloned into a promoterless luciferase reporter gene vector and was shown to be activated in IFN-γ-treated murine macrophage cell line RAW264.7 in a similar manner as in human monocytes (Guyre et al. 1983, Pan et al. 1990a). The reporter was also similarly activated in Stat1 and PU.1 deficient U3A fibrosarcoma cells when cotransfected with Stat1 and PU.1 expression plasmids. A reporter construct containing a smaller region (-189 to -66) that conferred binding sites for both Stat1 and PU.1 was activated slightly less efficiently, and mutation of either binding site from this construct resulted in abrogation of transcriptional activity. The activity of the Stat1 binding motif was also investigated in a different promoter context, that is by inserting the element (-156 to -126) into a reporter construct with a TATA box element from the IFNβ gene. The IFN-γ-induced activity of this construct was extremely low, showing that the presence of the TATA element is not sufficient for Stat1-driven transcription and confirming the necessity of PU.1.

8.1.1. The DNA binding and transactivation functions of Stat1 and PU.1 are necessary for IFN-γ-induced FcγRI promoter activation

To delineate the structures of Stat1 required for the cooperation with PU.1 in the activation of the FcγRI promoter, different Stat1 mutant expression plasmids were tested in the Stat1 and PU.1 deficient U3A cells. For comparison, the same plasmids were also cotransfected with a reporter construct containing a GAS element from IRF-1 gene inserted upstream of a thymidine kinase promoter (GAS-luc) (Pine et al. 1994). The same structures of IFN-γ-activated Stat1 were shown to be required for the activation of both reporter constructs. The transactivation function of Stat1 was critical, since the splice variant Stat1β, which is devoid of C-terminal 38 amino acids that constitute the activation domain, was unable to activate the transcription of either of the reporters. Likewise, a Stat1 variant (Glu428Ala, Glu429Ala) which is able to dimerize and localise into the nucleus but unable to bind DNA (Horvath et al. 1995), could not activate transcription.

Another mutant Stat1 with a substitution of the single serine residue (Ser727Ala), whose phosphorylation is critical for IFN-γ responses (Wen et al. 1995, Bromberg et al. 1996), was significantly less potent than wild-type Stat1 in stimulating transcription of both reporters. Mutation of Ser727 has been shown

to affect various IFN-γ target genes to a differential degree at the level of both basal and induced expression, the effects being particularly strong for the GBP1 and TAP1 genes and very modest for the p21 gene (Kovarik et al. 2001). Its effect on FcγRI promoter activation is of level similar to that observed for the IRF-1 gene, analysed also from U3A-derived cells, in the pioneering study by Wen et al. (Wen et al. 1995). In contrast to several genes analysed in Stat1-reconstituted fibroblasts from Stat1-deficient mice (Kovarik et al. 2001), the basal activation of the FcγRI promoter was not affected by the mutation of Ser727.

The promoter-specificity of PU.1 and Stat1 cooperation was shown by transfecting GAS-luc and Stat1α into U3A cells together with PU.1. The amount of PU.1 that readily activates the FcγRI promoter markedly inhibited the IFN-γ induction of the GAS-luc reporter. This inhibition was not studied further, but several mechanisms can be envisaged. PU.1 may compete for the basal transcription machinery components, or recruit repressor proteins. It has been shown to associate with a complex containing histone deacetylase HDAC1 and down-regulate transcription of c-myc (Kihara-Negishi et al. 2001). Alternatively, PU.1 may directly bind to CBP and inhibit its acetylation activity, which has been demonstrated to be a mechanism underlying PU.1-mediated inhibition of erythroid differentiation (Hong et al. 2002).

Similarly, the structures of PU.1 critical in the IFN-γ-stimulated transactivation of the FcγRI promoter were determined in reporter assays, cotransfecting them into U3A cells along with Stat1 and FcγRI-luc. Deletion of either region 33-74 from PU.1 that contains acidic transactivation domains or another transactivation domain that is rich in glutamines (Δ75-100), significantly reduced the ability of PU.1 to activate both basal and IFN-γ-induced transcription, the effect of deleting the acidic domains being more dramatic. The DNA binding domain alone (Δ2-167) and on the other hand a DNA binding-deficient mutant protein (Δ245-272) were totally unable to transactivate the promoter. Deleting the PEST domain (Δ118-167) shown to mediate association with PU.1 and IRF-4/IRF-8 had only a slight effect, and a mutant with a substitution of Ser148 to Ala was as active as the wild-type protein. Previous studies indicate that the regions critical for PU.1-mediated gene regulation are dependent on promoter context. For example, in the *in vitro* differentiation of macrophages the glutamine-rich transactivation domain is necessary, whereas deletion of the acidic domains or the DNA binding Ets domain still supports differentiation (Fisher et al. 1998). On Ig κ 3' enhancer the transactivation functions of PU.1 are dispensable (Pongubala and Atchison 1997). In IL-1β promoter activation PU.1 Ets domain directly associates with cytomegalovirus IE2 protein and is alone sufficient for promoter activation, while IE2 provides the transactivation domains (Wara-aswapati et al. 1999).

