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Regulation of STAT6-mediated Transcription in IL-4-induced Signal Transduction

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of Finn-Medi 1,
Lenkkeilijänkatu 6, Tampere, on May 23rd, 2003, at 12 o'clock.

Acta Universitatis Tamperensis 940
University of Tampere
Tampere 2003

ACADEMIC DISSERTATION
University of Tampere, Institute of Medical Technology
Tampere University Hospital, Department of Clinical Microbiology
Finland

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http://granum.uta.fi

Cover design by
Juha Siro

Printed dissertation
Acta Universitatis Tamperensis 940
ISBN 951-44-5691-2
ISSN 1455-1616

Tampereen yliopistopaino Oy Juvenes Print
Tampere 2003

Electronic dissertation
Acta Electronica Universitatis Tamperensis 263
ISBN 951-44-5692-0
ISSN 1456-954X
http://acta.uta.fi

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1. ABSTRACT

Cytokines are a group of small, secreted glycoproteins that have a crucial role in the regulation of cellular events including proliferation and differentiation. Hydrophilic cytokines cannot penetrate through the cell membrane; instead they bind transmembrane receptor molecules that consequently trigger intracellular signaling pathways. Pleiotropic cytokine IL-4 serves as a critical mediator of humoral immune responses executed by B and Th2 type lymphocytes. The engagement of IL-4 receptor on the cell surface leads to the activation of JAK1 and JAK3 tyrosine kinases followed by tyrosine phosphorylation and dimerization of transcription factor STAT6. The activated STAT6 translocates to the nucleus where it binds to specific DNA elements and activates the transcription of IL-4 responsive genes such as IL-4Rα, CD23, MHC class II molecules, and germline Igε. The immediate signaling events that lead to the activation and DNA binding of STAT6 are well established, but relatively little is known about the molecular mechanisms of STAT6-mediated transcription and the regulation of STAT6 activity. The aim of this study was to evaluate the role of serine/threonine kinase activity in STAT6-mediated signaling and the cofactor requirements of STAT6 in Igε promoter activation. It was observed that IL-4 stimulation induced the phosphorylation of STAT6 on both serine and tyrosine residues in B cells, and STAT6-mediated gene activation was dependent on serine/threonine inhibitor H7-sensitive kinase activity. The serine phosphorylation of STAT6 was insensitive to H7 treatment, instead H7 downregulated the IL-4-induced phosphorylation of RNA polymerase II. CD40 antigen engagement provides an important costimulatory signal for IL-4 treatment by activating many intracellular signaling molecules, including p38 MAP kinase. p38 MAP kinase activity was found to enhance the STAT6 transactivation domain (TAD)-driven transcription without an effect on the immediate activation or phosphorylation events of STAT6. The transcriptional coactivator p100 was identified as a novel STAT6TAD interacting protein. p100 expression did not affect the immediate activation events of STAT6, but it enhanced IL-4-induced germline Igε gene transcription in B cells. The results from interaction studies suggested that p100 functions as a bridging factor between STAT6 and the basal transcription machinery. The STAT6 response element in Igε promoter was observed to be activated in a cell-type specific manner. A minimal low affinity core binding sequence for hematopoietic transcription factor PU.1 was identified within the Igε-STAT6 DNA-binding site. PU.1 restored the IL-4-inducibility of Igε-STAT6 response element in non-hematopoietic cells. The co-operation between PU.1 and STAT6 in the transactivation of the Igε gene represents a molecular mechanism for the fine-tuning of the cell-type restricted expression of IL-4-induced gene responses.

2. LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV:

I M Pesu, K Takaluoma, S Aittomäki, A Lagerstedt, K Saksela, PE Kovanen and O Silvennoinen: IL-4-induced transcriptional activation by STAT6 involves multiple serine/threonine kinase pathways and serine phosphorylation of STAT6, *Blood,* 2000 Jan 15; 95(2): 494-502

II M Pesu, S Aittomäki, K Takaluoma, A Lagerstedt and O Silvennoinen: p38 mitogen activated protein kinase regulates interleukin-4-induced gene expression by stimulating STAT6-mediated transcription*, J Biol Chem,* 2002 Oct 11; 277(41): 38254-38261

III J Yang, S Aittomäki, M Pesu, K Carter, J Saarinen, N Kalkkinen, E Kieff and O Silvennoinen: Identification of p100 as a coactivator for STAT6 that bridges STAT6 with RNA polymerase II, *EMBO J,* 2002 Sep 16; 21(18): 4950-4958

IV M Pesu, S Aittomäki, T Välineva and O Silvennoinen: PU.1 is required for transcriptional activation of STAT6 response element in Igε promoter*, Eur J Immunol*, 2003, in press

3. ABBREVIATIONS

γc	common γ-chain
15-LOX-1	15-lipoxygenase-1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSAP	B cell specific activator protein
C	Carboxy, COOH
CAK	Cdk activating kinase
CBP/p300	CREB-binding protein/p300
Cdk	Cyclin-dependent kinase
C/EBPβ	CCAAT/enhancer binding protein β
CSF	Colony stimulating factor
CTD	C-terminal domain
DMEM	Dulbecco's modified Eagle's medium
DRB	5,6-dichloro-1-β-D-ribofuranosyl benzimidasol, inhibitor of casein II-kinase
EMSA	Electrophoretic gel mobility shift assay
EPO	Erythropoietin
ER	Estrogen receptor
ERK	Extracellular regulated kinases
FERM	a band-four-point-one, ezrin, radixin, moesin
GAS	Interferon γ-activated site
GR	Glucocorticoid receptor
GST	Glutathione S-transferase
H7	1-(5-isoquinolinylsulfonyl)2-methylpiperazine, a common serine/threonine kinase inhibitor
HAT	Histone acetyl transferases
I4R	insulin IL-4 receptor motif
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4R	IL-4 receptor
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
ISG15	Interferon-stimulated gene 15
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-α stimulated response element
JAK	Janus kinase
JNK	c-Jun NH2-terminal kinase
kDa	Kilodalton
MAPK	Mitogen activated protein kinase
MEK	MAP kinase/ERK kinase, see MKK
MHC	Major histocompatibility complex
MKK	MAP kinase kinase, see MEK
N	Amino, NH2
n.d.	Not determined
NcoA-1	Nuclear coactivator 1
NES	Nuclear export elements
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal

NMI	N-myc interacting protein
p38AF	p38 MAPKα where Thr-180 and Tyr-182 have been replaced with Ala-180 and Phe-182, inactive/dominant negative p38 MAPKα
p38wt	Wild type p38 MAPKα
PAGE	Polyacrylamide gel electrophoresis
PD098059	2′-Amino-3′-methoxyflavone, a MEK/ERK inhibitor
PD1693116	4-(4-Fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole, a p38 MAPK/JNK inhibitor
PDGF	Platelet-derived growth factor
PEST	Proline, glutamic acid, serine and threonine rich region
PI-3-K	Phosphoinositide-3-kinase
PIAS	Protein inhibitor of activated STAT1
PK	Protein kinase
PMSF	Phenylmethylsulfonyl fluoride
POZ	Poxvirus and zinc finger
PP2A	Protein phosphatase 2A
RE	Response element
SB202190	4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI, a p38 MAPK inhibitor
SB203580	4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole, a p38 MAPK inhibitor
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SH	Src-homology
SN	Staphylococcal nuclease
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
SPI	Serine protease inhibitor
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain
TAFs	TBP-associated factors
TBP	TATA-binding protein
Th	T helper
Th0	Naïve T cells
TNF	Tumor necrosis factor
TPO	Thrompotoietin
UTR	Untranslated region

4. INTRODUCTION

Cellular events, for example, proliferation and differentiation are orchestrated by signals provided both by direct contacts between cells and secreted intercellular molecules such as cytokines. Cytokines are commonly described as small, soluble glycoproteins, which are not able to penetrate through the cell membrane due to their hydrophilic nature. A cytokine binding to its receptor on the surface of a target cell leads to the activation of multiple intracellular molecules, which ultimately results in the control of cell behavior. New cytokines continue to be identified and the understanding of their intracellular signaling cascades is rapidly growing.

Interleukin (IL)-4 is a pleiotropic cytokine originally identified as a growth factor for B cells. Subsequent studies have demonstrated its role in the regulation of a variety of cell types. Most importantly, however, IL-4 has been shown to serve as a critical mediator of humoral immune responses executed by B and T helper 2 (Th2) type lymphocytes. IL-4 stimulation leads to the tyrosine phosphorylation of receptor associated Janus kinase (JAK) 1 and JAK3 followed by the activation of transcription factor Signal Transducer and Activator of Transcription (STAT) 6. STAT6 functions as an indispensable mediator of IL-4-induced gene responses. Both IL-4 and STAT6 knockout mice share a phenotype that is characterized by impaired Th2 differentiation and lack of immunoglobulin E (IgE).

While the immediate signaling events that lead to the activation and DNA binding of STAT6 are well established (Nelms et al. 1999), relatively little is known about the molecular mechanisms of STAT6-mediated transcription and the signaling pathways that regulate STAT6 activity. Because STAT6 has a crucial impact in the regulation of Th2 type immunity and IgE production, further characterization of IL-4/STAT6 signaling events is of broad interest, not least due to the potential role of STAT6 as a target for future asthma and allergy medicines. This study was set up to investigate both the significance of serine/threonine kinase activity in the regulation of STAT6-mediated transcription and to characterize cofactors for STAT6 in Igε promoter activation.

5. REVIEW OF THE LITERATURE

5.1 General features of cytokine signaling

5.1.1 Hematopoietic cytokines

Soluble regulatory proteins can be roughly subdivided into systemically acting hormones, growth factors and cytokines. Cytokines are a large and diverse group of small secreted glycoproteins that predominantly function locally either in a paracrine or autocrine fashion. It is noteworthy that by definition the term cytokine refers to all humoral factors regulating cellular functions (Silvennoinen and Ihle 1996). In this study the word "cytokine" is used to refer to factors that regulate hematopoietic cells through their cognate transmembrane hematopoietic cytokine receptor (i.e. hematopoietic cytokines).

Cytokines are classified based either on their biological or structural properties, or more recently also by the characteristics of their receptor molecules (Ihle et al. 1995). One of the most common divisions is made on the basis of the properties that a cytokine has in inflammatory processes. Th1-type cytokines, such as IFN-γ and IL-12 promote cell-mediated immunity, while Th2-type cytokines IL-4 and IL-10 direct immune response towards antibody production thus provoking humoral immunity (O'Garra 1998, Lappin and Campbell 2000). Traditional classification into class I (including e.g. IL-2, -3, -4, -5, -6, -7, -8, -11, -12, -13, -15, -21, -23, EPO and thrompotoietin (TPO)) and class II (e.g. IFNs and IL-10) cytokines on the other hand separates the two cytokine superfamilies according to the number of α-helices in the structure; class I cytokines contain four and class II six α-helices (Ealick et al. 1991, Rozwarski et al. 1994). In addition, despite the fact that helical cytokines generally function as monomers, class II cytokines IFN-γ and IL-10 have also been shown to function as dimers (Walter et al. 1995, Josephson et al. 2001).

The proliferation, differentiation and functioning of mature blood cells are tightly regulated by hematopoietic cytokines. Due to their biological importance, a number of cytokines (e.g. erythropoietin (EPO) and interferon (IFN)-α) are currently being used or tested as therapeutic agents. Conversely, the maintenance of an adequate balance between cytokines is lost in many diseases. For example, accumulating data in pre-clinical studies demonstrate that the sustained expression of the central cytokine of this study, IL-4, on the targeted organs or tissues may provide

an effective means of therapy for a variety of diseases including cancers (e.g. melanoma) and immunologic disorders (e.g. autoimmune encephalomyelitis) (Ohira et al. 1994, Shaw et al. 1997, Okada and Kuwashima 2002).

5.1.2 Hematopoietic cytokine receptors

Hematopoietic cytokine receptors are transmembrane glycoproteins, composed of an extracellular ligand binding domain, a hydrophobic transmembrane part and a cytoplasmic intracellular domain (Silvennoinen et al. 1997). The superfamily of cytokine receptors fall into five subcategories (single-chain receptors, common β -chain family, gp130 family, common γ -chain family and IFN receptors) defined either by the number of subunits and/or the utilization of shared subunits (Ihle et al. 1995). Most cytokines can exert their function on a variety of cell types, and biological responses to cytokine stimuli can also vary depending on both the cell type and the stage of cell differentiation. Conversely, different cytokines can have similar functions on a particular target cell. At least part of this redundancy and versatility of cytokines can be explained by the use of shared subunits between cytokine receptor family members.

Unlike the receptors of the protein tyrosine kinase family that are used by hematopoietic growth factors, including stem cell factor (SCF) and colony stimulating factor (CSF)-1, the cytoplasmic domains of cytokine receptors do not have any intrinsic catalytic activity (Ihle et al. 1995). However, early studies in the 1980s demonstrated the importance of tyrosine kinase activity in cytokine responses thereby suggesting that cytokine binding to its receptor is able to activate intracellular signaling through receptor associated tyrosine kinases.

5.1.3 The JAK/STAT pathway

The quest for cytokine receptor associated kinases led to the identification of the Janus kinases (JAKs, alternatively also “Just Another Kinases”). Upon cytokine stimulus, receptors undergo conformational changes that bring JAKs into proximity with each other, which enables their activation through tyrosine transphosphorylation (Silvennoinen and Ihle 1996). To date, four mammalian members of the JAK family have been cloned (JAK1, JAK2, JAK3 and TYK2) and as the draft sequence of the human genome has now been completed the number is not expected to increase (O'Shea et al. 2002). JAKs are relatively large kinases (110-140 kDa) that are ubiquitously expressed with the exception of JAK3, the expression of which is restricted mainly to hematopoietic

cells (Park et al. 1995, Leonard and O'Shea 1998). Studies with gene-targeted mice and humans deficient in JAK3 have attested the non-redundant *in vivo* function of JAK family members (Notarangelo et al. 2001). The results from JAK knockout experiments are also in line with the reported functions of different cytokine receptor chains, thus confirming the essential role of JAKs in cytokine signaling.

Based on sequence conservation, JAK kinases can be divided into seven JAK homology (JH) domains. The most characteristic feature of JAK kinases is their unique tandem kinase structure composed of a tyrosine kinase domain (JH1) and a pseudokinase domain (JH2, alternatively termed also kinase-like domain) (Hubbard and Till 2000, Kisseleva et al. 2002). The JH1 domain possesses all the typical features of tyrosine kinase domains and it is catalytically active (Briscoe et al. 1996, Saharinen et al. 2000, Saharinen and Silvennoinen 2002). The pseudokinase region has sequence similarity with kinase domains, but many residues that are required for phosphotransferase activity are altered, and consequently the JH2 domain is catalytically inactive (Frank et al. 1995, Velazquez et al. 1995). Recently, the pseudokinase domain has been implicated in the autoregulation of JAK kinase activity (Chen et al. 2000, Saharinen et al. 2000, Yeh et al. 2000, Saharinen and Silvennoinen 2002). Amino (N) terminal JH3-JH7 domains have been suggested to mediate the association between JAKs and cytokine receptor chains through the band-four-point-one, ezrin, radixin, moesin (FERM) domain (Kisseleva et al. 2002).

In addition to ligand-induced autophosphorylation, JAKs also phosphorylate intracellular tyrosine residues on cytokine receptors, thus creating docking sites for proteins that contain the SH2 domain, such as STAT transcription factors. Attached STATs also become tyrosine phosphorylated by JAK kinases, which results in the homodimerization of STATs. Once activated, dimerized and tyrosine phosphorylated, STATs rapidly translocate to the nucleus where they bind to the promoters of cytokine responsive genes (Aaronson and Horvath 2002). The DNA bound STATs have intrinsic ability to recruit nuclear co-activators and subsequently induce the transcription of target genes. A schematic representation of the JAK/STAT pathway is shown in Figure 1. The structure, function and biology of STATs are reviewed in more detail in the following chapters.

