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Molecular Mechanisms of IL-4 Induced Transcription

Characterization of Coregulatory Proteins of STAT6

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ACADEMIC DISSERTATION
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Contents

List of original communications

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

I Pesu M., Aittomäki S., **Välineva T.**, Silvennoinen O. PU.1 is required for transcriptional activation of the Stat6 response element in the Igepsilon promoter. *Eur J Immunol* 33(6): 1727-35, 2003.[1]

II **Välineva T.**[2], Yang J.[2], Palovuori R., Silvennoinen O. The transcriptional co-activator protein p100 recruits histone acetyltransferase activity to STAT6 and mediates interaction between the CREB-binding protein and STAT6. *J Biol Chem* 280(15): 14989-96, 2005.

III **Välineva T.**, Yang J., Silvennoinen O. Characterization of RNA helicase A as component of STAT6-dependent enhanceosome. *Nucleic Acids Res* 34(14): 3938-46, 2006.

IV Yang J., **Välineva T.**, Hong J., Bu T., Yao Z., Jensen O.N., Frilander M.J., Silvennoinen O. Transcriptional co-activator protein p100 interacts with snRNP proteins and facilitates the assembly of the spliceosome. *Nucleic Acids Res* 35(13): 4485-4494, 2007.

[1] The publication No I has also been used in the doctoral thesis of Marko Pesu.

[2] Equal contribution.

Abbreviations

15-LOX-1	15-lipoxygenase-1
ADAR	Adenosine deaminases acting on RNA
AF1	Ligand-dependent activation function 1
AF2	Ligand-dependent activation function 2
ATP	Adenosine triphosphate
BAZF	Bcl6-associated zinc finger protein
BCGF1	B-cell growth factor 1
bp	Base pair, -s
BP	Branchpoint
BRE	TFIIB-Recognition element
BRCA1	Breast cancer 1
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
BSAP	B cell activator protein
C	Carboxy
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
C/EBP	CCAAT/enhancer binding protein
CoASt6	Coactivator of STAT6
CREB	cAMP response element binding protein
CRSP	Cofactor required for Sp1
CTD	C-terminal domain
DBD	DNA binding domain
DCE	Downstream core element
DDX9	DEAD/H box 9
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
EBNA2	The Epstein-Barr virus (EBV) nuclear antigen 2
EF-2	Elongation factor-2
EJC	Exon-exon junction complex
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
Ets	E-twenty-six
FBS	Foetal bovine serum
FcεRII	Fcε receptor II
GAS	Interferon-γ-activated sequence
GH	Growth hormone
GR	Glucocorticoid receptor
GST	Glutathione-S-transferase
GTF	General transcription factor

H4	Histone 4
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMG	High mobility group protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4R	Interleukin-4 receptor
Inr	Initiator element
IRF-4	IFN regulatory factor 4
ISGF-3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK	Janus kinase
KDa	Kilodalton
LCR	Locus control region
Luc	Luciferase
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time Of Flight mass spectrometry
MAR	Matrix attachment regions
MED	Mediator
Mle	Maleless
MR	Rat mineralocorticoid receptor
MBD2a	Methyl-CpG binding domain protein 2a
mRNA	Messenger RNA
MTE	Motif ten element
N	Amino
NCoA	Nuclear coactivator
NDHII	Nuclear DNA helicase II
NF-kB	Nuclear factor kB
NP-40	Nonidet P40
OB	Oligonucleotide/oligosaccharide binding
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribosyl)polymerase
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PGC-1α	PPARγ coactivator-1α
PIAS	Protein inhibitors of activated STAT
PIC	Pre-initiation complex
PMSF	Phenylmethylsulfonyl fluoride
PolII	RNA polymerase II
PP2A	Protein phosphatase 2A
PRL	Prolactin
PSF	Polypyrimidine tract-binding protein-associated splicing factor
P-TEFb	positive transcription elongation factor b
PTP	Protein tyrosine phosphatase
PTP-BL	PTP-basophil like

QPCR	Quantitative PCR
RE	Response element
RHA	RNA helicase A
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Real-time PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Src homology
SHP-1	Src homology phosphatase-1
siRNA	small inhibitory RNA
SN	Staphylococcal nuclease
SND1	Homo sapiens staphylococcal nuclease and tudor domain containing 1
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleoprotein
SAR	Scaffold attachment regions
SF1	Splicing factor 1
SKIP	Ski-interacting protein
SMN	Survival of Motor Neuron
SOCS	Suppressor of cytokine signalling
SRC	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
SWI/SNF	Switch/sucrose nonfermentable family of proteins
TAD	Transactivation domain
TAFs	TBP-associated factors
TBP	TATA-binding protein
TD	Tudor domain
TFII	Transcription factor for RNA Pol II
TPO	Thrombopoietin
Th	T helper
Th0	Naïve T cells
TMG	Trimethylguanosine
tRNA	Transfer RNA
TCPTP	T-cell PTP
TSN	Tudor-SN domain of p100
U2AF	U2 snRNP auxiliary factor

Abstract

Signal transducer and activator of transcription 6 (STAT6) is a main regulator of Interleukin-4 (IL-4)-induced gene responses. The promoter regions of IL-4-inducible genes contain *cis*-acting elements for several transcription factors that act in concert with STAT6 and are also likely to modulate lineage-specific gene expression. In addition, STAT6 is expected to interact with transcriptional coregulators, which function either by connecting STAT6 to the basal transcription machinery, or by modifying the chromatin structure at the promoters. The ability of STAT6 to activate transcription appears to be strongly dependent on the presence of other transcription factors and coregulators.