8.1.2. Stat1 and PU.1 do not physically interact

The possibility of direct physical interaction between Stat1 and PU.1 was tested by cotransfecting Stat1 and PU.1 into COS-7 cells and immunoprecipitating

Stat1 with anti-Stat1 antibodies and blotting PU.1 or vice versa. Similarly coimmunoprecipitation of *in vitro* translated Stat1 and PU.1 was performed and pull-down assays with GST-PU.1 and Stat1 were carried out from cell lysates. No association between the proteins was detected by any of the methods. When electrophoretic mobility shift assays (EMSA) were performed using the 190-bp FcγRI promoter as a probe, no Stat1-PU.1-DNA ternary complexes were observed. PU.1 binding to the probe was constitutive and not affected by treating the cells with IFN-γ for different time periods, whereas Stat1 DNA binding, not surprisingly, was strictly dependent on IFN-γ stimulation, and not influenced by the presence or absence of PU.1. Thus Stat1 and PU.1 appear to bind independently to their binding sites on the promoter and do not physically associate with each other. However, the presence of other factors associating with the proteins so weakly and bridging Stat1 and PU.1 *in vivo* that they cannot be detected in the assays used cannot be excluded.

8.1.3. PU.1 associates with general transcription factors TATA binding protein and RNA polymerase II

Since sequence-specific transcription factors activate transcription by tethering coactivator proteins and general transcription factors to the regulatory regions of genes, the ability of Stat1 and PU.1 to recruit other proteins to the FcγRI promoter was studied. Pull-down assays were performed with different GST fusion proteins and cell lysates, and as previously shown, PU.1 was found to interact with TBP and CBP histone acetyltransferase (HAT) domain (fragment of amino acids 1089-1758, (Bannister and Kouzarides 1996)) ((Yamamoto et al. 1999, DeKoter and Singh 2000) **II** and unpublished data). For IFN-γ-activated Stat1, on the other hand, no association to TBP was observed. Thus PU.1 may function as a bridging factor, recruiting TBP to the promoter. TBP recruitment, however, was not sufficient, since placing of the TATA element from the IFN-β promoter in FcγRI promoter reporter construct could not substitute for the PU.1 binding site, when the PU box was mutated from the construct. Some sequence-specific transcription factors may also facilitate transcription by directly associating with RNA polymerase II (RNAPII), and this possibility was studied by immunoprecipitating RNAPII from cell lysates and incubating the precipitate with *in vitro* translated Stat1 or PU.1. No interaction between RNAPII and Stat1 was found, whereas PU.1 associated with RNAPII, further supporting the putative role of PU.1 in functioning as a bridging factor for the general transcription factor machinery. The acidic transactivation region of PU.1 appeared to contribute to the interaction, as its deletion reduced the affinity of PU.1 to RNAPII. A high salt concentration was used in the immunopurification of RNAPII from cell lysates, but the presence of other proteins in the complex cannot be completely excluded. The demonstration of direct interaction between PU.1 and RNAPII would require an experiment performed with highly purified proteins.

8.1.4. Stat1 recruits coactivators CBP/p300

As previously shown, both Stat1 and PU.1 were able to interact with CBP in GST-pull-down experiments (unpublished data). The function of CBP/p300 in

the FcγRI promoter activation was examined in reporter gene assays using cotransfected adenoviral E1A protein that inhibits CBP/p300. E1A could only partially decrease the basal activity of the FcγRI-luc reporter but totally abolished IFN-γ-induced activation. E1A mutants incapable of inhibiting CBP/p300 failed to repress transcription of FcγRI-luc and another mutant that can no longer bind to and block the function of CBP/p300, but that is capable of directly inhibiting Stat1, abrogated the IFN-γ-stimulated activity of the reporter but not the basal transcription, indicating that E1A may repress Stat1-mediated transcription by inhibiting both CBP/p300 and Stat1. These results suggest that the role of Stat1 is to recruit CBP/p300 coactivators to the promoter following IFN-γ stimulation. Since cotransfection of exogenous CBP or p300 in the reporter gene assays did not further enhance the transcription of FcγRI-luc (data not shown), the amount of CBP/p300 in the cells does not appear to be a rate-limiting factor. As the combinations of transcription factors required for transactivation are different for each gene, there are probably several ways that a given transcription factor can facilitate transcription, depending on the promoter context. Thus the recruitment of CBP/p300 coactivators by Stat1 may not be the only nor universal action mechanism of Stat1, and on other promoters other mechanisms are likely to act in place or in concert with the one specified in this study.

There is a multitude of candidate proteins among the dozens of proteins involved in transcriptional initiation with which Stat1 or PU.1 could interact on regulatory regions of different genes. For example, an essential role for the SWI/SNF chromatin-remodelling complex in IFN-γ-stimulated CIITA induction has recently been demonstrated (Pattenden et al. 2002), and it would not be surprising if this or analogous ATP-dependent chromatin-remodelling complexes were also required for the regulation of other IFN-γ-responsive genes. Other Stats have been reported to interact with additional coregulatory proteins; for instance NcoA/SRC1a interacts with Stat3 and functions as its coactivator, and following IL-6 stimulation Stat3, NcoA/SRC1a and CBP/p300 are simultaneously recruited to the $p21^{waf1}$ promoter (Giraud et al. 2002). Following stimulation with IFN-α Stat2 interacts with GCN5, and the histone acetyltransferase activity of GCN5 is necessary for the expression of several IFN-stimulated genes (oligoadenylate synthase, ISG15, ISG54, ISG56 and ISG60), whereas PCAF and CBP/p300, which also associate with Stat2, appear not to be required (Paulson et al. 2002). Similarly, IFN-α-dependent transcription is enhanced by a Mediator complex subunit DRIP150, which also interacts with Stat2 (Lau et al. 2003). CBP/p300 has also been shown to acetylate non-histone proteins, such as the general transcription factors TFEII and TFIIF, and several gene regulatory factors, including GATA-1 and EKLF. Stat6 has been demonstrated to be acetylated by CBP/p300, and its acetylation has been shown to be critical in the IL-4-dependent activation of 15-lipoxygenase-1 gene (Shankaranarayanan et al. 2001). There are no reports on the acetylation of Stat1 or PU.1, and the requirement of CBP HAT activity for FcγRI transactivation was not addressed in our experiments.