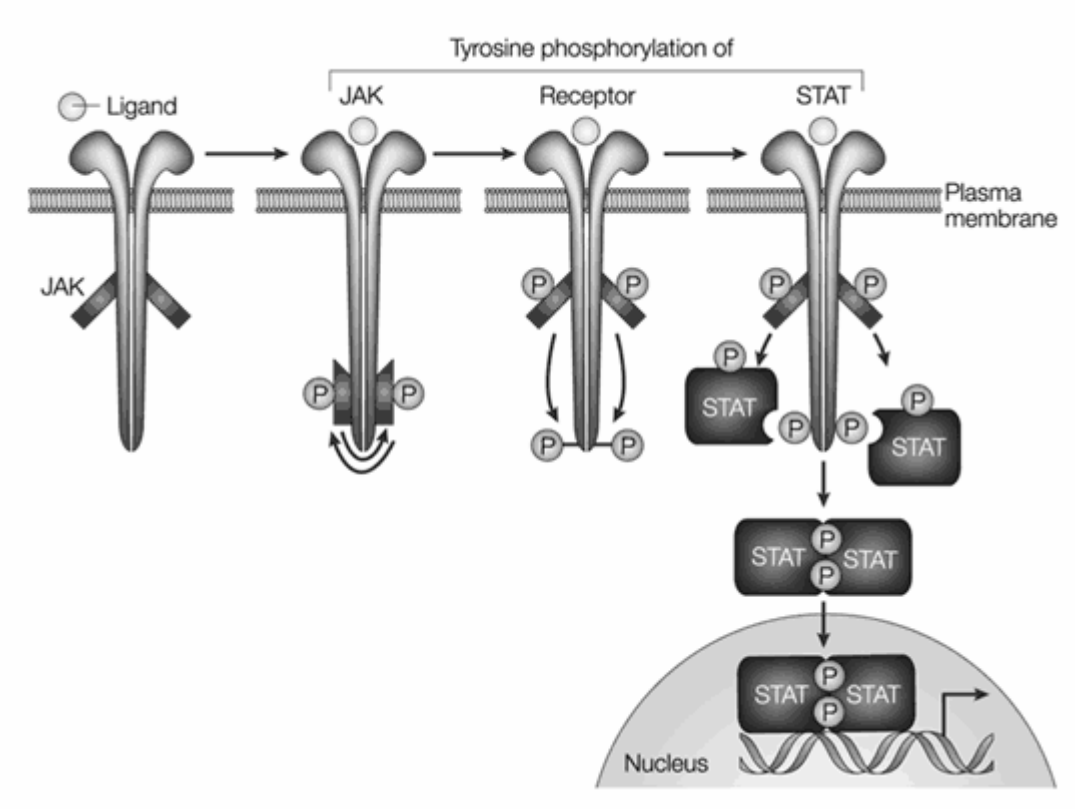

Figure 1. Schematic representation of the JAK/STAT pathway. Reprinted from (Levy and Darnell 2002), with permission from Nature Publishing Group, Copyright (2002).

5.2 STATs

The STAT family of transcription factors was initially recognized as ligand induced transcription factors in IFN-treated cells. In human, STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) are encoded by seven genes mapped in chromosomes 2, 12 and 17 (Darnell 1997). Although both the chromosomal distribution and studies with more primitive eukaryotes suggest that evolutionally the STAT family arises from a single gene, their tissue distribution and amino acid sequences are sufficiently unique to provide diversity in their responses to extracellular stimuli (Aaronson and Horvath 2002, Kisseleva et al. 2002).

5.2.1 Structure of STAT proteins

STATs 1, 3, 4, 5A and 5B consist of 750 to 795 amino acids, while STATs 2 and 6 are larger, approximately 850 amino acids in size (Darnell 1997). Through structural and functional analysis it has become evident that all seven STAT family members share six conserved domains: N-terminal domain, coiled-coil domain, DNA binding domain, linker domain, Src homology (SH) 2-domain and carboxy (C) terminal transactivation domain (TAD). The schematic domain structure and a tertiary model of a DNA bound STAT dimer are presented in Figure 2.

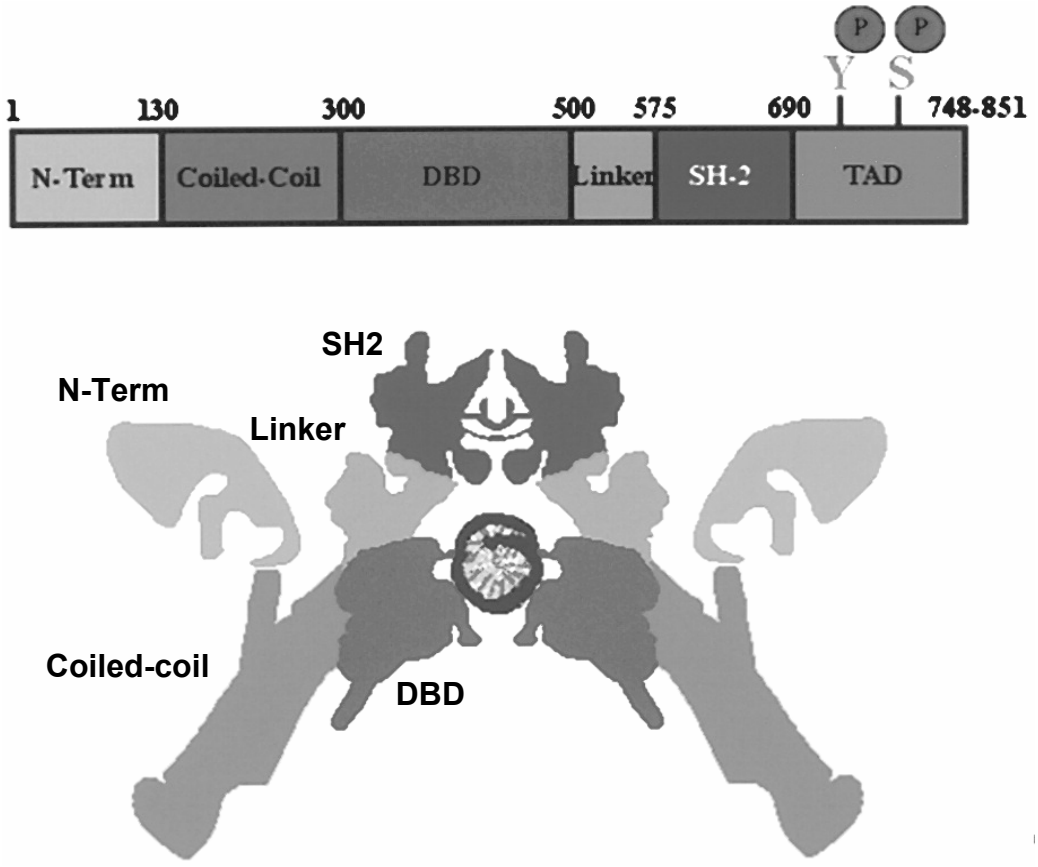

Figure 2. The domain structure of STATs. Schematic representation (upper figure) and the structure of DNA bound STAT dimer (lower figure). Reprinted from (O'Shea et al. 2002), with permission from Elsevier, Copyright (2002).

In the promoter regions of several cytokine inducible genes, two STAT-binding sites are located about 20 base pairs from each other, and these can be occupied by tetramerized STAT dimers. The 130 conserved amino terminal residues have been implicated in the tetramerization of STATs and consequently they further stabilize the STAT-DNA interactions (Vinkemeier et al. 1998, Kisseleva et al. 2002). The N-terminal part also has a role in receptor recognition and in the regulation of the nuclear transport and dephosphorylation of STATs (Strehlow and Schindler 1998) The coiled-coil domain is located adjacent to the N-terminal domain (amino acids ~135 to ~315) and it consist of four α-helices forming a large, principally hydrophilic surface, which mediates the interactions with other helical proteins such as N-myc interacting protein (NMI) and interferon regulatory factor (IRF) 9 (Zhang et al. 2000, Kisseleva et al. 2002). Recently, the coiled-coil domain has also been demonstrated to take part in the nuclear export of STATs (Schindler 2002).

The STAT DNA binding domain positioned carboxyterminal to the coiled-coil domain (residues ~320 to 490) contains several β-sheets that are folded similarly to the DNA binding domains of

nuclear factor κb (NF-κB) and p53 (Becker et al. 1998, Chen et al. 1998). The adjacent linker domain (amino acids ~490 to ~580) connects the DNA binding domain with the SH2 domain and it has also been implicated in the nuclear export (Schindler 2002). The SH2 domain recruits STATs to tyrosine phosphorylated receptor units and is also involved both in the STAT association with activating JAK kinase and STAT dimerization (Greenlund et al. 1994, Greenlund et al. 1995, Gupta et al. 1996). SH2 domain (amino acids ~580 to ~680), the most highly conserved region of STATs, is a pocket-like structure of antiparallel β-sheets flanked by two α-helices. A conserved arginine residue that mediates interactions with phosphate is positioned at the base of this pocket (Kisseleva et al. 2002).

With the exception of STAT2, the phosphorylation of single C-terminal tyrosine residue that is located between SH2 and transactivation domains (amino acid ~700) has been shown to mediate the homodimerization of STATs (Levy and Darnell 2002). The phosphorylated tyrosine residue associates with the SH2 domain of reciprocal STAT resulting in dimer formation. The tertiary structure of transactivation domain (TAD), the most carboxyterminal part of STAT proteins, has remained elusive but it is known to vary considerably both in length (38 to 200 amino acids) and sequence between family members (Schindler 2002). The TAD is indispensable in the activation of transcription, and C-terminally truncated isoforms identified for STATs 1, 3, 4, 5 and 6 appear to function as dominant-negative regulators (Kisseleva et al. 2002).

5.2.2 Nuclear transport of STATs

In resting cells, STATs appear as latent cytoplasmic monomers, but cytokine stimulus enforces the rapid nuclear accumulation of phosphorylated STAT dimers. Because STAT dimer is a relatively large protein complex, an active energy-dependent mechanism is required for its nuclear import. An undisputed, autonomously functioning nuclear localization signal (NLS) element that would be conserved among STAT family members remains unidentified, and the understanding of the structural and functional characteristics of the nuclear localization of STATs continues to evolve (O'Shea et al. 2002). The nuclear import of STAT1 has been shown to be dependent on tyrosine phosphorylation, and the point mutations in the DNA binding domain abolish the critical association with a subunit of nucleocytoplasmic transport machinery, importin-α5 (Fagerlund et al. 2002, Levy and Darnell 2002, McBride et al. 2002). However, the discrepancy of the field is demonstrated by a recent study with human cancer cells in which the nuclearilization of STAT1

was shown to occur independently of tyrosine phosphorylation and also to be unaffected by mutations in the DNA binding domain (Meyer et al. 2002).

Control for the duration of transcriptional responses is provided by the nuclear dephosphorylation of the tyrosine residues of STAT dimers. Dephosphorylated STAT proteins are exported from the nucleus by a mechanism that is dependent on the presence of different nuclear export elements (NES) (McBride et al. 2000, Mowen et al. 2001, Kisseleva et al. 2002). Currently, it has become evident that STATs are constantly cycled between the cytoplasm and the nucleus (phosphorylation, nuclear localization, dephosphorylation, nuclear export, re-phosphorylation and re-import); in fibroblast, the cycle time for an individual STAT molecule is approximately 20 minutes (Levy and Darnell 2002).

5.2.3 STAT DNA binding

As discussed above, in most cases, cytokine stimulus results in the activation and homodimerization of STAT proteins. On some occasions, heteromeres between STATs 1 and 3, and 5A and 5B are also known to be formed (Horvath 2000). Activated STAT dimers recognize a palindromic motif defined as (Interferon) γ activated site (GAS) with a consensus sequence of 5'-TT(N_{4-6})AA-3'. The most preferable spacing between AA and TT residues varies between STAT family members. STAT1 binds the DNA element with five spacing nucleotides, whereas STAT3 and STAT6 favor elements where n=4 and n=6 respectively (Mikita et al. 1996, Kisseleva et al. 2002). Though the structure of full length STAT remains yet to be crystallized, studies with the DNA bound STAT1 and 3 molecules have suggested a nutcracker-like structure to be established between dimerized STATs and DNA (Becker et al. 1998, Chen et al. 1998, O'Shea et al. 2002). A schematic structure of the DNA bound STAT dimer is represented in Figure 2.

Notably, STAT2 does not bind DNA as a homodimer, but it functions as a component of the multimeric IFNα-induced IFN-stimulated gene factor 3 (ISGF3) complex (Bluyssen et al. 1996). The ISGF3 complex consisting of STAT1, STAT2 and IRF9 transcription factors recognizes a unique IFNα stimulated response element (ISRE, consensus sequence 5'-AGTTN_3TTTCC-3') but it fails in binding to GAS elements (Horvath 2000).

5.2.4 Modulation of STAT-mediated transcription

The rate and specificity of STAT-mediated transcription is regulated by diverse mechanisms, including posttranslational modifications of STAT proteins and interactions between STATs and other cellular proteins and nuclear regulators. Several cytoplasmic tyrosine phosphatases (e.g. SHP1, SHP2, PTP1B) dephosphorylate cytokine receptors and activating kinases, thus controlling STAT activity (O'Shea et al. 2002). The suppressor of cytokine signaling (SOCS, alternatively termed also as JAK-binding protein, JAB or STAT-induced STAT-inhibitor, SSI) family proteins were originally identified as cytokine-induced negative regulators of cytokine signaling (Endo et al. 1997, Naka et al. 1997). SOCSs are generally thought to directly interact and inhibit activated JAKs, but they can also cause the turnover of cytokine receptors through ubiquitin-proteasome–mediated processes (Kovanen and Leonard 1999, Levy and Darnell 2002).

STATs have been shown to interact with a wide variety of other factors that include proteins with chromatin modifying activity, transcriptional co-activators/repressors and other DNA-binding transcription factors. Predominantly, the interaction takes place in the C-terminal TAD of STATs, but some interactions between the cofactors and different STAT domains have also been described (e.g. N-terminal domain interacts with Protein Inhibitor of Activated STAT1 (PIAS) -family proteins) (Shuai 2000). The biological impact of interactions largely depends on the nature of the interacting protein. For example, well-established interaction between STATs and histone acetyl transferases (HATs), such as CREB-binding protein/p300 (CBP/p300), promotes STAT-mediated transcription by providing a functional connection between STATs and basal transcription machinery (Korzus et al. 1998). On the other hand, the physical interaction with PIAS inhibits STAT DNA-binding activity, thus resulting in the inhibition of STAT-mediated gene activation (Shuai 2000).

Posttranslational modifications in the STAT structure provide another important regulative mechanism for STAT activity. The requirement of tyrosine phosphorylation was discussed above, and the following chapter (5.2.4.1) concentrates on the serine phosphorylation of STATs. In contrast to phosphorylation events, rather sparse information exists about the role of other posttranslational modifications of STAT proteins. Arginine methylation of STAT1 N-terminus promotes transcription by preventing contact with inhibitory proteins that inhibit STATs, (Mowen et al. 2001) and STAT6 has been shown to undergo acetylation by associated coactivator proteins resulting in enhancement in DNA binding (McDonald and Reich 1999, Shankaranarayanan et al.

2001). The known characteristics of the transactivation via STAT6 are reviewed more thoroughly in chapter 5.3.

5.2.4.1 Serine phosphorylation of STATs

As previously discussed, tyrosine phosphorylation is required for the initial activation of STATs. In addition to tyrosine phosphorylation, all other STATs but STAT2 have been shown to be phosphorylated also in their C-terminal serine residues (Decker and Kovarik 2000, Wick and Berton 2000, Visconti et al. 2000). The phosphorylated serine residues, the stimuli provoking STAT serine phosphorylation and the pathways that have been reported to be involved are summarized in Table I. The best-characterized target of serine kinase activity in the STAT structure has been shown to be the conserved P(M)SP motif found in the TADs of STAT 1, 3, 4, 5A and 5B (Decker and Kovarik 2000). The P(M)SP motif is generally positioned between amino acids 720 and 730, but in STAT5A it formed around Ser780 (Ramsauer et al. 2002). However, it is apparent that other less studied serine residues in STATs are also phosphorylated both constantly and upon cytokine stimulus.

The biological outcome of STAT serine phosphorylation is largely dependent on the cell-type as well as the promoter context and the way of induction. STAT1 serine phosphorylation has been shown to promote STAT1-mediated transcription by recruiting positive transcriptional cofactors to STAT1TAD (Zhang et al. 1998, Ouchi et al. 2000). Furthermore, the serine phosphorylation of STAT1 has also a role in the specificity of STAT1-mediated gene responses (Kovarik et al. 2001). In the case of STAT3, serine phosphorylation and serine kinase activity in general have been reported to have both positive and negative effects on STAT3-mediated gene activation (Decker and Kovarik 2000). While the mutation of Ser727 into alanine represses STAT3-mediated transcription about 50 %, the serine phosphorylation of STAT3 has also been shown to downregulate the tyrosine phosphorylation and thus the DNA binding activity of STAT3 (Wen et al. 1995, Chung et al. 1997). IL-12-induced STAT4-mediated transcription is also enhanced upon the phosphorylation of Ser721 in STAT4TAD (Visconti et al. 2000), while the IL-2-induced phosphorylation of Ser780 in STAT5A does not have effect on STAT5A-dependent gene activation (Xue et al. 2002). The serine phosphorylation of STAT6 is discussed in the following chapters.