The main aim of this thesis project has been to investigate the mechanism of STAT6 mediated transcriptional activation by identifying and characterizing transcriptional coregulators of STAT6. We identified a minimal low-affinity PU.1-core-binding sequence within the STAT6 DNA-binding site in the Igε promoter. Our results suggest that STAT6 and PU.1 co-operate in transactivation of the Igε gene, and the presence of PU.1 in transcription activation complex contributes to the cell type-specific expression of IL-4-responsive genes. We have also identified novel STAT6 transactivation domain (TAD) interacting nuclear proteins. The first of the identified proteins was transcriptional coactivator protein p100 which was found to be an important regulator of STAT6 mediated transcription. In the present study, we identified another novel member of STAT6 enhanceosome, RNA helicase A (RHA). Our results suggest that RHA acts as a coactivator for STAT6 in IL-4 induced STAT6 mediated transcription, and the coactivating function is dependent on the helicase activity of RHA. Previously identified coactivator p100 was found to bridge STAT6 and RHA. To further investigate the mechanism of STAT6 mediated transcriptional activation, we investigated, how STAT6 recruits chromatin modifying activities to the promoter. Coactivator proteins CBP/p300 have been shown to be required for STAT6-mediated gene activation, but the underlying molecular mechanisms are still elusive. Our results suggest that p100 has an important role in the assembly of STAT6 enhanceosome, and that p100 stimulates IL-4-dependent transcription by mediating interaction between STAT6 and CBP and recruiting chromatin modifying activities to STAT6-responsive promoters. These studies demonstrated that p100 has a central role in STAT6 enhanceosome. p100 is composed of four Staphylococcal nuclease (SN)-like domains and a C-terminal TSN domain. The SN-like domains of p100 have been shown to function in transcription, but the function of TSN domain has remained elusive. To analyze the function of TSN domain, we identified by MALDI-TOF TSN domain interacting proteins. p100 TSN was found to interact with small nuclear ribonucleoproteins (snRNPs) that function in pre-mRNA splicing. TSN domain of p100 was also found to enhance the kinetics of the spliceosome assembly.

These results suggest that p100 protein functions as a dual function regulator of gene expression that participates via distinct domains in both transcription and splicing processes.

Lyhennelmä

Interleukiini (IL) -4 on sytokiini, jolla on useita tehtäviä hematopoeettisten solujen kasvun ja immunologisen vasteen säätelyssä. IL-4:n vaikutukset solussa välittyvät pääasiassa STAT6 (Signal transducer and activator of transcription 6) transkriptiotekijän aktivoitumisen kautta. Aktivoitunut STAT6 sitoutuu IL-4-indusoituvien geenien säätelyalueille ja käynnistää geenien luennan. STAT6:n lisäksi säätelyalueille sitoutuu lukuisia muita transkriptiotekijöitä, joiden arvellaan osaltaan vaikuttavan geenien ilmentymiseen eri solu- ja kudostyypeissä. Transkriptiotekijöiden lisäksi geenien ilmentymistä säätelee joukko erilaisia koregulaattoriproteiineja. Nämä sitovat STAT6:n basaalitranskriptiokoneistoon, tai vaikuttavat kromatiinirakenteeseen säätelyalueilla. Koregulaattoriproteiineilla ja säätelyalueelle sitoutuvilla transkriptiotekijöillä on tärkeä merkitys STAT6-välitteisen geeniluennan käynnistymisessä.

Tämän tutkimusprojektin tärkein tavoite on ollut tutkia STAT6-välitteisen transkriptioaktivaation mekanismeja pyrkimällä tunnistamaan ja tutkimaan muita STAT6-välitteisessä transkriptiossa toimivia säätelyproteiineja. Tutkimuksessa identifioimme immunoglobuliini E-geenin (Igε) promoottorialueella STAT6:n sitoutumiskohdan sisällä sijaitsevan uuden sitoutumiskohdan PU.1-transkriptiotekijälle. Tulostemme perusteella PU.1 ja STAT6 toimivat yhteistyössä Igε-geenivasteen käynnistämiseksi, ja PU.1:n ilmentyminen vaikuttaa IL-4-responsiivisten geenien spesifiseen ilmentymiseen tietyissä solutyypeissä. Olemme myös identifioineet uusia tumaproteiineja jotka sitoutuvat STAT6:n transaktivaatiodomeeniin (TAD). Ensimmäinen identifioiduista proteiineista oli koaktivaattoriproteiini p100, joka osoittautui tärkeäksi STAT6-välitteisen geeniaktivaation säätelijäksi. Tässä tutkimuksessa olemme tunnistaneet toisen STAT6-transkriptiokompleksissa sijaitsevan tumaproteiinin, RNA helikaasi A:n (RHA). Tulostemme mukaan RHA toimii p100:n tavoin STAT6:n koaktivaattorina. RHA:n toiminta koaktivaattorina on riippuvainen sen helikaasiaktiivisuudesta. Aiemmin tunnistamamme koaktivaattori p100 toimii transkriptiokompleksissa adapterina RHA:n ja STAT6:n välillä. Selvittääksemme tarkemmin STAT6-välitteisen geeniaktivaation mekanismeja, tutkimme miten STAT6 toimii yhteistyössä kromatiinirakennetta muokkaavien proteiinien kanssa. Koaktivaattori CBP/p300:n on aiemmin osoitettu olevan välttämätön STAT6-välitteisen geeniluennan käynnistymiselle, mutta mekanismi, jolla CBP/p300:n histoniasetylaatioaktiivisuus välittyy promoottorille, on jäänyt avoimeksi. Tulostemme perusteella koaktivaattori p100 toimii adaptorina myös CBP:n ja STAT6:n välillä, ja välittää siten CBP:n entsyymiaktiivisuutta transkritiokompleksissa. Tutkimuksemme perusteella p100:lla on erittäin keskeinen rooli STAT6-transkriptiokompleksissa. p100 koostuu neljästä