In addition to the recruitment of acetyltransferases to promoter regions, Stats have been reported to recruit deacetylase enzymes. The acetylation of C/EBPβ

diminishes its DNA-binding activity, and following IL-3 stimulation, Stat5 mediates the deacetylation of C/EBPβ by recruiting HDAC1 to the Id-1 enhancer, resulting in increased DNA binding affinity of C/EBPβ and transactivation of the Id-1 gene (Xu et al. 2003). Deacetylase inhibitor treatment also inhibited IL-3-dependent activation of CIS and Pim-1 genes (Xu et al. 2003).

Despite the fact that the binding of two factors, Stat1 and PU.1, to the FcγRI promoter has been verified, additional factors may well be required *in vivo.* There are several putative elements in the immediate promoter region including motifs for C/EBPβ, IRF proteins and GATA factors, and additional regulatory elements may reside further upstream. The regulatory region of the myeloid-specific $gp91^{phox}$ gene has been analysed in several studies and a recent report identifies a Stat1 response element in the promoter several dozen base pairs upstream of an element that binds a multiprotein complex containing PU.1 and additional factors whose identities have not yet been defined (Kumatori et al. 2002). As it has been shown that PU.1, IRF-1, and ICSBP-mediated recruitment of CBP to the promoter enhances $gp91^{phox}$ transcription (Eklund and Kakar 1999), the authors hypothesize that Stat1 and PU.1 could cooperate in cell type-specific activation of the gene by IFN-γ through physical interaction with CBP, Stat1 N-terminal region associating with the CREB binding domain of CBP towards the N-terminal end, and PU.1 transactivation domain with another region of CBP containing the E1A binding site (Zhang et al. 1996, Yamamoto et al. 1999, Kumatori et al. 2002). Thus IFN-γ-induced transcription of the $gp91^{phox}$ gene would involve binding of a large complex composed of Stat1, IRF-1, PU.1, ICSBP, and CBP to the proximal promoter (Kumatori et al. 2002).

In reporter assays the proximal promoter regions confer the regulatory motifs for both the cell type-specific and IFN-γ-induced transcription of FcγRI, but on chromatinized DNA templates additional proteins may be necessary. It will be of interest to study the binding of putative transcription factors *in vivo* to the FcγRI gene regulatory regions in chromosomal environment instead of using transient transfection assays. Using chromatin immunoprecipitation assays, for example, it will be possible to study the *in vivo* recruitment of coactivators and general transcription factors to the promoter. In addition, the kinetics of transcription factor binding and factor recruitment can be assessed.

8.2. Glucocorticoid-mediated enhancement of IFN-γ-induced transcription of the natural FcγRI promoter involves PU.1 and indirect cross-talk between Stat1 and GR and requires PU.1 (III)

Glucocorticoids are among the most widely used anti-inflammatory and immunosuppressive drugs and are thought to exert their immunomodulatory function largely by inhibiting the synthesis of pro-inflammatory proteins, such as certain cytokines. Glucocorticoids can also interact with cytokine signalling pathways by other means. In T cells they repress IL-2 receptor β-chain and Jak3 expression, resulting in inhibition of IL-2 signalling (Bianchi et al. 2000). They inhibit the responsiveness of activated blood mononuclear cells to IL-12 by

down-regulating the IL-12 receptor β_1- and β_2-chain expression, and IL-12-stimulated phosphorylation of Stat4, resulting in suppression of IL-12-induced IFN-γ secretion in natural killer cells and T lymphocytes (Wu et al. 1998, Franchimont et al. 2000). Glucocorticoids also have positive effects on cytokine signalling. Glucocorticoid receptor (GR) has been shown to cooperate with Stats. IL-6-activated Stat3 interacts with ligand-bound GR to augment glucocorticoid signalling without association with a Stat DNA binding motif, and IL-6 and glucocorticoids synergistically activate the expression of acute phase proteins (Kim and Baumann 1997, Zhang et al. 1997, Takeda et al. 1998). GR and Stat5 interact physically and cooperate in prolactin-stimulated β-casein gene activation, whereas prolactin- or IL-2-activated Stat5 inhibits glucocorticoid-dependent transcription (Stocklin et al. 1996, Stoecklin et al. 1997, Biola et al. 2001). The effects of glucocorticoids on a particular gene may also vary in different cell types. In monocytes the synthetic glucocorticoid dexamethasone (Dex) enhances the IFN-γ-induced expression of FcγRI gene, whereas in immature myeloid leukaemia cell lines its effect is inhibitory (Guyre et al. 1983). Thus the mechanism of interaction between glucocorticoid and cytokine signalling pathways depends on the cell type and cytokine in question.