A plethora of serine kinases activated by either environmental agents (e.g. ultraviolet light) or soluble mediators (e.g. cytokines) have been implicated in the induction of the serine

phosphorylation of STATs (Table I). The C-terminal P(M)SP motif in the STAT structure confers the consensus sequence for mitogen activated protein kinase (MAPK) phosphorylation site, and thereby the roles of MAPK subgroups, extracellular regulated kinases (ERK), c-Jun NH2-terminal kinases (JNK) and p38 MAPKs, have been extensively investigated (Decker and Kovarik 2000). Activated by a variety of stimuli, MAPKs are attested to directly induce the C-terminal serine phosphorylation of STATs 1, 3, 4 and 5 with varying biological implications. For example, the growth factor-induced and ERK-mediated phosphorylation of Ser727 in STAT3 inhibits its tyrosine phosphorylation, thus consequently inhibiting STAT3-dependent gene activation, whereas IL-6/STAT3-mediated transcription is promoted by p38 MAPK activity (Chung et al. 1997, Zauberman et al. 1999). The complexity of this field is also demonstrated by studies of IFNγ/p38 MAPK/STAT1. Although p38 MAPK has been shown to mediate the cellular stress-induced phosphorylation of Ser727 in STAT1, IFNγ-induced Ser727 phosphorylation was found to occur independently of the p38 MAPK pathway (Kovarik et al. 1999). In addition, p38 MAPK has been shown to enhance IFNγ/STAT1-dependent transcription independently of Ser727 phosphorylation (Ramsauer et al. 2002). The biological importance of STAT serine phosphorylation is clearly attested by findings from cancer research. The constitutive phosphorylation of Ser727 in STAT3 has been linked to cellular growth control in v-Src transformed cells and in B cell derived tumors (Frank et al. 1997, Bromberg et al. 1998).

STAT PROTEIN	PHOSPHORYLATED SERINE	STIMULI PROMOTING SERINE PHOSPHORYLATION	PATHWAYS REPORTED TO BE INVOLVED
STAT1	Ser 727	IFN-γ, IFN-α, PDGF, lipopolysaccharide, combination of IL-2 and -12, ultraviolet-light, TNF-α, B cell receptor	JAK2-dependent, H7-sensitive, p38 MAPK-dependent
STAT3	Ser 727 and n.d.	Epidermal growth factor, insulin, steel factor, granulocyte-colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-2, T cell receptor, IL-6, oncostatin M, leukemia-inhibitory factor, combination of IL-2 and –12, complement C5a, TNF-α, ansiomycin, sodium arsenide, hyperosmolarity, v-Src transformation	MEK/ERK-dependent, PKCδ-dependent, p38 MAPK-dependent, JNK-dependent, SEK1-, Rac1- and Vav-dependent
STAT4	Ser 721 and n.d.	IL-12, IFN-α	p38 MAPK-dependent
STAT5A	Ser 725, Ser 780	Unregulated event, IL-2	MEK/ERK-dependent
STAT5B	Ser 730	Prolactin	-
STAT6	n.d.	IL-4	PP2A–sensitive

Table I Serine phosphorylation of STATs, modified from (Decker and Kovarik 2000).

5.2.5 STAT functions *in vivo*

Due to their cellular function as transcription factors, STATs were prone to have profound functions in the regulation of biological events. Hitherto, gene-targeted removal of all seven STAT proteins has been accomplished in mice (phenotypes of nullizygous mice are summarized in Table II) (O'Shea et al. 2002). All STAT-deficient mice with the exception of $STAT3^{-/-}$ have a phenotype bearing distinct defects mainly due to impaired responses to growth factor/cytokine stimuli. The embryonic lethality of STAT3 knockout mice implicating its crucial role in development has been

circumvented by Cre-*lox*P-mediated removal of STAT3 in individual tissues. Recent studies on tissue specific STAT3-deficient mice have revealed its diverse and to some extent contradictory roles in biological processes (Levy and Lee 2002). For example, while the neurons of $STAT3^{-/-}$ mice suffer from impaired cell survival, the mammary epithelium was found to undergo defective apoptosis.

Another way of obtaining information about STAT biology has emerged from cancer research. A wide variety of different tumors, including hematopoietic, head and neck, breast, lung, kidney, prostate and ovarian malignancies, are shown to express activated STAT molecules, mainly STATs 3 and 5 (Bowman et al. 2000). For example, the abrogation of the STAT3 function leads to increased apoptosis and specific downmodulation of Bcl-X_L in head and neck tumors (Song and Grandis 2000). Conversely, on the basis of its growth inhibitory function, STAT1 has been speculated to attenuate uncontrolled cell proliferation (Levy and Gilliland 2000, O'Shea et al. 2002). Yet another proof of the importance of STATs in human biology is demonstrated by findings showing that mutations in the STAT1 germline gene result in hampered immunity against mycobacterial infection (Dupuis et al. 2001).

STAT PROTEIN	PHENOTYPE OF NULLIZYGOUS MICE
STAT1	Impaired responses to interferons, increased susceptibility to tumors, impaired growth control
STAT2	Impaired responses to interferons, reduced STAT1 expression in some tissues
STAT3	Embryonic lethality, conditional knockout: multiple defects in adult tissues including changes in cell survival (both positive and negative), impaired response to pathogens
STAT4	Impaired Th1 differentiation owing to loss of IL-12 responses, increased susceptibility to intracellular pathogens
STAT5A	Impaired mammary gland development owing to loss of prolactin responses
STAT5B	Impaired growth owing to loss of growth hormone responsiveness
STAT6	Impaired Th2 differentiation and lack of IgE owing to loss of IL-4 responsiveness, increased susceptibility to helminthic infestation

Table II Phenotypes of STAT-deficient mice. Modified from (Levy and Darnell 2002) and (O'Shea et al. 2002).

5.3 IL-4/STAT6-mediated gene activation

5.3.1 Interleukin-4

IL-4 is a pleiotropic cytokine predominantly produced by mast cells, T helper 2 (Th2) cells and basophils. IL-4 is implicated in the regulation of the growth and function of various cell types, including B and T lymphocytes, eosinophils, keratinocytes, epithelial cells, hepatocytes and myeloid cells (Paul 1991). The IL-4 gene that encodes cDNA for a glycoprotein of 129 amino acids is mapped in the human cytokine cluster found in chromosome 5q23-31 (Coffman et al. 1986). In the hematopoietic cell compartment, IL-4 functions as a key regulator of humoral immune responses executed by Th2 and B lymphocytes.

In contrast to its original and well-established role as a B cell growth factor, IL-4 has been shown to promote apoptosis in pre-B leukemia cells (Howard et al. 1982, Farrar et al. 1983, Manabe et al. 1994). IL-4 stimulates the transcription of the unrearranged immunoglobulin heavy-chain germline Igε and Igγ4 genes, which ultimately leads to the secretion of immunoglobulin subclasses G_4 and E by terminally differentiated human plasma cells (Warren and Berton 1995). The B cell differentiation stage thus serves as an important determinant for the biological outcome of IL-4 treatment. Notably, IL-4 stimulus alone is not sufficient to drive the deletional switch recombination of immunoglobulin, but a second signal must also be provided to B cells by surface antigen CD40 engagement (Oettgen 2000). In B cells, IL-4 also induces the expression of other immunomodulatory genes, such as major histocompatibility complex class II (MHC II) and the low affinity receptor for IgE (FcεRII or CD23) (Rousset et al. 1988, Glimcher and Kara 1992).

The fate of antigen-activated naïve T cells (Th0) is dictated by the surrounding cytokine milieu. In T cells, IL-4 is generally considered a hallmark cytokine for humoral immunity due to its potential of skewing T helper cells into Th2 direction. IL-4 is capable of upregulating its own production and it acts as an autocrine growth factor for Th2 cells. Simultaneously, IL-4 suppresses the IFNγ-driven Th1 polarization and thereby the events of cell-mediated immunity (Rengarajan et al. 2000). IL-4 also directly inhibits the monocytic expression of proinflammatory cytokines like IL-1, IL-6 and tumor necrosis factor (TNF) (Silvennoinen and Ihle 1996). Recently, much work has focused on defining the mechanistic background of Th cell polarization resulting in the identification of novel IL-4 target genes in Th2 cells (Ho and Glimcher 2002).

IL-4 deficient mice have normal B and T cell development but greatly diminished levels of IgG_1, and they completely lack IgE (Kuhn et al. 1991). Some of the biological functions of IL-4-deficiency can be substituted by a functionally related cytokine IL-13. The IL-13 gene is located within 50 kb of the IL-4 gene and similarities in the intron-exon structure suggest evolution from a common ancestral gene by gene duplication (Morgan et al. 1992, Minty et al. 1993, Silvennoinen and Ihle 1996). Although IL-4 and IL–13 share only 25 % homology in primary structure, their biological properties are largely overlapping mainly due to a shared receptor subunit (IL-4 receptor α chain, discussed below). However, differences both in the kinetics of cytokine secretion as well as in the regulation of immune responses against parasitic infections have been reported, and generally the biological effects of IL-13 are more restricted than those of IL-4 (McKenzie et al.

1998). Emerging evidence is also suggesting that IL-13 has a specific, IL-4-independent role in the pathogenesis of bronchial asthma (Grunig et al. 1998, Wills-Karp et al. 1998).

5.3.2 The IL-4 receptor complex

The receptors for IL-4 are ubiquitously expressed on a wide variety of cell types (Nelms et al. 1999). One cell can bear a widely ranging IL-4 receptor (IL-4R) copy number (from 100 to 5000 receptors per cell), which thus serves as a determinant for IL-4-inducibility. The IL-4 receptor complex is predominantly composed of the ligand-specific IL-4 receptor α-chain (IL-4Rα) and the common γ -chain (γc). Alternatively IL-4Rα forms heterodimers with the IL-13 receptor (IL-13Rα and IL-13Rα' chains), and in some circumstances two copies of IL-4Rα can also homodimerize (Fujiwara et al. 1997, Nelms et al. 1999). Because of the usage of a common subunit shared by the receptors for interleukins 2, 4, 7, 9, 15 and 21, the IL-4 receptor is logically categorized into the γc-family of cytokine receptors. IL-13 does not utilize the γc chain, but the redundant functions of IL-4 and IL-13 cytokines are largely explained by IL-4Rα-IL-13Rα or IL-4Rα-IL-13Rα' receptor heterodimers, which can activate cellular signaling in response to both of the cytokines (Wurster et al. 2000, Kawakami et al. 2001).

The IL-4Rα gene in human encodes a 140-kDa glycoprotein with typical features (e.g. WSXWS motif in the extracellular portion and the Box1 and Box2 motifs in the intracellular domain) of a cytokine receptor (Idzerda et al. 1990, Silvennoinen and Ihle 1996). The cytoplasmic domain of IL-4Rα contains five highly conserved tyrosine residues with an important function in IL-4-induced cellular signaling. The most membrane proximal Tyr497 mediates proliferative signals while Tyr575, Tyr603 and Tyr631 are important for STAT6 activation and C-terminal Tyr713 serves as a docking site for phosphatases (Nelms et al. 1999). Interestingly, mutations in IL-4Rα have been reported to have clinical significance. Ile50Val substitution in the IL-4Rα extracellular motif has been linked to increased IgE production and atopic diseases (Mitsuyasu et al. 1998, Mitsuyasu et al. 1999), and a polymorphism Gln576Arg in the intracellular core STAT6 binding region has been found in patients with hyper-IgE syndrome and atopic dermatitis (Hershey et al. 1997).

Similarly to IL-4Rα, the extracellular part of γc also shows typical characteristics of a cytokine receptor. The γc gene encoding a 64-kDa glycoprotein is widely expressed in hematopoietic cells but generally absent in other cell types. The critical role of γc in the development of lymphocytes is

well illustrated by individuals suffering from severe combined immunodeficiency (SCID) on the basis of functionally mutated γc (Noguchi et al. 1993). Two different human IL-13 receptor α chains (IL-13Rα and IL-13Rα' or alternatively termed IL-13Rα1 and IL-13α2) have been cloned (Aman et al. 1996, Caput et al. 1996). Both components are 55–70 kDa in size and they bind IL-13 with different affinities. IL-13Rα initially binds IL-13 with the subsequent recruitment of IL-4Rα to efficiently transduce signal. IL-13Rα' can bind IL-13 in the absence of IL-4Rα, but its role in IL-13 signaling is still unclear. Importantly, IL-13Rα' is reported to serve as a component of the functional IL-4 receptor complex in γc-deficient, non-hematopoietic cells (Murata et al. 1998).

5.3.3 Immediate signaling events upon IL-4 stimulus

IL-4 stimulus results in the dimerization of the IL-4 receptor chains. Characteristically for cytokine receptors, none of the components of the IL-4R complex has intrinsic kinase activity; rather the activation of associated tyrosine kinases is required for the initiation of signaling events. All the members of JAK kinase family have been shown to be activated upon IL-4 stimulus. JAK1 and (in certain cell lines) JAK2 are shown to be associated with the IL-4Rα chain, while JAK3 is exclusively bound to γc (Murata et al. 1996, Nelms et al. 1999). When IL-4 signaling occurs through IL-4Rα-IL-13Rα complex, Tyk2 kinase can also be activated as an IL-13Rα associated JAK family member (Orchansky et al. 1999). Bound to dimerized receptor chains, JAKs get rapidly activated and transphosphorylated followed by the phosphorylation of critical tyrosine residues in the cytoplasmic domains of the IL-4 receptor complex. The phosphorylated tyrosine residues can thereafter bind adapter proteins such as insulin receptor substrates (IRSs) or transcription factors such as STAT6 (discussed below).

The recruitment and activation of IRS proteins is dependent on the Insulin IL-4 Receptor (I4R) motif (amino acids 437 and 557) present in the membrane proximal cytoplasmic region of IL-4Rα. To date, four IRS proteins (IRS-1-4) with at least partly redundant functions have been cloned (Giovannone et al. 2000). IRS proteins are large adapter proteins (approximately 170 kDa) that become tyrosine phosphorylated by JAKs on multiple (approximately 20) tyrosine residues upon cytokine treatment (Nelms et al. 1999). The phosphotyrosines on IRS proteins serve as docking sites for SH2 domain containing signaling molecules, including the regulatory subunit of phosphoinositide-3-kinase (PI-3-K) and a Ras/MAPK pathway adaptor molecule Grb-2 (Nelms et al. 1999). IRS downstream signaling via activated PI-3-K and its substrate Akt/protein kinase B is

implicated in the regulation of IL-4-mediated cell growth and the prevention of apoptosis (Wang et al. 1993, Franke et al. 1997). PI-3-K also phosphorylates serine residues on IRS proteins providing a negative feedback loop via diminished I4R-IRS-interaction. In contrast to the insulin activated phosphorylation events of IRS proteins, IL-4 stimulus generally fails to activate the Grb-2 linked Ras/MAPK pathway (Satoh et al. 1991). However, some cell-type dependency exists and, for example in keratinocyte, IL-4-induced IL-6 secretion is dependent on the rapid activation of p38 MAPK (Wery-Zennaro et al. 2000). Furthermore, at least in certain cells (e.g. myeloid progenitor cell line 32D), the activation of cell growth regulation and Ras/MAPK pathway by IL-4 has been shown to occur independently of IRS-1/2 proteins by the recruitment and phosphorylation of another adaptor protein termed Shc (Nelms et al. 1999).

5.3.4 Structure of STAT6

STAT6 (previously also termed as IL-4 STAT, STF-IL-4 and IL-4 NAF) was originally identified as a GAS binding protein in extracts derived from IL-4-treated cell lines (Kotanides and Reich 1993, Hou et al. 1994, Schindler et al. 1994). In addition to IL-4, the functionally related cytokine IL-13 is able to trigger STAT6 (Lin et al. 1995). More unconventional stimuli that activate STAT6 include angiotensin II, platelet-derived growth factor (PDGF), leptin and surface Ig or CD40 crosslinkings (Wurster et al. 2000). The biological significance of STAT6 activation in these alternative pathways remains ambiguous.