Staphylococcal Nuclease (SN) like-domeenista ja C-terminaalisesta TSN-domeenista. p100:n SN-like-domeenit toimivat tutkimustemme mukaan transkriptiossa, kun taas C-terminaalisen TSN–domeenin toimintaa ei tunneta. Selvittääksemme TSN-domeenin toimintaa, identifioimme massaspektrometrian avulla tähän domeeniin sitoutuvia proteiineja. Havaitsimme, että lähetti-RNA:n silmukoinnissa toimivat snRNP-proteiinit (small nuclear ribonucleoproteins) sitoutuvat TSN-domeeniin. TSN:n havaittiin toimivan mRNA:n silmukoinnissa, nopeuttaen silmukointikompleksin kokoamista. Näiden tulosten perusteella p100 säätelee geenien ilmentymistä sekä transkriptiossa että mRNA:n silmukoinnissa eri domeeniensa kautta.

Introduction

Eukaryotic genomes contain several thousands of protein- and RNA-encoding genes. The genes may be ubiquitously expressed or expressed in a tightly controlled manner under particular conditions during development or disease, or in a certain part of the organism. The expression is regulated at multiple levels, including transcription and mRNA processing.

At the level of transcriptional activation the gene expression is mainly regulated by sequence-specific DNA binding transcription factors, which are expressed and activated in response to extra- and intracellular stimuli. Enhanceosome complexes that are formed around sequence specific transcription factors recruit the basal transcription machinery to the gene promoters. The spatial and temporal differences in gene expression are largely determined by the protein composition of these complexes. The modular nature of enhanceosomes provides the cell versatility to respond to changing signals and conditions in its environment.

Transcription and RNA editing, such as capping, polyadenylation and splicing, are integrated processes and several proteins have been identified in both transcription initiation and different RNA processing complexes. Especially RNA Polymerase II has been solidly established to function in both of these processes. Increasing evidence also connects transcriptional coactivators into RNA editing processes. However, the mechanisms have still remained largely unknown.

The present work focuses on the Signal Transducer and Activator of Transcription 6 (STAT6) mediated transcriptional regulation. STAT6 is a member of STAT protein family, and an important mediator of interleukin-4 (IL-4) responses. Activated STAT6 binds to the promoter elements of IL-4 responsive genes regulating their expression. The transcriptional regulation on the IL-4 responsive promoters requires binding of STAT6 to a conserved sequence motif, but STAT6 binding alone is usually not sufficient for transcriptional activation. A set of other transcription factors and coregulators specific for each cell line is necessary for gene activation. Specific interactions and functional cooperation between different regulators are required to obtain efficient transcriptional response to IL-4 stimulus. The main aims of the present investigation have been to gain information on the composition of the STAT6 enhanceosome complex and the mechanisms of function of its individual members. Identification and characterization of transcriptional coregulators in enhanceosome complexes will help in understanding both the mechanisms of IL-4 regulated gene activation and also general gene regulation in cells.

Review of the literature

1. Transcriptional regulation of protein coding genes

1.1. Basal transcription machinery

Eukaryotic mRNA synthesis is a complex multistep process, in which the RNA polymerase II (PolII) containing basal transcription apparatus is the key player. In addition to PolII the basal transcription machinery consists of several other general transcription factors (GTFs), TFIID, TFIIA, TFIIB, TFIIF, TFIIH and TFIIE. During the initiation of transcription, PolII and general transcription factors assemble into a large protein complex, transcription pre-initiation complex (PIC), which specifies the transcription start site (Dvir, Conaway and Conaway, 2001, Hahn, 2004).

Human PolII consists of 12 subunits, which have diverse functions. Different subunits are responsible for start site selection, determining the transcription elongation rate and interactions with transcriptional activators. RNA PolII contains a highly conserved domain (CTD) at the C-terminus of the largest subunit. CTD contains multiple copies of a consensus sequence YSPTSPS. Several kinases can phosphorylate serines in these sequences, and the CTD mainly exists in two different phosphorylation states. The phosphorylation state largely determines the function of PolII in different phases of gene expression, and an important switch in the phosphorylation state occurs between transcription initiation and elongation (Myer and Young, 1998).