8.2.1. Dexamethasone increases IFN-γ-stimulated FcγRI expression by enhancing Stat1-dependent transcription

The previously documented effects of glucocorticoids on the surface expression of the FcγRI protein on human peripheral monocytes were first verified by FACS analysis. Dexamethasone (Dex) treatment alone did not affect the basal FcγRI expression, but further enhanced the increase in the expression stimulated by IFN-γ treatment. The effect of Dex was mediated through GR, since pre-treatment of monocytes with GR antagonist RU486 abolished Dex-mediated enhancement of FcγRI expression. The mechanism by which glucocorticoids enhance IFN-γ-stimulated expression of the FcγRI gene was first investigated by studying the effects of Dex on the activation of Stat1 DNA binding, employing electromobility shift assay (EMSA) and human peripheral blood monocytes. Three different DNA probes were used: GAS element from the IRF-1 gene, the gamma response region (GRR) from the FcγRI promoter, and the 190-bp insert from the FcγRI-luc reporter. IFN-γ treatment induced DNA binding complexes to all the probes tested, and these complexes were not affected by the treatment of cells with Dex for either 30 minutes or 20 hours, nor did Dex treatment induce any novel DNA binding complexes to any of the probes. The intensities of the IFN-γ-stimulated DNA complexes were reduced significantly following the 20-hour treatment with IFN-γ, in accordance to previous results in fibroblasts (Shuai et al. 1992). The probe excised from the FcγRI-luc reporter also contains the PU box, and the binding of PU.1 to the probe was constitutive, not influenced by treatment of monocytes with IFN-γ and Dex. Likewise, the PU.1 protein levels were unaffected by Dex stimulation, as examined by Western blotting (data not shown). Thus it would appear that Dex does not regulate the levels or activities of the transcription factors binding to the FcγRI promoter. The immediate IFN-γ signalling events also appear unaffected by Dex, as suggested by the unaltered DNA binding of Stat1 and by a recent study in which two or three-day treatment

of blood mononuclear cells with Dex did not change Jak1 and Jak2 protein levels or IFN-γ activation of Jak1 (Hu et al. 2003).

The effects of Dex treatment on IFN-γ-induced transcription were studied with reporter gene assays and cotransfection of GR expression vector into HepG2 cells that are normally unresponsive to Dex. The activity of the IFN-γ responsive reporter GAS-luc was not affected by treatment with Dex alone, but co-stimulation with both IFN-γ and Dex significantly increased the activity compared to the induction by IFN-γ alone. The increase was dose-dependent, i.e. it correlated to the amount of transfected GR plasmid. A reporter construct with a mutated Stat1 binding site could not be activated by any treatments, confirming that GR has a direct effect on Stat1-responsive transcriptional activation.

8.2.2. Transactivation and DNA binding functions of Stat1 and glucocorticoid receptor are required for cooperation

To characterize the structural determinants of Stat1 and GR proteins necessary for cooperation, GR and different Stat1 mutants were transfected into the Stat1-deficient U3A cells along with the GAS-luc reporter. Activation of the reporter required cotransfection of transcriptionally active Stat1α isoform, and as in HepG2 cells, the activation increased significantly following stimulation with both IFN-γ and Dex. The other splice variant Stat1β was incapable of either stimulating transcription in response to IFN-γ or of cooperating with GR. The inactive non-phosphorylated mutant Stat1 Tyr701Phe and the DNA-binding deficient mutant Stat1 Glu428Ala, Glu429Ala were likewise inactive. The requirement of Stat1 serine phosphorylation was studied by employing the Stat1 Ser727Ala mutant. In accordance with the results of (**II**), the overall activation of GAS-luc following IFN-γ stimulation was reduced compared to that with cotransfected Stat1α, but the enhancement of transcription in response to treatment with both IFN-γ and Dex was comparable to that obtained in cells transfected with Stat1α. In summary, the DNA binding and transactivation domains of Stat1, but not its serine phosphorylation, are required for cooperation between Stat1 and GR. In that respect, the mechanism is different from that of the synergism between Stat5 and GR, as a C-terminal deletion mutant of Stat5 lacking the transactivation domain was able to cooperate with GR in activating the transcription of a β-casein reporter (Stoecklin et al. 1997).

Analogously, various GR mutant plasmids were cotransfected into HepG2 cells together with the GAS-luc reporter. Two different mutants incapable of binding to DNA (Ala458Thr and D4x containing amino acid substitutions Asn454Asp/Ala458Thr/Arg460Asp/Asp462Cys) were unable to increase IFN-γ-dependent GAS-luc activity following treatment with IFN-γ and Dex. A mutant GR (GR 407-795) devoid of the N-terminal ligand-independent activation function (AF-1) was less potent than the wild-type protein in cooperating with Stat1, and a constitutively active GR variant GR 3-556 lacking the ligand binding domain enhanced Stat1-dependent reporter activity equally well in the absence and presence of Dex. These results suggest that GR DNA binding is

absolutely necessary and the AF-1 domain is needed for optimal stimulation of IFN-γ-induced transcriptional activation.

8.2.3. Stat1 does not influence dexamethasone-mediated transcription

The effect of Stat1 on Dex-stimulated transcription was explored by cotransfection of U3A cells with GR-responsive reporter GRE-luc and Stat1 expression plasmids. Dex treatment activated the reporter and the activation was greatly increased by cotransfection of exogenous GR. None of the Stat1 plasmids tested (Stat1α, Stat1β, Stat1 Ser727Ala or Stat1 Tyr701Phe) had any effect on the activity of GRE-luc. In interaction studies with Stat5 and GR it has been shown that prolactin-activated Stat5 may inhibit glucocorticoid-dependent transactivation (Stocklin et al. 1996). Cooperation between Stat3 and GR differs from that between Stat5 and GR and between Stat1 and GR, as Dex and IL-6 can synergistically activate a reporter construct containing multiple GREs in the absence of any Stat3 binding element (Zhang et al. 1997). Thus different Stats seem to have distinct effects on glucocorticoid-mediated gene responses.