The STAT6 gene is mapped to the human 12q13-14.1 and murine 10 chromosomes and it encodes a single 4 kb transcript for a 94 kDa protein (Silvennoinen and Ihle 1996, Kriebel et al. 1999, Ihle 2001). STAT6 shares a 34 % structure homology with STAT5, but is less identical with other STAT family members (17-20 % homology). STAT6 consists of the typical functional domains of STAT family members described in chapter 5.2.1. The functionally indispensable tyrosine phosphorylation upon IL-4/IL-13 stimulus occurs at Tyr641 positioned adjacent to the SH2 domain (Mikita et al. 1996). However, the substitution of two residues at positions 547 (Val) and 548 (Thr) in the SH2 domain by alanine has been reported to result in an IL-4-stimulus independent constitutively active STAT6 mutant, which suggests that small changes in the STAT6 gene could cause hyperactivation of the protein and thereby the constitutive expression of STAT6-dependent genes (Daniel et al. 2000). The most unique feature of the STAT6 structure is formed by 170 C-terminal amino acids that serve as an autonomous transactivation domain (Mikita et al. 1996). As the TADs of other family members, STAT6TAD is also rich in proline residues and it contains

eight serine residues that are positionally conserved between mouse and human species (Moriggl et al. 1997). However, compared to the TADs of STATs 1, 3, 4 and 5, STAT6TAD is considerably longer in size and it lacks the functionally important consensus phosphorylation site for MAPK kinases (Decker and Kovarik 2000, Horvath 2000). Two distinct portions of the STAT6 C-terminus (amino acids 661–715 and 753–810) are able to activate IL-4 responsive reporter constructs when fused as a duplicate with the C-terminally truncated STAT6 mutant (Goenka et al. 1999). Another proof of the IL-4-independency of STAT6TAD in the initiation of transcription came from experiments with conditionally active STAT6-estrogen receptor (ER) chimera. Estrogen treatment results in the dimerization and nuclear translocation of STAT6-ERs, which consequently results in the activation of transcription without IL-4 treatment or Tyr641 phosphorylation (Kamogawa et al. 1998).

In addition to the full-length STAT6 protein, naturally existing isoforms on the basis of alternate splicing of mRNA have also been described. STAT6b has a deletion at the N-terminus while STAT6c lacks a portion of SH2 domain, and both isoforms can attenuate IL-4 responses when overexpressed in cells (Patel et al. 1998). STAT6c was particularly reported to effectively reduce IL-4-driven mitogenesis and the immediate activation events of STAT6. Furthermore, in addition to conventional STAT6, mast cells co-express another C-terminally truncated 65-kDa STAT6 isoform due to selective proteolysis (Sherman et al. 1999, Suzuki et al. 2002). Lacking the whole TAD 65-kDa STAT6 also functions as a dominant negative regulator of STAT6-mediated transcription.

5.3.5 Modulation of STAT6-mediated transcription

Recently, the negative regulation of the immediate events of cytokine signaling via the JAK/STAT pathway has been intensively investigated. A negative feedback loop mechanism for IL-4-induced JAK1/STAT6 activation in both monocytes and non-hematopoietic cells has been proposed to take place by SOCS 1 and 3-mediated inhibition (Dickensheets et al. 1999, Losman et al. 1999, Haque et al. 2000). The magnitude of the inhibition of STAT6 by SOCSs is controlled both by the SOCS expression level that can be upregulated by the IL-4 counteracting cytokine IFNγ and Pim protein kinase activity (Chen et al. 2002). Furthermore, IL-4-induced STAT6 activation is also a target for protein phosphatase activity. Hematopoietically expressed Shp-1 downregulates the STAT6 phosphorylation and DNA binding, thereby eventually suppressing IL-4-induced gene responses (Haque et al. 1998).

When detached from the IL-4 receptor complex, tyrosine phosphorylated STAT6 dimer has been shown to undergo secondary post-translational modifications such as serine phosphorylation (Wick and Berton 2000, Woetmann et al. 2002) and acetylation (McDonald and Reich 1999, Shankaranarayanan et al. 2001). The acetylation of STAT6 in the nucleus was reported to occur as a consequence of the IL-4-induced acetyl transferace activity of CBP/p300. CBP/p300-mediated acetylation is required for STAT6 binding to reticulocyte-type 15-lipoxygenase (15-LOX-1) promoter, and thus acetylation has a role in the regulation of STAT6-mediated gene activation (Shankaranarayanan et al. 2001).

Collaboration between STAT6 and other nuclear transcription factors and transcriptional co-regulators provides an important regulative mechanism for STAT6-mediated transcription. STAT6 has been reported to cooperate with several different kinds of coregulators, including both acetyl transferases and a steroid receptor, and therefore the mechanisms and biological impacts of these interactions are divergent. In Igε promoter, a binding site for a constitutively active transcription factor C/EBPβ is positioned adjacent to the STAT6 core binding sequence (Mikita et al. 1996). Although there is no direct evidence about physical interaction between the transcription factors, functional synergism exists as C/EBPβ enhances STAT6-mediated transactivation by reducing the fast dissociation rate of the DNA-bound STAT6 (Mikita et al. 1998). Another Igε promoter binding transcription factor NF-κB has been reported to directly associate physically with STAT6 (Shen and Stavnezer 1998). The function of the STAT6-NF-κB complex is dependent on intact DNA binding sites and it serves as a more potent transactivator than individual transcription factors alone. Furthermore, the physical association between IRF-4 and STAT6 has been suggested to enhance IL-4-driven CD23 expression in B cells by an additive mechanism which does not require cooperative DNA binding (Gupta et al. 1999).

The interaction between STAT6 and other nuclear factors can also repress the transcription by STAT6. STAT6 interaction with glucocorticoid receptor (GR) has been reported to suppress IL-4-induced gene activation, but the underlying mechanism of the inhibition remains unknown (Biola et al. 2000). A zinc finger protein Bcl-6, another negative regulator for STAT6 activity, is expressed in germinal center B cells and T lymphocytes (Cattoretti et al. 1995). Bcl-6 belongs to the poxvirus and zinc finger (POZ) family of transcriptional repressors and it is able to recognize and bind STAT6 DNA binding site on IL-4 responsive promoters (e.g. CD23 and Igε) consequently preventing STAT6-mediated gene activation (Harris et al. 1999). A different Bcl-6 family member,

BAZF, was recently cloned and it is also suggested to possess a similar STAT6-inhibitory function as Bcl-6 (Hartatik et al. 2001). Bcl-6 deficient mice have elevated IgE levels and a Th2 type inflammatory disease confirming the repressive role of Bcl-6 in STAT6 biology (Dent et al. 1997). Intriguingly, double-deficient mice that lack the expression of both STAT6 and Bcl-6 ($STAT6^{-/-}Bcl\text{-}6^{-/-}$ phenotype) do not have upregulated IgE production but they still develop a lethal Th2 inflammatory response *in vivo* revealing an additional yet uncharacterized STAT6-independent pathway for Th2 differentiation (Dent et al. 1998, Harris et al. 1999).

The functional connection between the transactivation domain of the DNA bound STAT6 and the basal transcription machinery is also accomplished by interaction with nuclear co-regulators. Proteins with intrinsic HAT activity such as CBP and p300 are generally implicated in loosening the super-coiled chromatin structure, thereby making it more feasible for transcription (Giles et al. 1998). CBP/p300 are not able to bind DNA; instead they serve as a bridging factor between specific transcription factors and the RNA polymerase II-driven basal transcription machinery. The RNA polymerase II holoenzyme complex consists of several components including general transcription factors (e.g. TFIID, TFIIB, TFIIF, TFIIH, TFIIE), TATA-binding protein (TBP), and TBP-associated factors (TAFs) (Lau and Horvath 2002). The complexity of the RNA polymerase II machinery allows broad regulatory possibilities for transcription by providing several different interaction surfaces for both transcriptional repressors and activators. CBP/p300 HAT proteins have been shown to interact and promote transcription by STAT family members 1, 2, 3, 5 and 6 (Bhattacharya et al. 1996, Zhang et al. 1996, Horvai et al. 1997, Pfitzner et al. 1998, Gingras et al. 1999, Paulson et al. 1999). Each member of the STAT family makes the contact in a unique fashion; in the case of STAT6, the connection is made between amino acids 1850-2176 of CBP and TAD of STAT6 (Gingras et al. 1999). An adenoviral-transforming protein E1A binds to CBP/p300 and sequesters its coactivator functions. The importance of CBP/p300 in STAT6-mediated transcription is thus further confirmed by the findings that show IL-4/STAT6-mediated transcription to be repressed in the presence of E1A (Gingras et al. 1999, Goodman and Smolik 2000). The N-terminal part of Nuclear coactivator 1 (NCoA-1), a member of the p160/steroid receptor coactivator (SRC) family, has also been shown to associate with the LXXLL motif in STAT6TAD (Litterst and Pfitzner 2002). Like CBP/p300, NCoA-1 also has intrinsic HAT activity and its expression enhances the transcriptional activity of STAT6TAD (Litterst and Pfitzner 2001). Finally, Nmi protein has been demonstrated to enhance the recruitment of coactivators (e.g. CBP/p300) to STATs (Zhu et al. 1999). STAT6 interacted with Nmi in a GST-pulldown assay, but the functional significance of this interaction remains elusive.

5.3.6 STAT6 regulated promoters

In contrast to other STAT family members, STAT6 displays strong binding preference to GAS-like sequences with longer, a six-base pair spacer between dyads (5'-TT(N_6)AA-3', also termed N4 site: TTCNNNNGAA) (Horvath 2000). In *in vitro* experiments none of the other STAT family members was capable of recognizing the N4 site, but conversely, STAT6 was observed to bind other GAS-like sites with lower affinity (Mikita et al. 1996). Somewhat conflictingly to the commonly agreed requirement of STAT dimerization, a report described STAT6 binding to DNA also as a monomer (Lee and Park 2001). Most of the STAT6 responsive genes, however, have been characterized by a unique subset of STAT binding sites (N4 sites), which generally become occupied with an activated STAT6 dimer upon IL-4/IL-13 treatment. STAT6 binding sequences are present in the promoter regions of several human and murine genes, including Igε, IL-4, IL-4Rα, CD23a, CD23b, polymeric Ig receptor, eotaxin, 15-LOX-1, μ-opioid receptor, Igγ1, Igγ3 and Igγ4 genes (Kotanides and Reich 1993, Berton and Linehan 1995, Seidel et al. 1995, Kotanides and Reich 1996, Curiel et al. 1997, Richards and Katz 1997, Matsukura et al. 1999, Schaffer et al. 1999, Kraus et al. 2001, Shankaranarayanan et al. 2001). More STAT6 responsive promoters are likely to be identified in the future; a recent report where gene chip technology was used to survey STAT6 responsive genes demonstrated that the transcription of at least 70 mRNAs is regulated by STAT6 expression in B cells (Schroder et al. 2002).

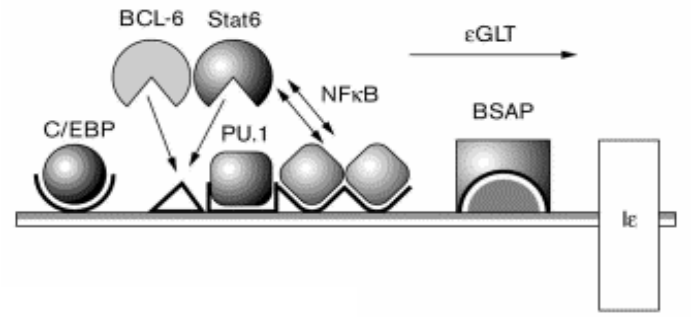

Figure 3. Structure of the germline Igε promoter. Reprinted from (Oettgen 2000), with permission from Elsevier, Copyright (2000).

The immunoglobulin heavy-chain germline Igε gene promoter is unequivocally the best characterized of IL-4/STAT6 responsive promoters thus far. The IL-4 responsive region of Igε gene

promoter has been shown to contain several *cis*-acting elements that mediate the DNA binding of transcription factors STAT6, Bcl-6, CCAAT/enhancer binding protein β (C/EBPβ), NF-κB, PU.1 and B cell specific activator protein (BSAP/Pax5) (Delphin and Stavnezer 1995, Thienes et al. 1997, Harris et al. 1999, Stutz and Woisetschlager 1999). Some differences between the murine and human Igε promoters have also been reported, for example AP-1 transcription factors are indispensable for mouse but not for human Igε promoter activation (Shen and Stavnezer 2001). As discussed above, both combinational binding and functional cooperation between different transcription factors is required to obtain efficient transcriptional response to IL-4 stimulus. Transcription factors PU.1, C/EBPβ and BSAP are constitutively expressed and unresponsive to cytokine stimulus, while the activation of STAT6 is regulated by IL-4. Notably, several reports have shown that adequate induction of both human and murine germline Igε transcripts by IL-4 requires a co-stimulatory signal provided by surface CD40 antigen engagement in B cells (Jabara et al. 1990, Warren and Berton 1995, Iciek et al. 1997). CD40 engagement leads to the activation and nuclearilization of NF-κB transcription factor, which, as described above, cooperates with STAT6 in the initiation of transcription (Iciek et al. 1997, Shen and Stavnezer 1998). Recently, CD40-mediated activation of NF-κB has been shown to occur through the p38 MAPK pathway (Brady et al. 2001).

5.3.7 Biological impact of STAT6

Several lines of studies *in vitro* have demonstrated the essential role that STAT6 has as a mediator of IL-4/IL-13-induced gene responses. The analysis of targeted STAT6 gene disruption in mice has further enlightened the profound impact of STAT6 on the expression of IL-4 inducible genes *in vivo* (Kaplan et al. 1996, Shimoda et al. 1996, Takeda et al. 1996). Lymphocytes isolated from $STAT6^{-/-}$ mice fail to express MHC class II, CD23 and IL-4Rα genes upon IL-4 stimulus. STAT6-deficient B cells do not produce detectable levels of IgE, and the IgG1 production is also greatly diminished. Th cells isolated from STAT6 deficient animals do no differentiate into the Th2 phenotype under a proper skewing cytokine milieu. Furthermore, in conflict with *in vitro* findings that emphasize the role for IRS proteins in IL-4-induced cell proliferation, studies with gene-targeted mice uncovered the function of STAT6 also in the regulation of mitogenesis (Wang et al. 1993, Kaplan et al. 1996). Cell cycle analysis showed that STAT6-deficient lymphocytes are defective in the progression from the G1 to the S phase of the cell cycle due to significantly increased expression levels of the cyclin-dependent kinase (cdk) inhibitor p27Kip1 protein (Kaplan et al. 1998).

STAT6-deficient animal models have also enabled a more clinically oriented evaluation of the function of STAT6 in the pathogenesis of the experimental models of human diseases. STAT6$^{-/-}$ mice fail to expel helminthic parasites such as *Nippostrongylus brasiliensis* and have an exaggerated severity of Th1 type diseases due to the absence of Th2 type responses (Urban et al. 1998, Chitnis et al. 2001). The predominance of cell-mediated Th1 type responses also results in increased tumor immunity (Kacha et al. 2000, Ostrand-Rosenberg et al. 2000, Terabe et al. 2000). Much effort has been aimed at assessing how STAT6 is implicated in the pathogenesis of atopic diseases, asthma and allergy. In the lungs, STAT6-deficiency downregulates experimental allergen induced inflammation, bronchial hyperresponsiveness and mucus overproduction (Akimoto et al. 1998, Pernis and Rothman 2002). More indirect evidence of STAT6 in the pathogenesis of asthma is suggested by a finding where patients with elevated airway STAT6 expression levels have more severe disease outcome (Mullings et al. 2001).

In genetic linkage studies with atopic diseases the exons, flanking introns and the promoter region of the STAT6 gene have been screened for single nucleotide polymorphisms (SNPs). Associations between a GT repeat in allele A4 and enhanced eosinophilic inflammation, and a SNP in intron 18 and an increase in total IgE levels were found (Duetsch et al. 2002). However, neither the SNP nor the GT repeat showed linkage/association to asthma as a disease. In contrast, another study has showed that polymorphism in the 3' untranslated region (UTR) of the STAT6 gene is associated with the susceptibility to and the severity of nut allergy (Amoli et al. 2002).

6. AIMS OF THE STUDY

Several lines of study have unequivocally demonstrated the biological importance of the IL-4/STAT6 signaling pathway, especially its function in allergic responses. The immediate events that lead to the IL-4-induced activation of STAT6 are relatively well established. However, little is known of how STAT6 activation is regulated in the cell. Furthermore, the mechanisms by which STAT6 initiates the transcription are also elusive.