GTFs assist RNA PolII in the promoter recognition. TFIID consists of TATA binding protein (TBP) and TBP-associated factors (TAFs), which are required for promoter selection. TFIID recognizes TATA box, Initiator (Inr) and downstream promoter element (DPE) of the core promoter (Orphanides, Lagrange and Reinberg, 1996). TFIIB bridges promoter bound TFIIE and PolII and modulates the catalytic center of PolII, and may be a target for transcriptional activators that stimulate PIC assembly (Deng and Roberts, 2007).

1.2. Regulatory elements

Eukaryotic protein coding genes are regulated by *cis*-acting regulatory elements, DNA sequences in the vicinity of the structural portion of a gene that via *trans*-acting elements or factors regulate the expression of genes on the same chromosome. These elements include promoters, which consist of a core promoter and the proximal promoter elements, and distal regulatory elements, such as enhancers, silencer, insulators or locus control regions (LCRs). The concerted action of these regulatory elements determines the spatial and temporal expression pattern of a gene (Maston, Evans and Green, 2006).

The core promoters are located around the transcription start site. Many genes may have multiple start sites, which reside close to each other (Sandelin et al., 2007). The core promoters serve as recognition sequences for the basal transcription machinery and as platforms for PIC assembly. The core promoters may contain a conserved TATA box, but also other regulatory elements, such as the Initiator element (Inr), Downstream Promoter Element (DPE), Downstream Core Element (DCE), TFIIB-Recognition Element (BRE) or Motif Ten Element (MTE). These elements function as binding sites for general transcription factors (Maston, Evans and Green, 2006, Smale and Kadonaga, 2003, Smale, 2001).

TATA boxes can be found from strong, tissue specific promoters, and they often occur together with an Inr element. TATA box comprises of the TATAA consensus sequence, and it functions as the binding site for TATA binding protein (TBP), a member of PIC. TATA boxes are located ~30bp upstream of the transcription start site (Schug et al., 2005). Core promoters may also contain CpG islands, genomic stretches rich in CG nucleotides. CpG islands are more often linked to more widely expressed genes, such as housekeeping genes (Sandelin et al., 2007, Schug et al., 2005). Overall, the core promoter diversity may contribute to the combinatorial regulation with other regulatory elements (Maston, Evans and Green, 2006, Smale and Kadonaga, 2003, Smale, 2001).

The proximal promoter elements are located immediately upstream of the core promoter, and function as binding sites for different transcriptional activators (Maston, Evans and Green, 2006, Walhout, 2006). Eukaryotic enhancer elements are functionally similar to proximal promoters, and are composed of a cluster of transcription factor binding sites. However, enhancers, on the contrary to the proximal promoters, are located long genomic distances away from their target genes. Transcription factors bound to enhancers and the basal transcription machinery located at the core promoter can interact as a result of looping of the intervening DNA. Different enhancer elements may act in concert with a single promoter in response to changing conditions and stimuli (Blackwood and Kadonaga, 1998). Enhancers are also able to activate several genes located in the same chromosomal region (West and Fraser, 2005). It has been suggested that PIC assembly may in some cases be initiated at the enhancers, and the pre-assembled complex may then contact the core promoter (Szutorisz, Dillon and Tora, 2005).

Silencers mediate the opposing effect on transcription as enhancers, and function as binding sites for repressors. Silencers can be located at the proximal promoter, as a part of an enhancer element, in gene introns or as independent regulatory modules (Maston, Evans and Green, 2006).

LCRs can be defined as groups of regulatory elements, such as enhancers, silencers, insulators and nuclear-matrix or chromosome scaffold attachment regions (MARs or SARs), that direct tissue-specific, physiological expression of a gene. They function in the regulation of an entire gene locus or gene cluster, and consist of multiple *cis*-acting elements, which bind transcription factors and transcriptional coregulators. LCRs regulate gene expression from a distance, and their mode of action may also involve DNA looping (Maston, Evans and Green, 2006).

Insulators are boundary elements that prevent certain genes from being activated by other regulatory elements, such as enhancers or LCRs. The precise mechanisms of insulator function are not known, but insulators are often located between distal regulatory elements and promoters, and may function by blocking the communication between them. Since LCRs and enhancers have a potential to activate all the neighbouring genes in a gene locus, insulator function is crucial in restricting the unnecessary activation. Another suggested mechanism of function may be in regulation of self-propagating chromatin condensation processes. Transcriptionally repressed condensed chromatin has been suggested to be buffered from more open chromatin structures by insulator elements, which prevent the spread of the silencing effect, when positioned between closed chromatin and a gene promoter (West and Fraser, 2005).

1.3. Epigenetic regulation of gene expression

Eukaryotic DNA is packaged into nucleoprotein complex called chromatin to fit to the limited amount of space available in the nucleus. The most abundant proteins in the chromatin are the histones termed H1, H2A, H2B, H3 and H4. In a nucleosome DNA is wrapped around a histone octamer consisting of two copies of H2A, H2B, H3 and H4. Positively charged histones interact with the phosphate backbone and the deoxyribose moieties of DNA. Linker DNA connects adjacent nucleosomes into a beads-in-a string –like structure. These structures are further coiled to higher-order chromatin structures as a result of nucleosome interactions. The N-terminal histone tails protrude from nucleosomes, and can form contacts with other nucleosomes or linker DNA (Kadam and Emerson, 2002).