8.2.4. Dexamethasone-mediated enhancement of Stat1-stimulated transcription is indirect and involves new protein synthesis

Since GR has been shown to associate with both Stat3 and Stat5 (Stocklin et al. 1996, Zhang et al. 1997), the ability of Stat1 to interact physically with GR was tested. In a control experiment cotransfected Stat5 and GR coimmunoprecipitated from cell lysates even in the absence of stimulation. However, when Stat1 was transfected along with GR, no association between the proteins was observed either in non-treated cells or in IFN-γ and Dex treated cells. As the cooperation appeared not to involve direct interaction between Stat1 and GR, and the GAS-luc reporter used in the experiments harboured no consensus GRE elements that could bind GR directly, the necessity of *de novo* protein synthesis for the Dex-stimulated enhancement of Stat1-dependent transcription was investigated. Inhibition of protein synthesis with cycloheximide during Dex stimulation resulted in failure of GR to enhance GAS-luc activation after washing away the inhibitor and treating the cells with IFN-γ, whereas the IFN-γ response was unaffected. Thus the GR effect seems to be indirect and require the expression of new protein or proteins, possibly those with coactivator activity. The effect of glucocorticoid treatment on the expression of various steroid receptor coactivators was studied in several rat tissues (Kurihara et al. 2002). SRC-1 mRNA was down-regulated in the tissues examined (heart, stomach, kidney, liver, and cerebrum), whereas GRIP-1, p/CIP, CBP and PCAF did not show significant changes in mRNA expression. Haematopoietic cells, however, were not included in the study, and thus the influence of glucocorticoids on coactivators in monocytes cannot be evaluated on the basis of the results of (Kurihara et al. 2002). Our studies do not confirm the involvement of CBP in the cooperation between Stat1 and GR. Overexpression of CBP in HepG2 cells did not further augment IFN-γ-induced transcription or the Dex-stimulated enhancement of IFN-γ-stimulated reporter activity (data not shown), suggesting that CBP was not a rate-limiting component. However, coexpression of E1A completely blocked both IFN-γ and

Dex enhancement of transcription showing that CBP is required as a coactivator for Stat1 (unpublished data).

8.2.5. The stimulatory effect of glucocorticoids on FcγRI promoter activation is dependent on Stat1 and PU.1

The effect of activated GR on the expression of the natural FcγRI promoter was investigated by using the FcγRI-luc reporter construct. Transfection of FcγRI-luc and GR into HepG2 cells failed to activate the reporter without cotransfection of PU.1, indicating that in the context of the FcγRI promoter PU.1 is necessary for the GR action. The effect of GR on FcγRI promoter activation was not as great as on the activation of GAS-luc, suggesting that the promoter context plays a role. Glucocorticoids have long been known to suppress IFN-γ-stimulated expression of MHC class II molecules (Warren and Vogel 1985). Stat1, however, does not directly regulate MHC II expression but induces the transcription of Class II transactivator protein CIITA that is necessary for class II induction (Steimle et al. 1994). Glucocorticoids inhibit the transcription of MHC class II genes by causing GR to translocate to the nucleus and compete with CIITA for a limited amount of CBP (Fontes et al. 1999). It would be interesting to study the effects of glucocorticoid treatment on other IFN-γ-responsive genes in macrophages. In addition to being gene-specific, the effects of glucocorticoids are distinct in different cell types. According to previous results (Guyre et al. 1983), in myeloid leukaemic HL-60 cells IFN-γ-induced FcγRI surface expression was down-regulated by Dex as determined by FACS analysis (data not shown). Dex treatment did not affect Stat1 activity or the PU.1 and GR protein levels, thus GR may possibly act by inducing a repressor or competing of coactivators. The former alternative is suggested by the kinetics of Dex inhibitory effect on IFN-γ-stimulated FcγRI mRNA levels, which was seen after 3 to 6 hours of Dex treatment in U937 cells, whereas the IFN-γ induction occurred within one hour (Pan et al. 1990a). The requirement of *de novo* protein synthesis was also demonstrated for the Dex effect, further supporting the possibility that Dex up-regulates a repressor protein. A schematic representation of the IFN-γ and glucocorticoid-regulated activation of the FcγRI promoter is shown in Figure 6.

A novel mechanism of GR action was proposed in a study showing that the steroid-bound ligand-binding domain of GR was able to enhance C/EBPβ-dependent transactivation of C/EBPα gene through induction of proteosomal degradation of an mSin3A/HDAC1-containing corepressor complex that restrains the onset of C/EBPα transcription in adipocyte precursors (Wiper-Bergeron et al. 2003). Since the DNA binding domain of GR was found to be necessary in the cooperation between Stat1 and GR in IFN-γ-induced transcription, HDAC1 targeting by GR is not a likely explanation in our studies.