The detailed aims of this study were:

1. To study the role of serine/threonine kinase activity in IL-4/STAT6-mediated gene activation.

2. To characterize novel co-factors for the STAT6-mediated transcription of the immunoglobulin germline ε promoter.

7. MATERIALS AND METHODS

7.1 Cell culture and transfections

All the cell lines were obtained from American Type Culture Collection, Manassas, VA. HepG2, 293T and Cos7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. 32D myeloid cells, and BJAB, BJAB-p100, Daudi and Ramos 2g6 B cells were grown in RPMI medium (Invitrogen) containing 10% FBS and antibiotics (Tong et al. 1995). Transfections of 293T and HepG2 cells were done by the calcium phosphate co-precipitation method. Daudi, Ramos 2g6 and Cos7 cells were electroporated with a Bio-Rad gene pulser and at 220 V/960 μF, 200 V/960 μF and 260 V/960 μF, respectively. In some experiments Daudi cells were also transfected with DEAE-dextran as previously described (Berberich et al. 1994). Stable PU.1 expressing HepG2 cell lines were established by transfecting a 10 cm dish of subconfluent HepG2 cells with 5 μg of human wild type PU.1 in pCIneo expression plasmid (Promega, Madison, WI) by the calcium co-precipitation method. 24 hours after transfection the cells were placed in fresh growth medium + Geneticin antibiotic at 1 μg/ml (Sigma). Cells were grown in selection for several weeks and positive clones were picked up and screened for PU.1 expression with western blot with α-PU.1 antibody (Santa Cruz biotechnology inc, Santa Cruz CA.).

7.2 DNA constructs

Reporter constructs were made by directly cloning specific promoter sequences into HindII and/or SalI sites of pfLUC-plasmid containing c-fos minimal promoter in front of the *photinus pyralis* luciferase gene. Furthermore, GAL4-RE reporter plasmid and GAL4-STAT6TAD (amino acids from 642 to 847) (Moriggl et al. 1997), STAT6 and STAT5 expression vectors, STAT5-JAK2-VP16 construct (Berchtold et al. 1997), STAT5 responsive serine protease inhibitor (SPI) luciferase construct (Wood et al. 1997) were kind gifts from Drs. M. Heim, B. Groner and T. Wood. Dr. Tuula Kallunki kindly provided HA-tagged p38 MAPKα expression plasmid. The dominant negative p38 MAPKα (p38AF) and constitutively active MAP kinase kinase (MKK)6b (MKK6b(E)) (Raingeaud et al. 1996) were kindly provided by Dr. J.Han. PU.1 expression plasmids (Fisher et al. 1998) were made by subcloning the PU.1 mutants into pCIneo vector. pCMV-β-galactosidase plasmid for

HepG2, Cos7 and 293T cells and pEBB-β-galactosidase plasmid (Tanaka et al. 1995) for Daudi and Ramos 2g6 cells were used to monitor transfection efficiency.

7.3 Antibodies

α-phosphotyrosine antibody (clone 4G10) came from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal α-JAK1 and α-JAK3 antibodies have been previously characterized (Witthuhn et al. 1994), and were a kind gift from Dr. J. Ihle. Monoclonal α-JAK1 antibody from Transduction Laboratories (Lexington, Kentucky) was used for Western blotting. Antibodies against IRS-1 and IRS-2 were from Upstate Biotechnology (Lake Placid, NY); mouse monoclonal antibody against human CD40 from Immunotech (Marseille, France) and α-phosho-p38 antibody from New England Biolabs inc. (Beverly, MA). α-STAT6 (M-20), α-RNA polymerase II (C-21) α-p38 (N-20) and α-C/EBPβ (C-19) antibodies were purchased from Santa Cruz Biotechnology inc. (Santa Cruz, CA).

7.4 Immunoprecipitation and Western blotting

Cells were lysed in either Triton-X lysis buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4), in NP-40 buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 50 mM NaF, 1 mM Na_3VO_4) or in RIPA lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS) supplemented with phenylmethylsulfonyl fluoride (PMSF) and aprotinin, and immunoprecipitations from equal protein amounts were carried out as previously described (Saharinen et al. 1997). The protein concentrations of the cell lysates were measured using the BioRad Protein Assay system (Bio-Rad Laboratories, Hercules, CA). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Micron Separation Inc, Westborough, MA). Immunodetection was performed using specific primary antibodies, biotinylated α-mouse or α-rabbit secondary antibodies (Dako A/S, Denmark) and streptavidin-biotin horseradish peroxidase-conjugate and ECL detection (Amersham Life Science, UK).

7.5 Electrophoretic Gel Mobility Shift Assay (EMSA)

For electrophoretic gel-mobility shift assay either nuclear extracts or whole cell extracts were prepared as described earlier (Saharinen et al. 1997). Annealed Igε (5'-GATCAAGACCTTTCCCAAGAAATCTATC-3') and mutated Igε (5'-GATCAAGACCTTTCCCATGAAATCTATC-3') oligonucleotides were end-labeled by T4 polynucleotide kinase using [γ-^{32}P]-adenosine triphosphate (ATP). Nuclear or whole cell extracts (6-20 μg), poly-dI-dC (240 ng/μL), bovine serum albumin (BSA; 1.5mg/mL) and ^{32}P-labeled Igε oligonuclotide (0.5 ng) were incubated for 30 minutes at room temperature, and the reactions were resolved in 4.5% TBE (0.25X) PAGE, followed by autoradiography. For competition assays lysates were preincubated for 15 minutes with 100 or 1000-fold molar excess of unlabeled PU.1 oligo, which represents the PU.1 element in the human FcγRI promoter (nucleotides between -104 and –79). For supershift assay 1 μl of either α-PU.1 or α-STAT6 antibody (Santa Cruz Biotechnology) was added to the EMSA reactions for 30 minutes.

7.6 Luciferase assay

HepG2 and 293T cells were transfected on six well plates by the calcium phosphate coprecipitation method, Daudi and Cos7 cells by electroporation as described above. Cells were grown for 24 to 48 hours and starved overnight in medium containing 1% FBS. After starvation the concentration of Daudi cells was adjusted to 1x10^6 cells per mL. Cells were stimulated with 10 ng/mL recombinant human IL-4 (PeproTech EC Ltd., London, UK), 4 IU/ml erythropoietin (Eprex^{TM}, Janssen-Cilag) or 1 μg/mL α-human CD40-antibody (Immunotech). p38 MAPK inhibitor 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI (SB202190) (Sigma Chemical Co, St. Louis, MO), H7 (1-(5-isoquinolinylsulfonyl)2-methylpiperazine) (Sigma) and wortmannin (Sigma) were added to the cultures at varying concentrations 30-60 minutes before stimuli. After stimulation for 6-16 hours, cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI) and luciferase activity was determined using Luciferase Assay System (Promega) according to the manufacturer's instructions. The luciferase values were normalized against measured β-galactosidase activities or against the total protein concentration in Daudi cells when the DEAE-dextran method was used for transfection.

7.7 Phospho-amino acid analysis and phosphopeptide map

Phospho-amino acid analysis and phosphopeptide maps were done as described earlier (Boyle et al. 1991, Valmu and Gahmberg 1995, Valmu et al. 1999). Briefly, 32D, Ramos 2g6 and Daudi cells were starved overnight and labeled with ^{32}P-orthophosphate (Amersham) in phosphate free medium (Sigma) for three hours. STAT6 was immunoprecipitated from Triton-X cell lysates; immunocomplexes were separated on SDS-PAGE and transferred onto a nitrocellulose membrane. Proteins were visualized by autoradiography and bands corresponding to STAT6 were excised for further analysis. For the phosphopeptide map, proteins were digested with Sequencing grade Trypsin (Promega). Washed fragments were applied onto thin layer cellulose plates, separated in the first dimension by electrophoresis at pH 1.9 and in the second dimension by ascending chromatography using isobutyric acid buffer (isobutyric acid: n-butanol: pyridine: Hac: water 1250:38:96:58:558 v/v/v/v/v/v) or Phospho-buffer (37,5 % n-butanol, 25% pyridine, 7,5% acetic acid). Phosphopeptides were visualized by autoradiography. For the phospho-amino acid analysis approximately 10% of digested proteins were hydrolysed in HCl for 1 hour at 110°C. Lysates were lyophilised and resolved in pH 1.9 buffer containing standard phospho-amino acids (o-Phospho-DL-serine, o-Phospho-DL-threonine, o-Phospho-DL-tyrosine from Sigma). The phospho-amino acids were separated by two-dimensional electrophoresis. Standard amino acids were visualized by ninhydrin staining and autoradiography was used to detect phosphorylated amino acids.

7.8 RNA polymerase II phosphorylation *in vivo*

Ramos 2g6 cells were labeled in phosphate-free medium containing 10 μCi/mL ^{32}P-orthophosphate as described above, and treated with IL-4 and H7, and nuclear extracts were prepared (Kumahara et al. 1999). 10 μg of nuclear extract was separated with 5% SDS-PAGE and transferred onto PVDF-membrane. Phosphorylated proteins were detected with autoradiography. The RNA polymerase II was detected by immunoblotting with α-RNA polymerase II antibody using unlabeled lysates from the same experiment. For immunoprecipitation, ^{32}P orthophosphate labeled Ramos cells were lysed in RIPA lysis buffer, and 1000μg of the lysate was immunoprecipitated with 2 μg of α-RNA polymerase II antibody. Immunoprecipitates were separated with 6% SDS-PAGE and phosphorylated proteins were detected with autoradiography.

7.9 FACS analysis

Ramos 2g6 cells were suspended in RPMI+10 % FBS and stained for 30 minutes at 4°C with 20 μL FITC-conjugated mouse α-human-CD23 antibody (PharMingen, San Diego, CA) or with 20 μL PE-conjugated mouse α-human-CD23 antibody (Becton-Dickinson). Unspecific staining was monitored with isotype matched control antibodies, FITC-conjugated mouse α-human-CD64 antibody (Immunotech, Marseille, France) or PE-conjugated mouse α-human-CD13 antibody (Becton-Dickinson). For some experiments, unconjugated mouse α-human-CD23 antibody (2 μg) (Pharmingen, San Diego, CA) and control antibody, isotype matched mouse α-human-Nef MoAb (a kind gift from Dr. V. Ovod) together with secondary antibody, 5 μg FITC-conjugated goat anti-mouse antibody (Dako), were also used. After staining cells were washed twice with PBS and analyzed with FACSscan (Becton-Dickinson).

7.10 Glutathione S-transferase (GST) pull-down assay

GST and GST-PU.1 proteins were produced in BL21 bacteria and purified with Glutathione Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. COS7 cells were transfected with 5 μg of STAT6 or pSG5 plasmid DNA, allowed to grow for 24 hours, and lysed in 0.5 % NP-40 lysis buffer. 500 μg of total cell extract from COS7 extracts was then incubated overnight at 4°C with the GST fusion proteins bound to the Glutathione Sepharose beads in 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. Proteins were boiled in SDS-PAGE sample buffer, separated by 10% SDS-PAGE and analyzed by immunoblot assay with α-STAT6 antibody (Santa Cruz Biotechnology).

7.11 Quantification of Igε gene expression using real-time PCR

Total RNA was isolated from BJAB and BJAB-p100 cells using Trizol reagent (Life Technologies, Inc.) and used for the first-strand cDNA synthesis with M-MLV reverse transcriptase (Gibco-BRL) and random hexamers (Amersham Pharmacia) according to the manufacturer's protocol. Total RNA from BJAB cells that had been stimulated for 48 h with IL-4 (100 ng/ml) was used to prepare standard curves. After the first-strand cDNA synthesis, serial dilutions were made to correspond to

cDNA transcribed from 750, 75, 7.5 and 0.75 ng of total RNA. Primers for the Igε and TBP have been described previously (Thienes et al. 1997, Linja et al. 2001).

The PCR reactions were performed in the LightCycler apparatus using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The cycling conditions (45 cycles) consisted of denaturation at 95°C for 15 s (Igε) or 3 s (TBP), annealing at 55°C for 8 s (Igε) or 58°C for 13 s (TBP) and elongation at 72°C for 16 s (Igε) or 7 s (TBP). The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the elongation step. After the PCR reaction, the logarithmic values of the fluorescence were plotted against the cycle number, and the relative Igε expression was calculated as described previously (Linja et al. 2001). PCR samples were also run in 1.2% agarose electrophoretic gels to ensure that a right-sized product was amplified in the reaction.

8. RESULTS

A schematic representation that summarizes the main findings of this study is presented on the last page of the discussion (Figure 4 on page 60).

8.1 STAT6 is phosphorylated on both tyrosine and serine residues (I, II)

Cytokine stimulus leads to the JAK-mediated tyrosine phosphorylation and activation of STATs. In addition, the serine phosphorylations of STAT1, STAT3 and STAT4 have been described to regulate their transcriptional activities by diverse mechanisms (Decker and Kovarik 2000). To study the serine/threonine phosphorylation of STAT6 Ramos and Daudi B cells were metabolically labeled with ^{32}P orthophosphate. The cells were stimulated with IL-4 for 20 minutes, and STAT6 was immunoprecipitated and separated on SDS-PAGE. Proteins were transferred onto a nitrocellulose filter and visualized by autoradiography. IL-4 stimulation was observed to induce the total phosphorylation of STAT6. The band corresponding STAT6 was excised and phosphorylation events were further studied in phospho-amino acid analysis and with tryptic phosphopeptide mapping. In unstimulated cells STAT6 was phosphorylated at low levels only on serine residues. IL-4 stimulation resulted in increased phosphorylation on both serine and, as previously reported, tyrosine residues, while no phosphorylation on threonine residues was detected in phospho-amino acid analysis. When the phosphorylation of STAT6 was further investigated by tryptic phosphopeptide mapping, two to three weakly phosphorylated peptides were detected in unstimulated cells, and IL-4 stimulation resulted in enhancement in the phosphorylation of these peptides, and also in the occurrence of several additional phosphopeptides.

In phospho-amino acid analysis, the magnitude of IL-4-induced serine phosphorylation of STAT6 was relatively low, about twofold, but this is similar to the level of induction of serine phosphorylation observed in other STATs (Chung et al. 1997, Yamashita et al. 1998). In addition, the majority of the IL-4-induced phosphopeptides were observed to be phosphorylated only on their serine residues when further analyzed with the hot-sequencing method (data not shown). Thus, the results from phosphorylation studies with *in vivo* labeled B cell lines demonstrate that STAT6 is constitutively phosphorylated on its serine residues, and that IL-4 treatment induces both the serine and the tyrosine phosphorylation of STAT6.

8.2 Serine phosphorylation of STAT6 occurs independently of the IRS pathway and is not regulated by H7-sensitive kinases or p38 MAPK (I, II)

IRS downstream signaling via activated PI-3-K has generally been implicated in the regulation of IL-4-mediated cell growth. To study whether STAT6 serine phosphorylation was occurring through IRS/PI-3-K activation, additional phosphorylation experiments were performed in IRS-deficient 32D myeloid cells (Wang et al. 1993). In untreated cells, a low level of basal serine phosphorylation of STAT6 was detected. Similarly as in B cell lines, IL-4 induced both the serine and the tyrosine phosphorylation of STAT6, indicating that the kinase responsible for STAT6 serine phosphorylation is independent of the IRS/PI-3-K signaling pathway (data not shown).

A broad-spectrum serine/threonine kinase inhibitor H7 was used to characterize the STAT6 serine kinase. H7 binds to the ATP binding site in the kinase domain, and inhibits many but not all serine/threonine kinases. For example the cytokine induced ERK and p70 S6 kinases are not affected by H7 (Boulton et al. 1995, Engh et al. 1996). The treatment of metabolically labeled Ramos cells with H7 for 30 minutes before IL-4 stimulation did not have any significant effect on the IL-4-induced total phosphorylation of STAT6 or on the level of serine and tyrosine phosphorylations as evaluated by phospho-amino acid analysis. Furthermore, in phosphopeptide mapping, the phosphorylation pattern of STAT6 was similar in both IL-4 and H7/IL-4 treated cells, thus confirming that the phosphorylation of STAT6 is mediated by H7-insensitive kinase(s).

CD40 cell surface molecules mediate important costimulatory signals for IL-4-induced gene responses, and activate protein kinases, including p38 MAPK (Sutherland et al. 1996). p38 MAPK mediates the lipopolysaccaride and cellular stress induced phosphorylation of Ser727 in STAT1, and the IL-12-induced phosphorylation of Ser721 in STAT4 (Kovarik et al. 1999, Visconti et al. 2000). To investigate the possible role of p38 MAPK in the phosphorylation of STAT6, overnight-starved Daudi B cells were metabolically labeled with ^{32}P-orthophosphate for 3 hours and stimulated with IL-4 and/or α-CD40 for 20 minutes in the presence or absence of p38 MAPK inhibitor SB202190. Cell permeable pyridinyl imidazole compounds SB202190 and SB203580 are specific inhibitors of the α and β isoforms of p38 MAPK, and they do not inhibit the kinase activity of other mitogen activated protein kinases such as ERKs and JNKs (Davies et al. 2000). As described above, in resting cells STAT6 was phosphorylated at low levels only on serine residues, and IL-4 induced phosphorylation on both tyrosine and serine residues. In phospho-amino acid analysis, CD40 engagement did not have any marked effect on either the total phosphorylation or

the tyrosine or the serine phosphorylation of STAT6. Also, the pre-treatment of Daudi cells with SB202190 did not inhibit the tyrosine or the serine phosphorylation of STAT6. To further confirm that the phosphorylation of STAT6 was unaffected by p38 MAPK activation, a part of the purified STAT6 protein was used for tryptic phosphopeptide mapping. The CD40 engagement alone or in combination with IL-4 and SB202190 treatments did not change the phosphopeptide pattern of STAT6, and there were no consistent changes in the relative intensities of individual phosphopeptides. Taken together, the results indicated that p38 MAPK does not mediate the phosphorylation of STAT6.