The organization of DNA into nucleosomes is essential in regulating gene expression. The packaged chromatin is transcriptionally repressed, because of the decreased accessibility of regulatory proteins and the basal transcription machinery for their binding sites within the nucleosome. Transcriptionally active chromatin forms as a result of multiple structural modifications that are catalyzed by ATP-dependent chromatin remodeling or histone modifying protein complexes. Core histones of the nucleosomes can also be substituted by variant histones, resulting in changes in gene activation (Felsenfeld and Groudine, 2003).

Chromatin remodeling enzymes exist as large protein complexes, and function by disrupting nucleosomal structure by ATP-dependent hydrolysis. Several families of mammalian chromatin remodeling complexes have been identified, one of the best studied is SWI-SNF. SWI-SNF can function selectively to regulate specific genes. The specificity is conveyed by specific protein-protein interactions between distinct subunits of SWI-SNF and DNA-binding transcription factors, which direct the remodeling activity to a specific gene (Sudarsanam and Winston, 2000, Vignali et al., 2000).

Histone proteins can be subjected to multiple posttranslational modifications on histone tails or within the body of the histone octamer that can activate or repress gene expression. The modifications, such as acetylation, deacetylation, methylation, demethylation, phosphorylation and ubiquitination, are catalyzed by specific enzymes that function in concert with additional factors affecting chromatin structure and function (Felsenfeld and Groudine, 2003, Kadam and Emerson, 2002).

Acetylation and deacetylation of histone proteins H3 and H4 is strongly linked to the transcriptional activity. Histones associated with actively transcribed genes exist mainly in acetylated state, whereas hypoacetylation of histones is generally linked to the transcriptionally repressed form of chromatin, heterochromatin (Roth, Denu and Allis, 2001). The protruding histone tails contain positively charged lysine groups, which can be reversibly acetylated or deacetylated by specific enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs). Acetylation neutralizes the positive charge of lysines, preventing DNA binding and reducing chromatin condensation. Therefore, histone acetylation opens up the chromatin structure making the DNA more accessible to the proteins required for transcription (Shahbazian and Grunstein, 2007).

Histone acetylation and other modifications may also affect the flexibility of chromatin, and therefore assist in chromatin loop formation. Chromatin loop formation is associated with gene activation by remote enhancers. Histone modifications have been suggested to modulate the distribution of the preferential looping sites in the chromatin and determine the propability of interaction between an enhancer and the target gene (Li, Barkess and Qian, 2006).

Histone modifications and chromatin remodeling processes occur in a coordinated fashion. In many cases, both processes are required, and the order of effector recruitment is crucial for appropriate timing of gene expression (Felsenfeld and Groudine, 2003).

1.4. Transcription factors and transcriptional coregulators

In addition to the basal transcription apparatus, sequence specific transcription factors and various coregulatory proteins are necessary to recognize specific promoters and recruit the basal transcription apparatus to the transcription initiation site (Kingston, Bunker and Imbalzano, 1996, Ptashne and Gann, 1997).

1.4.1. Transcription factors

Differential gene expression is achieved in part during the initiation of transcription by the action of transcription factors, *trans*-acting proteins that control gene expression via intermediary proteins or RNA they encode. Transcription factors bind to *cis*-regulatory elements often located in or near their target genes. The transcription factors usually regulate many targets forming

complex regulatory networks, which integrate to control the development, function, and pathology of the system (Walhout, 2006). Unique composition and spatial arrangement of transcription factors bound to a promoter-enhancer region is required for transcription initiation, and contributes to the specificity on the level of transcription (Chen, 1999).

Structural and functional studies have indicated that transcription factors are modular proteins that employ distinct regions for different functions. Transcription factors may contain for example DNA binding domains, multimerization domains and effector domains, which regulate transcriptional activation or repression (Ptashne and Gann, 1998, Tjian and Maniatis, 1994). Transactivation domains (TADs) rich in proline, glutamine, or acidic amino acids are typical for eukaryotioc transcription factors. TADs often exist in an unstructured form before binding to the interacting proteins, which has hindered their structural characterization (Chen, 1999).

1.4.2. Coregulators

Sequence specific transcription factors activate or repress transcription of a gene. However, in many cases sequence specific DNA binding is not sufficient to engage or repress transcription, but the functional cooperation of transcription factors and coregulatory proteins is needed for efficient gene activation or repression. Cellular factors called coregulators (coactivators and corepressors) are necessary for this purpose (Naar, Lemon and Tjian, 2001).

Coregulators can be defined as factors that regulate transcriptional activity without binding to DNA. Coregulators usually exist as protein complexes, which comprise of number of different components with distinct activies and modes of action. Coregulators are modularly assembled as distinct complexes, which share different subunits. The composition of a complex is believed to vary between different genes, and to have a significant effect on the specificity and extent of the gene activation or repression (Naar, Lemon and Tjian, 2001).