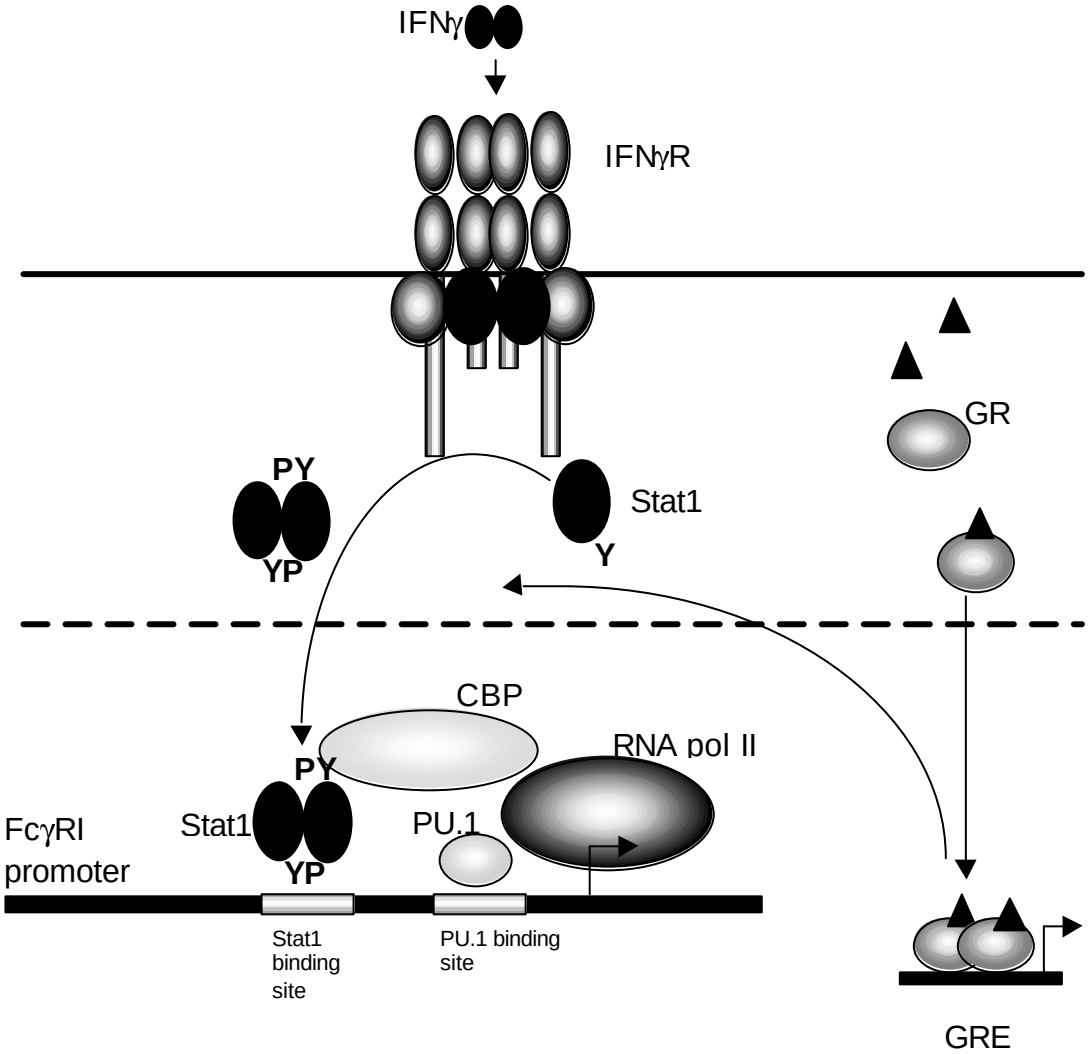

Figure 6. Schematic representation of the IFN-γ and Dex-regulated activation of the FcγRI promoter. Upon IFN-γ stimulation Stat1 molecules are activated subsequent to receptor oligomerization and JAK activation. Activated Stats are transported into the nucleus, where they bind to their target element in the promoter. They activate transcription in cooperation with PU.1 and coactivator protein CBP. Dex in turn binds to glucocorticoid receptor which is translocated into the nucleus and binds to glucocorticoid response elements (GREs) in the target genes. Some of the proteins induced are probably coactivators that augment IFN-γ-induced transcription of the FcγRI promoter.

In a recent study, glucocorticoid treatment was shown to inhibit IFN-γ signalling by down-regulating the expression of Stat1 mRNA in monocytes. However, this effect was seen only after 24-hour treatment with Dex and required coculturing the monocytes with lymphocytes, indicating that glucocorticoid treatment changes the interactions between lymphocytes and monocytes rather than directly inhibiting Stat1 expression (Hu et al. 2003). This result was consistent with that obtained from our EMSA experiment in which purified monocytes were cultured in a standard medium and no change in Stat1 DNA binding was observed after 20-hour treatment with IFN-γ alone versus IFN-γ plus Dex. The importance of studying the cross-talk between IFN-γ and glucocorticoids *in vivo*

is suggested by a study in which treatment of ulcerative colitis patients with systemic glucocorticoid prednisolone for 2–4 weeks decreased the levels of phosphorylated Stat1 in their colonic mucosae, and a two-hour stimulation of isolated monocytes with prednisolone also directly inhibited IFN-γ-induced phosphorylation of Stat1 *in vitro* (Schreiber et al. 2002). Thus investigation of the molecular mechanisms of cytokine and hormone action is vital but cannot replace studies on patients and animal models of inflammatory diseases.

8.3. PIAS (protein inhibitor of activated Stat) proteins in the regulation of Stat signalling (IV)

The PIAS family members PIAS1 and PIAS3 were originally described as specific nuclear inhibitors of Stat1 and Stat3, respectively (Chung et al. 1997, Liu et al. 1998), and the purpose of this work was to examine the roles of PIAS proteins in the modulation of transcription mediated by Stat1, 5 and 6. The work was part of a study in which the effects of different PIAS proteins on steroid hormone receptor-dependent transcription were compared, and thus the same conditions were used in all experiments. HeLa and HepG2 cells were transfected with various Stat-responsive reporter gene constructs and treated with appropriate cytokines, and the effects of different doses of cotransfected PIAS proteins on the reporter gene activities were monitored.