8.3 STAT6-mediated gene activation is regulated through H7-sensitive serine/threonine kinase activity (I)

The serine phosphorylation of STAT6 was unaffected by H7-sensitive kinases. Next, H7 was used to characterize the role of serine/threonine kinase activity in STAT6-mediated transcription. IL-4-induced gene activation was studied in the human Burkitt lymphoma B cell line Ramos 2g6, which expresses abundant IL-4R (Siegel and Mostowski 1990). In B cells, IL-4 stimulation induces the expression of the low-affinity IgE Fc receptor (CD23) in a STAT6-dependent manner (Kaplan et al. 1996, Shimoda et al. 1996, Takeda et al. 1996). Ramos cells were stimulated with IL-4 for 20 hours in the presence or absence of H7, and the surface expression of CD23 was analyzed by using FACS. The IL-4-induced CD23 expression was inhibited by H7 in a concentration-dependent manner, indicating that the IL-4 and STAT6-regulated expression of CD23 requires serine/threonine kinase activity.

To examine whether the effect of H7 on CD23 expression was due to the inhibition of IL-4R-induced immediate signaling events, the consequence of H7 treatment on the IL-4-induced JAK, IRS and STAT6 tyrosine phosphorylations was investigated. Prior to IL-4 stimulus, Ramos 2g6 cells were left untreated, or were pre-treated with H7 for 30 minutes. The specific proteins were purified from the cell extracts by immunoprecipitation, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antisera. The IL-4-induced tyrosine phosphorylations of JAK1, JAK3, IRS-1, IRS-2 and STAT6 were found to be insensitive to H7 treatment. Furthermore, the IL-4-induced DNA binding activity of STAT6 was also unaffected when cells we pre-treated with serine/threonine kinase inhibitor H7. This result suggested that the immediate activation events of STAT6 and the tyrosine phosphorylation of IRSs are independent of H7-sensitive kinase activity.

STAT6-dependent reporter constructs were used to study the observed serine/threonine kinase dependency of IL-4-induced gene expression in more detail. The analysis of the promoter region of the human Igε heavy-chain constant region gene has defined the minimal IL-4-responsive element, composed of the C/EBPβ and STAT6 binding elements, to the nucleotides -168 to -138 relative to the first RNA initiation site (Mikita et al. 1996). The IL-4-induced activation of reporter gene construct that contains the binding sites for both of these transcription factors (C/EBPβ-STAT6RE reporter, alternatively termed also as Igε-RE reporter) was observed to be sensitive to H7 treatment in a dose dependent manner. The role of PI-3-K-dependent pathways in the regulation of C/EBPβ-STAT6RE-RE activation was studied by using an irreversible PI-3-K inhibitor, wortmannin (Arcaro and Wymann 1993). Wortmannin did not have any effect on IL-4-induced reporter activation at concentrations that completely block PI-3-K catalytic activity (Zamorano et al. 1996). Finally, a STAT5-dependent reporter was used to determine whether the H7-sensitive kinase was regulated directly by the cytokine receptor or by the downstream JAK kinases. For this purpose, 293T cells were transfected with STAT5, together with either JAK2 or EpoR. In these experiments H7 completely inhibited both the EpoR and JAK2-induced activation of the STAT5-dependent reporter. These results suggested that the H7-sensitive kinase/kinases functioned downstream of JAK kinases in the signaling cascade.

Two reporter constructs carrying either three copies for C/EBPβ or STAT6 binding sites separately were also created, and their activation was also found to be dependent on the H7-sensitive kinase activity. The STAT6-dependent reporter construct was activated upon IL-4 stimulus in COS7 cells in the absence of the C/EBPβ binding sequence when STAT6 was overexpressed by transient transfection. The IL-4-induced activation of STAT6RE-reporter was somewhat more sensitive to H7 treatment than the constitutive activity of C/EBPβRE-reporter. In conclusion, the results from the reporter studies indicated that the H7-sensitive kinases, which function downstream of JAK kinases, regulate the STAT6-dependent transcription, and the PI-3-K-dependent pathway does not regulate these kinase.

Because the phosphorylation of STAT6 was insensitive to H7 treatment, but H7 was found to regulate STAT6-mediated gene responses, the possibility that H7 inhibited other phosphorylation events in the IL-4-induced transcriptional regulation than those of STAT6 was explored. The general transcription factor TFIIH-associated cdk activating kinase (CAK), as well as cdk7, cdk8, and cdk9, are sensitive to H7 treatment *in vitro* (Yankulov et al. 1995, Mitsui and Sharp 1999).

CAKs phosphorylate the C-terminal domain (CTD) of RNA polymerase II, resulting in enhanced transcription elongation (Serizawa et al. 1995). To investigate the regulation of RNA polymerase II phosphorylation in IL-4 signaling, ^{32}P-orthophosphate-labeled Ramos cells were treated with H7 and IL-4, and the phosphorylation of RNA polymerase II was analyzed both from nuclear cell lysates and α-RNA polymerase II immunoprecipitates. IL-4 stimulation enhanced the phosphorylation of RNA polymerase II, and this phosphorylation was inhibited by the pretreatment of the cells with H7. Thereby, H7 was observed to inhibit the IL-4-induced, direct activation of RNA polymerase II.

8.4 p38 MAPK regulates STAT6-mediated transactivation (II)

As mentioned above, p38 MAPK has been reported to directly phosphorylate STAT family members on serine residues (Decker and Kovarik 2000). In addition, p38 MAPK kinase activity is required for the optimal STAT1-mediated transactivation of GAS and ISRE promoters without an effect on the IFNα-induced phosphorylation of Ser727 in STAT1 (Uddin et al. 1999, Uddin et al. 2000). Furthermore, the IL-6-induced transcriptional activation of STAT3 is also dependent on the IL-6-stimulated p38 MAPK activity in hepatocytes (Zauberman et al. 1999). Thereby, the role of p38 MAPK activity in the IL-4-induced and STAT6-mediated transcription was investigated despite the negative findings regarding its role in STAT6 phosphorylation events.

Ramos 2g6 B cells were stimulated with IL-4 in the presence or absence of different concentrations of specific p38 MAPK inhibitor SB202190, and the surface expression of CD23 was measured with FACScan. IL-4 stimulation resulted in a strong induction of the CD23 expression. α-CD40 treatment alone did not increase the CD23 expression, but it slightly enhanced the IL-4-induced CD23 expression. Resting B cells expressed low levels of CD23 without any apparent stimulation, and SB202190 inhibited both the basal and the induced expression of CD23 in a dose-dependent manner. Inhibition in the CD23 expression was also obtained when Ramos 2g6 B cells were transfected with dominant negative p38 MAPKα (p38AF). The p38 MAPK activity was analyzed in differently treated B cell lysates by Western blot with an antibody that recognizes the Thr^{180}/Tyr^{182}-phosphorylated catalytically active form of p38 MAPK. The constitutive phosphorylation of p38 MAPK was detected from both Daudi and Ramos 2g6 B cells, IL-4 stimulation (from 5 minutes to 24 hours) did not activate p38 MAPK in any of the cell lines tested (Daudi, Ramos, HepG2, HeLa, and 293T). In Daudi cells, the cross-linking of CD40 resulted in the rapid induction and prolonged

activation of p38 MAPK, whereas in Ramos cells CD40 engagement only slightly increased (10-20%) p38 MAPK activity. SB202190 pretreatment markedly diminished the basal phosphorylation of p38 MAPK as well as the CD40-induced phosphorylation in both cell lines. Thus, in Ramos 2g6 B cells, both the basal and the IL-4/CD40-induced CD23 expression directly correlated with the level of p38 MAPK activation suggesting that the IL-4-induced gene activation is dependent on p38 MAPK activity.

The IL-4-responsive promoter regions of the human Igε and CD23 genes contain binding elements for several transcription factors, and reporter studies were used to evaluate the role of p38 MAPK directly in STAT6-driven transcription. Both SB202190 and p38AF were found to downregulate the IL-4 and IL-4/CD40-induced activation of the C/EBPβ-STAT6RE reporter construct, whereas the overexpression of wild-type p38 MAPKα (p38wt) resulted in the enhancement of the reporter activity. The STAT6 response element was analyzed individually, and the STAT6RE reporter was found to function similarly as the C/EBPβ-STAT6RE reporter in Daudi B cells. The IL-4 and IL-4/CD40-induced activities of STAT6RE were sensitive to SB202190 treatment, but the inhibitor treatment did not reduce the activity of the C/EBPβRE reporter construct in Daudi B cells. In conclusion, these results suggest that STAT6-mediated transcription is a target for p38 MAPK activity in B cells.

When the mechanism of the p38 MAPK-mediated regulation of STAT6 activation was analyzed in more detail, the tyrosine phosphorylation and DNA binding activity of STAT6 were found to be unaffected by SB202190 treatment in Daudi B cells. SB202190 pretreatment had no effect on the immediate activation events of STAT6 (up to 2 h) in EMSA. The findings which showed that immediate activation events and the phosphorylation of STAT6 were not regulated by p38 MAPK brought up the possibility that p38 MAPK might affect the transactivation potential of STAT6. A fusion construct containing the yeast GAL4 DNA-binding domain and the TAD of STAT6 (GAL4-STAT6TAD) was used to test this hypothesis. The GAL4-STAT6 fusion construct has been shown to be constitutively active and to bind DNA independently of extracellular stimuli (Moriggl et al. 1997). The GAL4-STAT6TAD fusion construct and GAL4 binding sites containing reporter constructs were transfected into Daudi B cells together with p38AF, p38wt, or empty pSG5 plasmid DNA. The cotransfection of p38AF and SB202190 treatment reduced reporter activity approximately 50-80 %, whereas the overexpression of p38wt was found to upregulate STAT6TAD-mediated transcription. Similar results were also obtained in experiments with non-

hematopoietic HepG2 cells. IL-4 treatment had no effect on STAT6TAD activity in Daudi or HepG2 cells. In conclusion, these results indicate that p38 MAPKα regulates STAT6TAD-mediated transcriptional activity in different cell types.

8.5 p100 enhances the STAT6-mediated transcription of the Igε gene in BJAB B cells (III)

To identify novel co-regulators for STAT6, the TAD of STAT6 was expressed as a GST fusion protein and used to purify interacting proteins from Ramos 2g6 B cell nuclear extracts. The captured proteins were analyzed by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and the 'ProFound' program was used to compare the obtained mass maps against theoretical tryptic peptide mass maps in the OWL protein sequence database. The comparison resulted in the unequivocal identification of the 100 kilodalton (kDa) STAT6-interacting protein as the human p100 protein.

p100 was initially identified as a transcriptional co-activator for the Epstein-Barr virus nuclear antigen 2 (EBNA2) (Tong et al. 1995). p100 has also been reported to interact with c-Myb, a transcription factor involved in the regulation of the differentiation and proliferation of immature hematopoietic and lymphoid cells (Leverson et al. 1998). The scope of this study was to analyze the functional consequences of the interaction between p100 and STAT6 on the IL-4-induced germline Igε mRNA expression by using real-time PCR analysis. BJAB B cells and BJABs that overexpress p100 (BJAB-p100) (Tong et al. 1995) were starved overnight, and left untreated or stimulated with IL-4 for 24 hours. The extracted RNA was analyzed using assay conditions that allowed a quantitative comparison of the Igε mRNA expression by normalizing the expression levels to the expression of the housekeeping TBP gene (Thienes et al. 1997, Linja et al. 2001). The stable overexpression of p100 was not found to affect the basal expression, but it enhanced the IL-4-induced expression of Igε mRNA in human BJAB-p100 cells when compared to the parental BJAB B cells.

8.6 PU.1 is required for the transcriptional activation of the STAT6 response element in the Igε promoter (IV)

The reporter construct consisting of the STAT6 response elements from the Igε promoter (Igε-STAT6RE) was observed to be readily activated upon IL-4 stimulation in B cells but not in non-

hematopoietic HepG2 cells. The STAT6 binding elements on various IL-4-responsive genes were compared and a minimal low affinity PU.1 core binding sequence (5'-AGAA-3') was identified within Igε-STAT6RE (Himmelmann et al. 1997). PU.1 belongs to the Ets family of transcription factors that are expressed specifically in hematopoietic cells, and it participates in the regulation of several genes involved in the development and functioning of myeloid and B cells, including CD11b, CD18, CSF-1 receptor, FcγRI, Btk, and immunoglobulin heavy and light chain genes (Simon 1998, Lloberas et al. 1999).

The role of PU.1 in Igε-STAT6RE-mediated transcription was investigated in HepG2 cells that were transiently transfected with PU.1 expression plasmid together with the STAT6REx3 reporter. As mentioned above, the STAT6REx3 reporter construct was not responsive to IL-4 stimulation in HepG2 cells, but the ectopic expression of PU.1 conferred the IL-4-inducibility (fold of induction increased from 2 to 40). In contrast, the IL-4-inducibility of the STAT6REx3 reporter derived from the IL-4Rα gene promoter was insensitive to the PU.1 expression (data not shown). To confirm the effect of PU.1 on the IL-4-induced STAT6REx3-reporter gene activation, HepG2 cell lines that stably express PU.1 at physiological expression levels were established (HepG2-PU.1). The IL-4-inducibility of various Igε promoter constructs encompassing binding sites for C/EBPβ, STAT6, and NF-κB/PU.1 transcription factors was analyzed in both parent and HepG2-PU.1 cell lines. All the reporter constructs that contained Igε-STAT6RE had increased sensitivity to IL-4 treatment in the PU.1 expressing cells. The transactivation of the GAL4-STAT6TAD fusion construct was also analyzed in both HepG2 and HepG2-PU.1 cell lines. The basal activity of GAL4-STAT6TAD was slightly higher in HepG2-PU.1 cells than in HepG2 cells, but the expression of PU.1 did not affect the IL-4-inducibility of STAT6TAD.

A reporter construct containing a point mutation (A→T) that disrupts the PU.1 core binding sequence of Igε-STAT6RE (mSTAT6REx3-reporter) was used to study whether the intact PU.1 core binding sequence is required for the PU.1-mediated enhancement of STAT6-driven transactivation. A point mutation in the PU.1 binding site abolished the IL-4-inducibility of the reporter in Daudi B cells and the co-transfection of PU.1 failed to enhance the IL-4-inducibility of mSTAT6REx3 in HepG2 cells, further confirming that the PU.1 core binding sequence is required for the IL-4-induced transcriptional activity of the Igε-STAT6 response element. However, when the germline Igε RNA expression was analyzed by RT-PCR in parent and HepG2-PU.1 cells, the expression was below detection level in both cell lines also after IL-4 treatment for 24 hours. This

result indicates that the expression of PU.1 alone is not sufficient to render HepG2 cells into Igε expressing cells (data not shown).

In EMSA, STAT6 was found to bind STAT6RE and the mSTAT6RE oligos equally well, but direct DNA binding of either cellular or *in vitro* translated PU.1 to the Igε promoter-derived STAT6RE oligo could not be detected. However, the results from reporter studies suggested that the functional promoter association of PU.1 could involve an interaction with STAT6, and in line with this hypothesis, PU.1 and STAT6 were found to physically interact in a GST pulldown assay independently of IL-4 stimulus.

The three major functional domains of the PU.1 protein are the C-terminal winged helix-loop-helix DNA binding domain, the N-terminal transactivation domain, and the central proline, glutamic acid, serine and threonine rich region (PEST) domain that mediates protein-protein interactions (Fisher et al. 1998). To define the structural features of PU.1 that are required for the STAT6-mediated transcription, HepG2 cells were transfected with STAT6REx3 reporter construct and with equimolar amounts of PU.1 deletion constructs. The reporter activities of IL-4-treated and PU.1-deletion-construct-transfected cells were compared to those of the PU.1 wild type transfectants.