Based on their function, transcriptional coregulators can be grouped to primary and secondary coregulators. Primary coregulators are often enzymes, which employ their activity to regulate transcription, and directly interact with transcription factors. Primary coregulators include many chromatin remodelling or modifying enzymes that open up chromatin structure helping other transcriptional regulatory proteins and PIC to access the promoter. These coregulators often unpack the chromatin structure by acetylating histones, or unwind the dsDNA with their helicase activity. Secondary coregulators function as adaptors or scaffolds that bridge primary coregulators, transcription factors and basal transcription machinery together (Spiegelman and Heinrich, 2004).

Corepressors reduce the transcription of a gene. Their mode of function is usually the opposite from chromatin modifying coactivators: corepressors can disrupt activating interactions, recruit inhibitory enzymes to promoters or otherwise prevent chromatin structure from opening. Many corepressors are

histone deacetylases, but also other mechanisms of corepressor mediated silencing exist (Kumar, Wang and Barnes, 2004).

Mediator is an important coactivator complex that consists of approximately 30 proteins (Sato et al., 2004). Mammalian mediator complex is phylogenetically related to the yeast Mediator complex, and similarly expected to be composed of a core sub-complex and several groups of proteins that constitute different functional modules. As in yeast, mammalian Mediator seems to be required for the expression of almost all the genes (Malik and Roeder, 2005).

The Mediator potentiates transcriptional responses by facilitating PIC assembly. However, certain mammalian Mediator modules have also been implicated in gene repression. The versatile modes of function and the combinatorial assembly of Mediator modules may have a role in fine-tuning the regulatory signals. Generally, it has been suggested that activator binding triggers the recruitment of preassembled Mediator, which in turn promotes PolII recruitment, and PIC assembly. Mediator may also generate a stable scaffold-like structure at the promoter that primes the system for multiple rounds of transcription (Malik and Roeder, 2005).

Mediator forms a network of interactions with basal transcription machinery, transcription factors and other cofactors. Mammalian Mediator interactions have been most extensively studied in the context of nuclear receptors. Mediator subunits have been shown to directly associate with the CTD of PolII (Naar, Lemon and Tjian, 2001), and possibly also with other domains of PolII (Gu et al., 1999, Sun et al., 1998). The AF2 (activation function 2)-containing activation domains of the nuclear receptors interact with LxxLL motifs of MED1 subunit to recruit the Mediator complex (Malik et al., 2004, Ren et al., 2000). In various mammalian species and nuclear receptor signaling pathways, Mediator recruitment has also been shown to be facilitated by interactions of E1A and Elk-1 with MED23 subunit (Stevens et al., 2002), VP16 with MED17 and MED25 (Mittler et al., 2003, Yang et al., 2004), and AF1 domain of nuclear receptor with MED14 (Hittelman et al., 1999). Although chromatin modifying coactivators have been shown to arrive at the promoters before Mediator, transient interactions may occur during the transitions between the different phases of transcription initiation. For example PPARγ coactivator-1α (PGC-1α) promotes the recruitment and function of chromatin modifying coactivators SRC1 and CBP/p300 (Puigserver et al., 1999). Through a distinct domain it also facilitates the Mediator recruitment by interacting with MED1 subunit (Wallberg et al., 2003).

1.4.3. Enhanceosomes

Higher-order three dimensional nucleoprotein complexes that consist of transcription factors, coregulators and enhancers are often called enhanceosomes. Enhanceosomes are assembled from vast array of enhancer and protein modules. They are often formed around sequence specific transcription factors that are endpoints of signal transduction pathways and become activated

in response to specific stimuli. The transcriptional activation by enhanceosomes is based on delicate and precise protein-protein and protein-DNA interactions. The specificity of gene expression is largely conveyed by the composition of the enhanceosome complexes (Merika and Thanos, 2001, Ptashne and Gann, 1998, Thanos and Maniatis, 1995).

One of the best studied enhancers is the the virus-inducible IFNβ enhancer (Thanos and Maniatis, 1995). This enhancer is positioned between two nucleosomes, and has been shown to contain binding sites for NF-κB, IRF, and ATF/c-Jun proteins. All these factors together with the high mobility group protein HMG I(Y) protein have been shown to be necessary for the transcriptional activation of the IFNβ gene. HMG I(Y) protein has an essential role in the precisely regulated and coordinated assembly of IFNβ enhanceosome. HMG I(Y) was shown to have three binding sites within the enhancer, and to promote the recruitment of other factors to the enhanceosome complex. The formed enhanceosome was then shown to recruit distinct coactivators with chromatin remodelling activity to the complex, as well as the members of the basal transcription machinery (Agalioti et al., 2000). The assembly of IFNβ enhanceosome may be characteristic to other inducible enhanceosomes, although the spatial organization of the enhancer elements and transcription factor binding sites vary considerably (Thanos and Maniatis, 1995).

1.5. Transcription and pre-mRNA processing

1.5.1. Splicing

The majority of eukaryotic genes are interrupted by introns. The primary transcript, pre-mRNA, formed in transcription, contains coding sequences, exons, disrupted by intervening sequences, introns. Prior to translation, the introns are removed from pre-mRNA and exons joined in the splicing process, which occurs in the nucleus. The transcript is also capped at the 5' end and polyadenylated at 3' end. Mature mRNA is then exported from the nucleus to the cytoplasm (Hastings and Krainer, 2001, Proudfoot, 2003).