The involvement of PIAS1, PIAS3 and PIASxα/ARIP3 in the regulation of endogenous Stat1 activity was examined using the GAS-luc reporter. In HeLa cells none of the PIAS proteins tested had any effect on the IFN-γ-induced reporter activation, and in HepG2 cells only PIAS1 was able to moderately repress the activation when transfected in large amounts. Similarly, PIAS1, PIAS3 and PIASxα/ARIP3 were tested for their ability to influence Stat5- and Stat6-mediated transcription. Cells were transfected with Stat5 and erythropoietin receptor and a Stat5-responsive Spi-luc reporter, or Stat6 and a reporter containing Stat6 binding sites (pfNεN4-luc), and stimulated with erythropoietin or IL-4, respectively. Cotransfected PIAS proteins only minimally affected Stat5- or Stat6-mediated transcription, apart from PIASxα/ARIP3, that could modestly enhance IL-4-responsive transactivation. PIAS proteins were also coexpressed together with Stat3, and no modulation of IL-6-induced reporter activation was observed with the amounts of PIAS expression plasmids used (unpublished results). Likewise, the other splice variant of PIASx, PIASxβ/Miz1, had no effect on any of the Stat-responsive reporter activities used in this study. We also tested the ability of PIASxα/ARIP3 to influence the cooperation between activated GR and Stat5 in the transactivation of Spi-luc reporter, but no effect was observed in HeLa cells (unpublished results).

The same cell lines and plasmid concentrations were used to study the effects of the PIAS proteins on transactivation mediated by various steroid receptors, namely androgen, oestrogen, glucocorticoid and progesterone receptors. Different PIAS proteins were different in their ability to activate transcription. The degree of activation or repression of transcription was dependent on the cell

line, PIAS protein, the steroid receptor in question and the promoter context. The mechanism of action of the PIAS proteins likely involves interactions with other coregulatory proteins, and the amounts of these and PIAS proteins in different cell and promoter contexts may explain the results obtained in this study. In summary, in the experimental conditions in which various PIAS family proteins efficiently modulated steroid hormone-dependent transcription, they only minimally affected transcriptional activation stimulated by the Stats examined.

PIASx has recently been shown to act as a corepressor for Stat4 (Arora et al. 2003). Stat4 and PIASx were found to associate in the cells after treatment with IFN-α or IL-12, and PIASx was present in the Stat4-DNA-binding complexes but did not interfere with Stat4 DNA binding ability. The authors suggest that the inhibitory activity of PIASx may involve the recruitment of a histone deacetylase (HDAC) to the promoter, since treatment of cells with the HDAC inhibitor trichostatin A could abrogate the inhibition (Arora et al. 2003). In line with our results, (Arora et al. 2003) showed that PIASxα/ARIP3 did not modulate IFN-γ-induced expression of a Stat1-responsive reporter gene in 293T cells. PIAS1 and PIASy, on the contrary, significantly repressed transcription under the same conditions.

In the study defining PIAS1 as a specific inhibitor for Stat1, transfection of equal amounts of Stat1 and PIAS1 resulted in a clear reduction of IFN-γ-activated transcription (Liu et al. 1998). Several reasons in our studies may account for obtaining results contradictory to those originally reported. The cell lines and transfection methods used in the experiments were different, i.e. Liu et al. transfected 293 cells with calcium phosphate coprecipitation, whereas in this work HeLa and HepG2 cells were transfected with FuGene 6 reagent. Additionally, the reporter constructs were different: a luciferase reporter (3x)Ly6 containing three copies of the Stat1 binding sequence from the murine Ly-6A/E gene (Khan et al. 1993) was used by Liu et al. while in this study we employed a construct encompassing a single Stat1 binding element from the human IRF-1 gene (Pine et al. 1994). The amounts of DNA transfected were also different. In experiments performed using calcium phosphate coprecipitation as a transfection method and endogenous Stats, we could also obtain modest reduction in Stat1- and Stat3-dependent reporter activities with large amounts of PIAS1 and PIAS3 plasmid in HepG2 cells, respectively (unpublished data). In the case of steroid-receptor-dependent transcription, the effect of the PIAS proteins was dependent on the promoter and cell type, and the gene- and cell-type restricted differences may also influence Stat-dependent transcription. The study by Arora et al. (Arora et al. 2003) compares the effects of PIAS1, PIASy and PIASx on IFN-γ-stimulated transcription of the (3x)Ly6 reporter in 293T cells. No protein levels are shown, but according to the DNA amounts transfected, PIASy appears a much more potent inhibitor of Stat1 than PIAS1, since three times the amount of PIAS1 plasmid (50 ng) compared to PIASy (15 ng) was required for a 50% reduction in IFN-γ-induced activity of the reporter. In an earlier study PIASy strongly repressed IFN-γ-stimulated activation of (3x)Ly6 reporter driven both by endogenous and exogenous Stat1 in HeLa cells, but comparison between PIAS1 and PIASy was not included (Liu et al. 2001). In this work, PIASy was not included in the study, but it would be interesting to see if it has a more

pronounced effect on transcription in the experimental conditions used in our work.

A recent study by Coccia et al. (2002) provides evidence for a functional role for PIAS1 in Stat1-mediated gene regulation. Murine macrophage cell lines exhibit different responses to IFN-γ depending on their maturation stage. Less mature P388.D1 cells express high levels of PIAS1, resulting in reduction in the binding activity of Stat1 and diminished response to IFN-γ compared to mature RAW264.7 cells. Forced expression of a PIAS1 homologue, the Gu binding protein (GBP), inhibits the Stat1-mediated gene activation in the RAW264.7 cells. Thus the expression levels of PIAS1 may account for the differential IFN-γ responsiveness in the cell lines (Coccia et al. 2002).