The PU.1S148A construct where Ser148 had been mutated into alanine retained its positive effect on STAT6-mediated gene activation suggesting that the serine phosphorylation of PU.1 does not regulate its function on the STAT6 response element. The deletion of the PEST domain resulted in a slight decrease in the IL-4-inducibility (15-20 % less efficient than PU.1wt). A construct with an N-terminal deletion in the glutamic acid rich domain showed a 50-60% reduced ability to activate Igε-STAT6RE compared to wild type PU.1. The deletion of the N-terminal acidic transactivation region entirely abolished the ability of PU.1 to activate STAT6-mediated transcription. Likewise, the PU.1 mutant lacking the carboxyl 10 amino acids of the DNA binding domain was found to be completely inactive. Furthermore, a construct consisting only of the DNA binding domain of PU.1 failed also to activate STAT6-driven transcription. Together these results indicated that the glutamic acid rich and acidic transactivation regions of PU.1 are critical for the augmentation of STAT6 response element mediated transcription. Furthermore, the DNA binding domain of PU.1 was essential but not alone sufficient for the activation of the Igε-STAT6 response element.

9. DISCUSSION

9.1 The role of serine/threonine kinase activity in IL-4/STAT6-mediated transcription

The principal transcriptional responses upon IL-4 stimulus are mediated through the activation of the JAK/STAT pathway (Hou et al. 1994, Quelle et al. 1995, Nelms et al. 1999). Albeit the activation of STAT transcription factors is critically dependent on the tyrosine kinase activity of JAKs, it has recently become evident that multiple serine/threonine kinases modutale STAT-mediated transcription both directly and indirectly (Decker and Kovarik 2000). One of the main purposes of this study was to evaluate the role of serine phosphorylation in IL-4-induced and STAT6-mediated gene activation events. IL-4 was found to induce the serine phosphorylation of STAT6, and STAT6-dependent transcriptional activation was regulated by a convergence of both tyrosine and serine/threonine kinase pathways.

9.1.1 H7-sensitive kinases regulate STAT6-dependent gene activation

The serine/threonine kinase inhibitor H7 efficiently abrogated STAT6-dependent CD23 gene expression in Ramos 2g6 B cells without affecting the IL-4-induced tyrosine phosphorylation of the JAK kinases or IRS proteins. Since the immediate activation of STAT6 was not inhibited by H7 treatment, the function of H7 was speculated to be directed to the inhibition of transcription. Since the phosphorylation of Ser727 in both STAT1 and STAT3 and Ser721 in STAT4 are required for their optimal transcriptional responses, H7 sensitive kinases were first thought to directly phosphorylate STAT6, consequently affecting its transcriptional potential (Wen et al. 1995, Visconti et al. 2000). However, as discussed in the following chapter, the serine phosphorylation of STAT6 was insensitive to H7 treatment, and thereby STAT6 itself was not the target for the observed transcriptional effect of H7. The promoter regions of IL-4-responsive genes contain, in addition to STAT6 binding sites, response elements for several other transcription factors, such as C/EBPβ, NF-κB, BSAP and PU.1 (Thienes et al. 1997, Tinnell et al. 1998, Stutz and Woisetschlager 1999), which could also be targets for the observed H7-mediated inhibition. Reporter gene studies with Igε-RE, STAT6-RE and C/EBPβ-RE reporters confirmed the previously reported synergistic role of C/EBPβ in STAT6-mediated gene induction (Delphin and Stavnezer 1995, Mikita et al. 1996) and demonstrated that the STAT6-dependent transcription was the target of the H7 inhibition in several different cell types. Furthermore, H7 diminished both Epo- and

JAK2-induced activations of a STAT5-dependent reporter, suggesting that the activation of the H7-sensitive kinases occurs downstream of JAK kinases.

In view of the fact that STAT6 was apparently not a direct target for the H7-sensitive kinases literature was explored for potential candidates for the H7 action. The inhibitory spectrum of H7 is rather wide, also including kinases such as CAK and cdks 7-9 (Yankulov et al. 1995, Mitsui and Sharp 1999). CAK and cdks directly control the general RNA polymerase II dependent transcription machinery by regulating the phosphorylation status of the CTD of RNA polymerase II. In Ramos 2g6 B cells, IL-4 stimulus was observed to result in the increase of RNA polymerase II phosphorylation in an H7-sensitive manner. Since the phosphorylation of the CTD of RNA polymerase II correlates well with the enhancement of transcription elongation (Dahmus 1996), the results indicated that the H7 inhibitory action on STAT6-mediated transcription was at least partly due to the inhibition of the IL-4-induced RNA polymerase II phosphorylation. However, the possibility that H7-sensitive kinases could also regulate other phosphorylation events in STAT6-dependent transcriptional activation cannot be excluded. Identifying such phosphorylation events could provide further insight into the regulation of IL-4-induced gene activation events.

9.1.2 p38 MAPK as a regulator of STAT6-mediated gene activation

The results described above suggested that the STAT6-mediated transcription is regulated by still unidentified serine kinases. The engagement of the CD40 surface antigen provides important costimulatory signals for IL-4/STAT6-mediated transcription (Jabara et al. 1990, Paterson et al. 1996) by promoting the rapid activation of several tyrosine and serine/threonine kinases and additional transcription factors, such as NF-κB (Faris et al. 1994, Sutherland et al. 1996, Hanissian and Geha 1997, Craxton et al. 1998, Brady et al. 2001). Lately, much interest has been focused on the potential role of p38 MAPK in the regulation of STATs. p38 MAPK directly phosphorylates STAT1 and STAT4, but also regulates the STAT1 dependent transactivation of the GAS and ISRE promoters independently of Ser727 phosphorylation (Kovarik et al. 1999, Uddin et al. 1999, Uddin et al. 2000, Visconti et al. 2000). Furthermore, the IL-6-induced transcriptional activation of STAT3 requires the IL-6-stimulated p38 MAPK activity in hepatocytes (Zauberman et al. 1999). Because p38MAPK is readily activated through CD40, the role of p38MAPK in STAT6-mediated transcription was investigated.

The inhibition of p38 MAPK, either by pharmacological inhibitors or by the dominant negative p38 MAPK, diminished both the basal and the IL-4/CD40-induced expression of CD23. IL-4 was found not to induce p38 MAPK activation in B cells, which is in accordance with previous studies on other hematopoietic cells (Foltz et al. 1997). CD40 engagement resulted in a rapid activation of the p38 MAPK in Daudi B cells, and enhanced kinase activation correlated directly with the upregulation of the IL-4-induced transcriptional events, such as the CD23 expression and the IL-4-dependent reporter gene construct transcription. Previously, CD40 engagement has been shown to regulate the transcriptional activity of NF-κB via the activation of p38 MAPK in the IgE isotype switching (Brady et al. 2001). However, the role of p38 MAPK in different response elements on the germline Igε promoter was dissected, and the NF-κB binding site was not required for p38 MAPK action. Instead, the STAT6-binding element on the Igε promoter was identified as a direct target for p38 MAPK activity. In addition, another STAT6 collaborating factor, C/EBPβ did not appear to be a substrate for p38 MAPK, and C/EBPβ-mediated transcription was not regulated by p38 MAPK in B cells. This suggests that p38 MAPK is not a general regulator of transcription.

The effect of p38 MAPK activating and inhibiting treatments on STAT6 phosphorylation events was also evaluated. In phospho-amino acid analysis and phosphopeptide mapping experiments the phosphorylation of STAT6 was unaffected by CD40 engagement and SB202190 treatment, as discussed below. Also, the immediate activation events and the IL-4-induced DNA binding of STAT6 occurred independently of p38 MAPK activation. Therefore, the results from the reporter gene and phosphorylation studies suggested that the effect of p38 MAPK is directed to the transcriptional activity of STAT6. In agreement with this hypothesis, the GAL4-STAT6TAD fusion construct was directly stimulated by p38 MAPK activation and inhibited by the expression of the dominant negative p38 MAPK or SB202190. These results suggest a role for yet unidentified p38 MAPK-mediated phosphorylation events in the regulation of interaction between STAT6TAD and transcriptional coregulators. The findings regarding p38 MAPK as a regulator of STAT6 activity bear some resemblance to the effect that p38 MAPK has on NF-κB (Carter et al. 1999). p38 MAPK modulates transcriptional activity, but not the immediate activation events of NF-κB, by promoting the phosphorylation of TATA binding protein (TBP), thus facilitating the interaction between NF-κB and TBP. The role of p38 MAPK in possible TBP-STAT6 interaction was also studied, but in B cells, p38 MAPK did not regulate the TBP phosphorylation events.

The exact molecular mechanism by which STAT6 is connected to the basal transcriptional machinery is relatively poorly understood at present. STAT6 has been shown to interact with several general transcriptional coactivators such as CBP/p300, NCoA-1, Nmi and p100, but currently there is no information that would indicate these interactions to be regulated by serine phosphorylation (III, Gingras et al. 1999, McDonald and Reich 1999, Zhu et al. 1999, Litterst and Pfitzner 2001). However, it is likely that more transcriptional cofactors for STAT6 will be identified in the future, and also the analysis of the role of serine/threonine kinase activity in the regulation of currently identified transcription factors warrants further studies. Furthermore, the intriguing possibility remains that, in addition to the effects on the transcriptional activation of STAT6, p38 MAPK could modulate transcriptional events by directly molding the chromatin structure through the regulation of histone phosphorylation by the downstream kinase MSK-1 (Thomson et al. 1999).

In addition to the well-established regulative function of p38 MAPK in Th1 type pro-inflammatory cytokine signaling events, several reports have also demonstrated its essential role in eosinophilic inflammation (Birrell et al. 2000, Underwood et al. 2000). In this study, p38 MAPK was shown to regulate cellular responses in B cells upon Th2 type cytokine, IL-4, stimulation by regulating STAT6TAD. Considering the effects of p38 MAPK on both the STAT6 and NF-κB-mediated responses in lymphocytes, as well as its promoting role in eosinophilic inflammation, inhibitors of p38 MAPK kinase activity could potentially serve as an effective medicine for allergic diseases and asthma.

9.1.3 Serine phosphorylation of STAT6

Quiescent STAT6 was detected to be constitutively phosphorylated on its serine residues, and IL-4 stimulation resulted in the increase of the total phosphorylation of STAT6. When evaluated further, the augmentation in the total phosphorylation was observed to consist of both tyrosine and serine phosphorylation events. It is well established that the IL-4-induced JAK activation leads to the tyrosine phosphorylation of STAT6 (Nelms et al. 1999). One of the aims of the current study was to characterize the kinase(s) responsible for the serine phosphorylation of STAT6. The major serine phosphorylation site in STAT1 and STAT3 (Ser727) is located within the conserved PSMP target motif for MAP kinases located in the C-terminal part of the proteins (Wen et al. 1995). Similarly, the IL-12-induced Ser721 phosphorylation of STAT4 occurs within the PSMP motif, whereas the PD098059-resistant/sensitive serine phosphorylation of STAT5 isoforms utilize the conserved C-terminal PSP motif, also targeted by MAPKs (Kirken et al. 1997, Visconti et al. 2000, Yamashita et

al. 2001). Different MAPK family members, including ERKs and p38 MAPK, have been shown to serine phosphorylate STATs in an activating signal and cell-type dependent manner (Chung et al. 1997, Kovarik et al. 1999, Visconti et al. 2000). The vertebrate STAT6 structure contain the SSPD/E sequence at the position of the conserved PMSP or PSP motifs in STAT1, STAT3, STAT4 and STAT5s, and the results from phosphorylation studies showed that the serine phosphorylation of STAT6 was resistant to both p38 MAPK activation (CD40 engagement) and inhibition (SB202190) treatments (Decker and Kovarik 2000). Thereby, p38 MAPK is unlikely to directly regulate the phosphorylation of STAT6. Furthermore, IL-4-induced STAT6 serine phosphorylation events have been found to be insensitive to SB203580, PD098059, PD1693116, DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidasol), H7, HA1004, wortmannin and LY294002 treatments suggesting that kinases, such as MEK, JNK, casein II-kinase, PI-3-K, protein kinase C (PKC), PKA, PKG and cdks, that can be blocked by these compounds are not serine phosphorylating STAT6 (I, Wick and Berton 2000, Woetmann et al. 2002). However, the methodology used for the analysis of protein phosphorylation is not absolutely quantitative, and thus the existence of minor phosphorylation events in STAT6 that are sensitive to the inhibitors tested cannot strictly be excluded.

The functional role of STAT serine phosphorylation has been a matter of some controversy. For example, the phosphorylation of Ser727 in both STAT1 and STAT3 is required for their optimal transcriptional response, but the serine phosphorylation of STAT3 by the MAP kinases has also been reported to downregulate STAT3 activity due to the inhibition of tyrosine phosphorylation and DNA binding (Wen et al. 1995, Jain et al. 1998). Several other reports have also showed that the consequence and mode of STAT serine phosphorylation events seems to be dependent on both the cell-type and the promoter context, as well as on the triggering stimuli (Decker and Kovarik 2000). The IL-4-induced serine phosphorylation of STAT6 has been shown to occur in the C-terminal transactivation domain, but the exact Ser residues have not been identified (Wick and Berton 2000). In STAT1, Ser727 phosphorylation regulates the interaction with the transcriptional coregulators MCM5 and BRCA1 (Zhang et al. 1998, Ouchi et al. 2000). Based on this study, the significance of STAT6 serine phosphorylation remains elusive, but it is possible that the phosphorylation mediates an increase in negative net charge, and thereby endorses protein interactions also in STAT6TAD. Another possible functional role for the serine phosphorylation of STAT6 arose from a recent study in which the inhibition of a serine/threonine-specific phosphatase PP2A was shown to augment STAT6 serine phosphorylation (Woetmann et al. 2002). The PP2A-dependent increase in STAT6

serine phosphorylation was shown to result in enhancement in the transactivation by STAT6 via the upregulation of its DNA binding capacity.

9.2 Transcriptional regulation of the human germline Igε gene promoter

During the initiation of transcription the activated STAT6 collaborates with other sequence-specific transcription factors and nuclear coregulators. For example, the human germline Igε promoter contains *cis*-acting regulatory DNA elements for STAT6, Bcl-6, C/EBPβ, NF-κB, PU.1 and BSAP transcription factors. The cooperation between STAT6 and other factors modulates transcriptional responses either by affecting the DNA binding of STAT6 or by directly regulating its transactivation potential (Oettgen 2000). Furthermore, STAT6 cooperation with coregulators bearing intrinsic HAT activity makes target DNA sequences more feasible for the initiation of transcription (Gingras et al. 1999, McDonald and Reich 1999, Litterst and Pfitzner 2001, Shankaranarayanan et al. 2001, Litterst and Pfitzner 2002). Another main objective of this study aimed at the characterization of novel STAT6 coregulators in the IL-4-induced transcription of the Igε promoter.

9.2.1 STAT6 response element in the Igε promoter is regulated by PU.1 expression

The initial finding that the STAT6 response element from the B cell-specific Igε promoter was observed to be readily activated upon IL-4 stimulation in B cells but not in non-hematopoietic cells suggested that an unidentified factor, critical for the direct IL-4-inducibility of Igε-STAT6RE, is expressed in B cells. A minimal low affinity PU.1 core binding sequence (5'-AGAA-3') was identified within Igε-STAT6RE, and in contrast, STAT6RE from the ubiquitously expressed IL-4Rα gene promoter does not contain the PU.1 core binding sequence (Kotanides and Reich 1996, Mikita et al. 1996, Himmelmann et al. 1997). The ectopic expression of myeloid and lymphoid cell specific PU.1 restored the IL-4-inducibility of Igε-STAT6RE in PU.1-deficient cells, but failed to upregulate IL-4Rα-STAT6RE. The transcription factor IRF-4 acts in concert with PU.1 in the activation of the immunoglobulin light chain (Igκ/λ), human IL-1β and interferon-stimulated gene 15 (ISG15) genes (Pongubala et al. 1993, Marecki et al. 2001, Meraro et al. 2002). However, the central IL-4 responsive region in the Igε promoter lacks the IRF binding element, and in line with this, the IRF-4 expression alone or in combination with PU.1 did not stimulate the transcription mediated by the Igε-STAT6 response element.

The effect of PU.1 on Igε-STAT6RE was verified in the experiments with HepG2 cell lines that stably express PU.1 at more physiological expression levels. Previously, PU.1 has been shown to bind a DNA sequence that overlaps with the distal NF-κB1 binding sequence on the Igε promoter (Stutz and Woisetschlager 1999). However, several different reporter constructs derived from the Igε promoter were analyzed, and PU.1-mediated enhancement in STAT6-driven gene activation was detected to be independent of the reported NF-κB/PU.1 binding site. The expression of PU.1 also enhanced the IL-4-induced transcription of reporter constructs carrying only STAT6 binding sites alone, or in combination with C/EBPβ response elements. In contrast, the PU.1 expression was not able to directly affect the IL-4-inducibility of STAT6TAD. These results suggested that the strong induction of STAT6 transactivation by PU.1 expression is dependent on the presence of the Igε-STAT6 response element.