The mechanism of splicing involves several stages that have been characterized *in vitro*. In the first stage the transcript is cut from 5' splice site, separating left exon and the right intron exon pair. Left exon exists as a linear molecule whereas the right intron exon pair forms a structure called a lariat. In the lariat the 5' point end of the intron becomes attached to a base within the intron. In the second stage of the splicing process, a cut is made to the 3' splice site, which releases the intron in lariat form from the right exon. Exons are ligated together, and the released intron is reverted to linear form and rapidly degraded (Brow, 2002, Kramer, 1996).

To define an intron, three *cis*-acting elements are required: the 5'splice site, the 3' splice site, and the branchpoint/polypyrimidine-tract just upstream of the 3'

splice site. The 5' and 3' splice sites define the exon/intron boundaries while the branch point provides an adenosine for nucleophilic attack on the 5' splice site.

Introns are removed from pre-mRNA transcripts by a splicing apparatus that recognizes short consensus sequences located at exon-intron boundaries and within the intron. Splicing apparatus is a complex protein-RNA complex, which consists of five uridine-rich ribonucleoproteins named U1, U2, U4, U5, and U6 snRNPs and several hundreds of non-snRNP proteins that function coordinatively as a complex called the spliceosome (Kramer, 1996). Over 300 putative protein components of the spliceosome have been identified by proteomic screens (Jurica and Moore, 2003) including many factors that have not been previously associated with splicing. Many of the proteins linked to splicing are involved in transcription, RNA processing or mRNA export, indicating that different steps of gene espression are tightly coupled (Sanford and Caceres, 2004). It is assumed that the vast amount of proteins are needed to position spliceosome accurately and to control different steps of the process (Kramer, 1996).

Spliceosomal assembly is a complex pathway involving numerous RNA-RNA and RNA-protein interactions. The spliceosomal assembly is initiated by U1 snRNP binding to 5' splice site. Branchpoint binding protein SF1 becomes associated with the branchpoint, and U2 snRNP auxiliary factor (U2AF) recognizes and binds to the pyrimidine tract and the 3' splice site. These factors form the E (early) pre-spliceosomal complex. SF1 dissociation and U2 snRNP binding to the branchpoint results in ATP-dependent formation of pre-spliceosomal A complex. B complex forms, when base paired U4-U6 di-snRNP binds to U5 snRNP, and associates with pre-spliceosome. The catalytically active spliceosome (C complex) forms as a result of massive RNA and protein rearrangements. As a result of these rearrangements, U2 snRNP and U6 snRNP interact, and U6 snRNP replaces U1 snRNP at the 5' splice site (Will and Luhrmann, 2001).

After splicing a stable protein complex called the exon-exon junction complex (EJC) binds to a region 20-24 nt upstream of the exon-exon junction in mRNA. The EJC stimulates mRNA export by functioning as a binding platform for export factors (Le Hir et al., 2001).

During RNA splicing, exons can also be targeted for removal in different combinations. This process is referred to as an alternative RNA splicing. Alternative splicing permits the production of multiple mRNAs from a single pre-mRNA allowing the synthesis of structurally and functionally different protein isoforms (Lopez, 1998). Differential exon selection in different tissues or during the development are thought to be caused both by differences in the local amounts or activities of general or gene-specific splicing factors, that can be activated or recruited by signal transduction pathways, and the transcription rate largely determined by the factors functioning in transcription initiation (Proudfoot, 2003).

Alternative splicing is regulated by splicing enhancers and silencers, which are *cis*-acting exonic or intronic sequence elements that can bind *trans*-acting

splicing regulators. Serine/arginine-rich (SR) proteins are important regulators of alternative splicing. SR proteins bind to splicing enhancers in exons to promote splicing (Caceres and Kornblihtt, 2002). Enhancer bound SR proteins have been suggested to function by binding to other splicing factors via their RS-domains, for example to U1 snRNP and U2AF, to recruit the splicing machinery. Recent reports however suggest, that RS-domains may directly bind to branchpoint sequence and the 5' splice site, promoting snRNA-pre-mRNA interactions and spliceosomal assembly (Shen, Kan and Green, 2004).

1.5.2. RNA Pol II and the integration of nuclear processes

The CTD of PolII is an important integrator of transcription and RNA editing. The CTD functions as a platform for transcriptional regulators and RNA processing factors that function in 5' capping, splicing, 3' end formation and mRNP assembly (Aguilera, 2005, Bentley, 2005). Truncation of CTD results in defects in all these processes (McCracken et al., 1997). The phosphorylation state of PolII CTD varies in different phases of gene expression, and many factors specifically associate with either unphosphorylated or hyperphosphorylated CTD. Several different RNA processing factors specifically associate with hyperphosphorylated CTD of elongating RNA Pol II (Hirose, Tacke and Manley, 1999, Zeng and Berget, 2000).

The connection between PolII and splicing has not been demonstrated as strongly as the linkage between other RNA editing processes, but there is accumulating evidence that the processes are coordinated and physically and functionally coupled. Previous view that these processes occur separately was based on observations, that synthetic pre-mRNAs can be accurately spliced *in vitro*. However, already the large gene size of eukaryotes and the general transcription rate supports the idea of co-transcriptional pre-mRNA splicing (Proudfoot, 2003).