Different PIAS proteins are differentially expressed in tissues. For example, PIAS1 expression is high in the thymus, PIASy in the spleen, and PIAS1 and PIASxα show the highest expression in the testis, whereas PIAS3 is a ubiquitous protein (Chung et al. 1997, Moilanen et al. 1999, Tan et al. 2000, Liu et al. 2001). The effects of PIAS proteins on transcription may be complicated by the expression of two or more family members in the same cell, suggested by studies in which PIAS1 could self-associate and directly interact with other family members in GST-pull-down assays, and in which cotransfection of other family members could inhibit the effect of PIAS1 on steroid-mediated transcription (Tan et al. 2002). A variety of functions has been attributed to the family members, and they inhibit Stat-mediated signalling through different mechanisms, PIAS1 and PIAS3 by directly interacting with Stats and inhibiting their DNA binding, and PIASy and PIASx by binding to Stats and possibly recruiting corepressor proteins, perhaps those exhibiting deacetylase activity (Chung et al. 1997, Liu et al. 1998, Liu et al. 2001, Arora et al. 2003). In contrast, the function of PIAS proteins in the steroid receptor-dependent transcription can be both coactivatory and repressory, depending on the receptor, gene and cell context. In subsequent studies by Kotaja et al. (Kotaja et al. 2002a, Kotaja et al. 2002b) it has been shown that PIAS proteins can act as SUMO ligases and enhance sumoylation of androgen receptor and its coactivator GRIP1 and further that sumoylation is critical for the coactivator function of GRIP1. Recently GR has also been shown to be sumoylated on several sites, and the mutation of two major SUMO acceptor sites differentially affects GR-mediated transcription from different promoters (Tian et al. 2002). Sumoylation has been demonstrated to enhance the acetylase activity of the histone deacetylases HDAC1 and HDAC4 (David et al. 2002, Kirsh et al. 2002). The enzymes involved in the SUMO attachment on HDAC1 were not identified, while HDAC4 was sumoylated *in vitro* by RanBP2, a SUMO E3 ligase structurally unrelated to the PIAS proteins, but not by PIAS1 or PIAS3 (David et al. 2002, Kirsh et al. 2002). The PIAS family members have been shown to act as SUMO ligases for other transcription factors as well, including p53, c-Jun and Smad4 (Schmidt and Muller 2002, Lee et al. 2003). It will be interesting to see if Stats and/or their coregulators are sumoylated by the PIAS proteins and whether their possible sumoylation plays a role in the modulation of Stat-dependent gene regulation.

8.4. Summary

Cytokines are central mediators of immune responses in the body, and in the last decade our understanding of their mechanism of action has greatly improved since the discovery of the signalling pathway they utilise. Cytokines mainly affect the gene expression profiles of their target cells at the level of transcription by activating Stat factors that modulate transcription of genes containing binding elements for Stats. Stats, like other eucaryotic transcription factors, are not capable of activating transcription alone, but need to interact with other activating transcription factors and cofactors on the regulatory regions of their target genes. The main focus of this thesis work was to define the functional roles of Stat1 and other cooperating proteins in the IFN-γ-induced activation of a myeloid cell-specific gene encoding the high affinity receptor for IgG, FcγRI.

Binding of Stat1 and a haematopoietic cell-specific factor PU.1 to the FcγRI promoter was found to be necessary for the IFN-γ-dependent activation of the gene. The transactivation and DNA binding functions of both proteins were critical, but these factors did not physically interact with each other. Stat1 and PU.1 were found to perform distinct functions in promoting the expression of the FcγRI gene: PU.1 appeared to mediate contacts with basal transcription machinery proteins TATA binding protein TBP and RNA polymerase II, and Stat1 served as a recruiter of coactivator CREB binding protein (CBP)/p300.

The mechanism by which glucocorticoid hormones enhance IFN-γ-induced transcription of the FcγRI gene in macrophages was studied in the same experimental system that was used for identifying the roles of Stat1 and PU.1. The cooperation between activated Stat1 and glucocorticoid receptor in activating FcγRI expression was found to require the transactivation and DNA binding domains of both factors. No physical association between the proteins was observed, and glucocorticoid receptor did not bind to the FcγRI promoter but rather enhanced FcγRI transcription indirectly through glucocorticoid-induced *de novo* synthesized proteins that may function as coactivators.

To date, four members of the family of protein inhibitors of activated Stats, the PIAS proteins, have been described as specific inhibitors of different Stats. The roles of PIAS1, PIAS3 and PIASxα/ARIP3 in regulating several cytokine-responsive promoters was analysed with the same experimental settings in which these PIAS proteins clearly influenced steroid hormone-mediated transcription. It was found that in the given conditions the PIAS proteins only marginally affected cytokine-stimulated activation of the reporter constructs used in the study. Thus the roles of the PIAS proteins are clearly not restricted to down-regulating cytokine signalling but also differentially modulate steroid hormone-driven transcription promoter and cell type-dependently.

The great diversity in cell specialisation in the human body is achieved by the tight regulation of some 30,000 genes so that only a subset of the genes is expressed in each cell type. The initiation of transcription is a major point at which genes are regulated, modulated by a vast number of transcription factors binding to regulatory sequences. The regulatory region of each gene is an

individual with different transcription factors binding to different regulatory regions. In addition, an individual transcription factor may have different functions depending on the regulatory region it is binding to. Thus no general rules how genes are regulated can be deduced by studying a single gene. In all cases, however, transcriptional activation requires the creation of a chromatin structure that is accessible to the transcription machinery. This is achieved by the activities of various chromatin remodelling complexes. In this work, CBP/p300, which possesses chromatin acetylation activity, has been shown to be recruited to the FcγRI promoter by Stat1. Whether recruiting coactivator complexes is a universal property of Stats and whether the protein acetylation activity of CBP/p300 is required for Stat1-mediated transcription of FcγRI as well as of other IFN-γ-responsive genes is an interesting subject for future study.

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