The mutation/function analysis of the minimal low affinity PU.1 core binding sequence within Igε-STAT6RE showed that the intact PU.1 binding site is required for the IL-4-induced transcriptional activity of the Igε-STAT6 response element. This suggested that PU.1 facilitates STAT6-mediated transcription by directly binding to its core DNA binding sequence within the STAT6 response element on the Igε promoter. This is further supported by the findings from studies with PU.1 deletion constructs showing that the DNA binding domain of PU.1 is a critical determinant for the positive effect of PU.1 on Igε-STAT6RE. Another Igε promoter binding transcription factor, C/EBPβ has been reported to enhance the transactivation of STAT6 by reducing the fast dissociation rate of DNA-bound STAT6 (Mikita et al. 1998). To investigate whether PU.1 also stabilizes the STAT6-DNA complex, PU.1 binding to both STAT6RE and mSTAT6RE was analyzed. Somewhat unexpectedly, PU.1 could not be identified from either STAT6 or mSTAT6RE binding protein complexes. The PU.1 core binding sequence in Igε-STAT6RE is similar to the low affinity PU.1 binding site on the CD20 promoter. PU.1 has only weak homology to the consensus Ets domain, which mediates sequence-specific DNA binding. The general Ets consensus sequence is being defined as GGAA/T, but PU.1 is able to bind with low affinity to a purine-rich region on the CD20 promoter containing the minimal sequence AGAA (Himmelmann et al. 1997). However, the flanking sequences are critical in defining the binding specificity; consistent with this notion, PU.1 binding to the CD20 promoter also requires the presence of IRF-4. The STAT6 responsive site in the Igε promoter lacks the IRF binding element, and in line with this, the IRF-4 expression did not stimulate the transcription mediated by Igε-STATRE. It is possible that the experimental

settings used in this study were not sensitive enough to detect this low affinity binding that PU.1 has in the absence of IRFs.

The existence of the functional cooperation between STAT6 and PU.1 was further supported by the detected physical interaction in GST-pull down assays. In supershift assays, however, the mobility shift of the STAT6 DNA binding complex with α-PU.1 antibody or by the ectopic expression of PU.1 could not be observed, which suggests that the *in vivo* interaction between PU.1 and STAT6 is rather weak. Thereby, the assembly of enhanceosome on Igε-STAT6RE may involve relatively low affinity STAT6-PU.1 protein interaction that is elicited by the DNA binding activities of the proteins. At present, there is only limited information available about the DNA bound structures of transcription factors on composite response elements, and the exact configuration of the STAT6-PU.1 complex remains elusive. However, the crystal structures of the DNA bound STAT dimers indicate that the DNA binding domain mediates relatively few direct interactions with the palindromic STAT target sequence (Becker et al. 1998, Chen et al. 1998). Particularly, the spacing nucleotides at positions +1, 0, -1 between the two half sites may not directly contact STAT protein. Interestingly, the spacing between the STAT6 dyad binding elements is longer containing four bases. This increase in space could allow the contact between the two highly conserved arginine residues in the recognition helix of the PU.1 DNA binding domain and the PU.1 recognition core sequence A/GGAA (Seidel et al. 1995, Kodandapani et al. 1996).

The regulatory role of PU.1 in IL-4/STAT6-mediated transcription bears some similarity to the regulation of IFNα-induced transcription. IFNα induces transcription through the activation of multimeric complex consisting of STAT1, STAT2 and IRF-9 proteins (Bluyssen et al. 1996). During the IFNα-induced activation of the ISRE promoter, STAT proteins need to cooperate with IRF-9, since ISRE lacks the complete STAT-dyad binding sequence. In the absence of IRF-9 STAT1 homodimerizes and activates other promoters carrying the GAS sequence, thus IRF-9 serves as a determinant for the IFN-α specific gene expression. The results presented here suggest that PU.1, like IRF-9, can modulate promoter-specific transactivation by STAT6 and thereby it is involved in the regulation of cell-type specific gene expression. The requirement for PU.1 in the STAT6 response element-mediated transactivation in the Igε, but not in the ubiquitously expressed IL-4Rα promoter demonstrates a mechanism for fine-tuning the cell-type restricted expression of IL-4-induced genes.

9.2.2 p100 connects STAT6TAD with the basal transcription machinery resulting in enhancement in germline Igε gene expression

Sequence-specific transcription factors, such as STAT6, are obliged to collaborate with nuclear coactivators during the initiation of transcription. Functionally, the transcriptional coactivators can roughly be divided into two overlapping categories. Coactivators can modify chromatin structure for example by using their HAT activity (e.g. CBP/p300, (Xu et al. 1999)) or through nucleosome remodeling complexes (e.g. SWI/SNF, (Muchardt and Yaniv 1999)). Alternatively, though not exclusively, some general coactivators, such as TBP-associated factors (TAFs), are also known to function as bridging factors mediating the complex formation between sequence-specific transcription factors and the basal transcription machinery (Green 2000).

Previously, CBP/p300 and NCoA-1, coactivators with intrinsic HAT activity, have been shown to interact with STAT6 and, as a consequence, promote STAT6-mediated transcription (Gingras et al. 1999, McDonald and Reich 1999, Litterst and Pfitzner 2001). However, the mode of interaction between STATs and the basal transcription machinery has remained elusive. p100, originally characterized as a coactivator for EBNA2, was identified as a novel STAT6TAD interacting protein from B cell nuclear extracts (Tong et al. 1995). The interaction between STAT6TAD and p100 was observed to occur both *in vivo* and *in vitro*, and it was dependent on the staphylococcal nuclease (SN)-like domain of p100. The mechanisms of p100-mediated transcriptional regulation are currently poorly understood. The SN-like domains of p100 lack the residues required for the catalytic function, and p100 does not posses HAT activity (Wang et al. 2000). p100 enhances transcriptional activation by STAT6TAD without affecting tyrosine phosphorylation or DNA binding events. Instead, the results from interaction studies suggested direct interaction between p100 and the large subunit of RNA polymerase II. Consistent with this notion, p100 has also been shown to be able to interact with the general transcription factor TFIIE *in vitro* (Tong et al. 1995) and to serve as a component of the RNA polymerase II holoenzyme (K.Carter and E.Kieff, manuscript in preparation). Although the interactions between sequence-specific transcription factors and their coactivators are generally rather weak, ternary complex formation between STAT6, p100 and RNA polymerase II was detected in the interaction studies. Together these results suggest that the observed increase in IL-4-induced germline Igε expression in p100 overexpressing BJAB-p100 cells is due to the function of p100 as a bridging protein, which results in an improvement in the connections between STAT6 and the basal transcription machinery.

9.3 Conclusions and perspectives

The postgenomic era has also begun in STAT research, since the large-scale sequencing efforts aimed at identifying novel STAT family members have failed (Ihle 2001). Therefore, STAT research will be able to concentrate on still puzzling problems, such as the regulative mechanisms of STATs. This study sought to gain more insight into the regulative mechanisms of STAT6-mediated gene activation. IL-4 stimulation was observed to induce the phosphorylation of both serine and tyrosine residues on STAT6, and the H7 sensitive kinase(s) and p38 MAPK were shown to promote STAT6-driven transcription. In addition, the evaluation of cofactors for STAT6 in the IL-4-induced activation of the human germline Igε promoter led to the identification of PU.1 and p100 as novel STAT6 interacting proteins, which promote STAT6-mediated transactivation. It is of note, however, that cell line specific differences may exist, and thus it is important to confirm the results in primary B cells. The main findings of this study are summarized in Figure 4, wherein the original communications are referred to by their Roman numerals I-IV.

Several intriguing questions concerning STAT6 regulation still remain unresolved. Laborious mutation experiments targeted at the serine residues in the STAT6 structure would be required to identify the phosphorylated serine residues, and importantly, also to determine the functional consequence of STAT6 serine phosphorylation. Furthermore, the identity of the IL-4-inducible serine kinase responsible for STAT6 phosphorylation is also unknown. Results from the phosphorylation experiments where several serine kinase inhibitors were tested suggest that the kinase is somewhat different from other serine kinases that phosphorylate STAT family members (Wick and Berton 2000, Woetmann et al. 2002). The exact target where the H7-sensitive kinases and p38 MAPK exert their effect is also worth further evaluation. The IL-4-induced phosphorylation of RNA polymerase II was observed to be inhibited by H7 treatment, and p38 MAPK regulated the transactivation by STAT6TAD. However, the particular molecule in the IL-4/STAT6 signaling that is affected by p38 MAPK is unidentified. Additionally, taking into account the broad spectrum of H7, it is also likely that regulative phosphorylation events in IL-4/STAT6-mediated transcription other than that of RNA polymerase II will be identified.

It is evident that STAT6 must cooperate with other transcription factors and nuclear coregulators to gain enough specificity and potency to activate transcription. In addition to p100, several other nuclear proteins were also found to associate with STAT6TAD in GST pull-down assays, and further studies aiming at evaluating their function in STAT6-mediated transcription are presently

ongoing. The function of the PU.1-STAT6 cooperation specifically affected the activation of the Igε promoter. Therefore, intervention on this event could provide an effective and specific tool to block IgE production, a hallmark of atopic diseases. It would also be of interest to study whether point mutation in the PU.1 core binding sequence that was found to abrogate the function of the STAT6 response element exists in humans as genetic polymorphism, and to investigate its impact on serum IgE levels and the prevalence of asthma and allergic diseases.

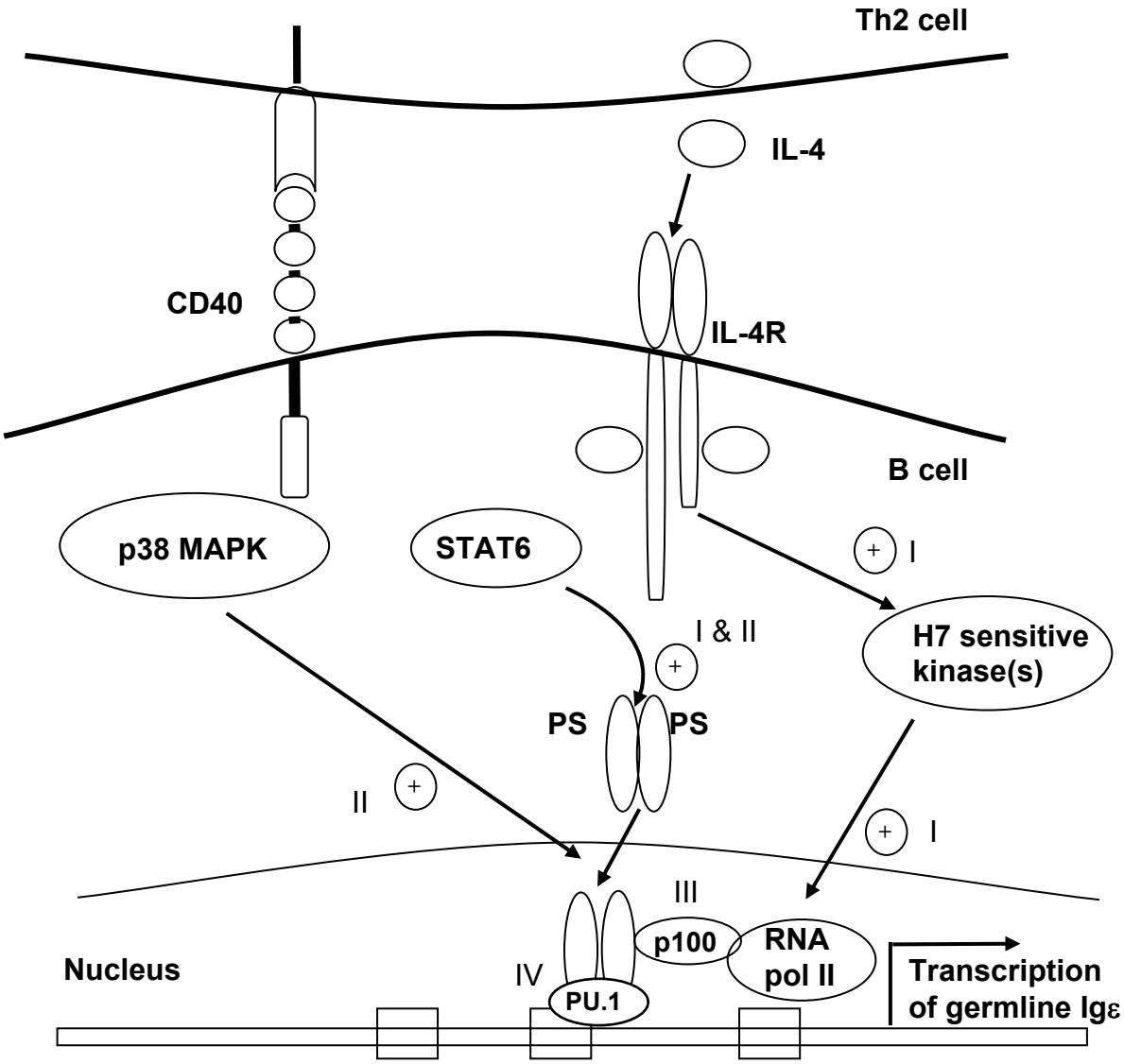

Figure 4. Main findings of the study. Original communications are referred to by their Roman numerals I-IV.

10. ACKNOWLEDGEMENTS

This study was carried out in the Laboratory of Molecular Immunology at the Institute of Medical Technology, University of Tampere. I wish to thank the head of the institute, Professor Olli Silvennoinen, M.D. Ph.D. for providing excellent working facilities and patient supervision. His positive attitude and vast knowledge on cytokine signaling got me through this journey. I am grateful to Olli for giving me the opportunity to evolve as a researcher under his guidance.

I am most thankful to my co-authors Professor Kalle Saksela M.D., Ph.D., Panu Kovanen, M.D., Ph.D., Jie Yang, M.D., Ph.D., Anssi Lagerstedt, M.D., Kati Takaluoma, M.Sc., Saara Aittomäki, M.Sc., Tuuli Välineva, M.Sc., Nisse Kalkkinen Ph.D., Juhani Saarinen, Ph.D., Kara L. Carter, Ph.D. and Elliott Kieff, M.D., Ph.D. for their contribution to the original publications. In addition, my deepest feelings of gratitude go to Ms. Paula Kosonen who has always been very kind and helpful.

Professors Riitta Lahesmaa M.D., Ph.D. and Jukka Pelkonen M.D., Ph.D. are acknowledged for their invaluable comments on this thesis.

I also want to thank the past and present fellow researchers at the Laboratory of Immunology, Panu Kovanen, M.D., Ph.D., Kati Takaluoma, M.Sc., Saara Aittomäki, M.Sc., Ilkka Junttila, M.D., Pipsa Saharinen, Ph.D., Kirsi Paukku, M.Sc., Matti Laakso, M.Sc., Jie Yang, M.D., Ph.D., Daniela Ungureanu, M.Sc., Claire Thompson, M.Sc., Sari Vanhatupa, M.Sc., Tuuli Välineva, M.Sc., Surinder Kaur Ph.D., Pia Isomäki, M.D., Ph.D., and others, for making the Lab years cozy. In particular, I am indebted to Kati and Saara for both countless pieces of advice and all the nice moments together. Panu has been a true friend and a scholar. Ilkka is cordially acknowledged for his continuous support and brainstorms that have kept me sane.

Janne Jauhiainen, B.A. is warmly thanked for the careful revision of the language, as well as for a long-time friendship.

I am obliged to Docent Ari Miettinen M.D., Ph.D. who initially gave me the privilege to get acquainted with the world of clinical microbiology. Working under the excellent supervision of Docent Risto Vuento, M.D., Ph.D. and Anna-Maija Haapala, M.D., Ph.D. in the friendly atmosphere of the Department of Clinical Microbiology has been very pleasant at all times.

During the course of this work I have had the pleasure to come together with most fascinating people. All the friends in IMT, UTA and TAUH are warmly remembered. Besides sharing the daily routines at the institute, I have greatly enjoyed the refreshing moments on play courts and in the cafeteria. Especially, Docent Hans Spelbrink, Ph.D. is acknowledged for being such an enthusiastic opponent in sport, and Kimmo Savinainen, M.Sc., for the talks on the prospects of the institute. Olli Kajander, M.D., Ph.D., has been a good friend during the years -thank you.

Finally, I extend my feelings of gratitude to my friends outside the scientific community and to my family. Most importantly, I thank my wife Kristiina for love, and our wonderful daughter Venla for everything.

This study has been supported by grants from the Medical Research Fund of the Tampere University Hospital, the Tampere University Foundation, the Finnish Cultural Foundation, the Finnish Society of Hematology, the Centenary Foundation of Kymi Corporation, the Finnish Medical Society Duodecim, AstraZeneca Finland, the Research and Science Foundation of Farmos and the Science Fund of the City of Tampere.

Tampere, April 2003

Marko Pesu

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