A growing number of detected protein-protein interactions between splicing and transcription complexes supports the idea of connected processes (Kornblihtt et al., 2004). The cotranscriptional recruitment of factors involved in splicing is often based on their interaction with PolII (Bentley, 2005). Many proteins involved in splicing, such as $p54^{nrb}$, PSF (polypyrimidine tract-binding protein-associated splicing factor)(Kameoka, Duque and Konarska, 2004), U snRNPs and SRs (Yuryev et al., 1996) either exist in PolII complex or bind directly to CTD. In addition to the CTD, some transcriptional coregulators have been shown to associate with splicing factors. For example, the coactivator protein NCoA-62/SKIP, a chromatin remodelling factor which regulates vitamin-D mediated gene responses interacts with splicing factors hPrp8, PSF, hPrp28, h200K (yeast Brr2p) and h116K (Kornblihtt et al., 2004).

The recruitment of snRNPs has been shown to happen sequentially in yeast, but it is currently not known, whether this is the case also in metazoans. Mechanisms of splicing factor recruitment may differ between organisms, involving more

protein-protein interactions in metazoans Accordingly, in yeast, the CTD is not as essential for RNA editing as in metazoans (Bentley, 2005).

The phosphorylated CTD of elongating PolII is known to enhance the splicing process (Fong and Bentley, 2001). Relatively little is known about the mechanism by which PolII enhances splicing, but it has been suggested that it may stimulate spliceosomal assembly by bringing mRNA processing factors close to growing transcript or by regulating their activity by promoting allosteric interactions between elongation complex and RNA processing factors. In addition, it may also have a direct role in exon recognition, and the CTD may facilitate spliceosomal assembly by bringing consecutive exons closer to each other (Kornblihtt et al., 2004). The CTD may also affect the splice site selection through the regulation of transcription elongation rate (Bentley, 2005, Hirose, Tacke and Manley, 1999, Howe, Kane and Ares, 2003, Zeng and Berget, 2000, de la Mata et al., 2003).

2. Cytokine signalling

2.1. Hematopoietic cytokines and cytokine receptors

Cytokines are soluble extracellular proteins or glycoproteins that function in intercellular signalling. Cytokines are released by many different types of cells and are important for cell growth, differentiation and development as well as for innate and adaptive immune responses and inflammation. Cytokines bind to specific cell-surface receptors engaging intracellular signalling cascades that can up- or downregulate transcription factors, other cytokines and cytokine receptors, and target genes (Oppenheim, 2001, Schindler, 1999).

Hematopoietic cytokines comprise a large cytokine subfamily, and function mainly in differentiation and proliferation of blood cells. According to their function, hematopoietic cytokines can be classified as T helper type 1- (Th1) and T helper type 2- (Th2) cytokines. Th1- and Th2-type cytokines are produced by Th1 and Th2 cells respectively. Th1-type cytokines, for example interleukin-12 (IL-12) and interferon-γ (IFN-γ) enhance cell-mediated immune responses, whereas Th2-type cytokines like IL-4, IL-5, IL-10 and IL-13 are involved in humoral immune responses (Mosmann and Coffman, 1989, Mosmann, 1991).

Most of the hematopoietic cytokines signal through the receptors of the cytokine receptor superfamily. These cytokine receptors can be structurally classified into class I and II receptors based on the differences in the extracellular domains of the receptors. Class I includes the receptors for most of the interleukins, EPO, PRL, GH and TPO, whereas e.g. receptors for interferons and IL-10 belong to the class II receptors. Hematopoietic cytokine receptors are transmembrane receptors, which consist of a single or multiple receptor chains. Ligand binding results in the activation of the receptor, often by dimerization of receptor

subunits, which initiates the intracellular signalling cascade (Karnitz and Abraham, 1995, Touw et al., 2000).

2.2. Signalling through the JAK-STAT pathway

The effect of a given cytokine is dependent upon the downstream signals that are activated by receptor binding. Hematopoietic cytokines employ JAK-STAT signalling pathway to elicit their biological effects (Figure 1.). Ligand binding results in dimerization of receptor subunits, allowing interactions between adjacent cytoplasmic domains. JAK kinases (Janus kinases) associate with the membrane-proximal cytoplasmic receptor domains, and their activation is required for downstream signalling events (Schindler, 1999).

JAK family of tyrosine kinases consist of JAK1, JAK2, JAK3 and TYK2. Different cytokine receptors employ a specific combination of JAKs, although JAKs may not have a significant effect on signalling specificity (Ihle, 2001, Kisseleva et al., 2002). Receptor dimerization allows JAKs to interact and initiate their transphosphorylation on specific receptor tyrosine motifs. Receptor phosphorylation creates docking sites for downstream signalling proteins, most importantly the members of Signal Transducer and Activator of Transcription (STAT) family of proteins. STATs are recruited to the receptor and phosphorylated on a single C-terminal activating tyrosine residue by JAKs. Phosphorylation results in the dimerization of STATs, and subsequent translocation into the nucleus. In the nucleus STATs bind to specific enhancer elements. STAT homodimers bind to a motif termed a GAS (γ activated sequence) element (TTTCCNGGAAA). Heterodimeric complexes of STAT1 and STAT2 may also bind to ISRE enhancers together with IRF-9 forming an ISGF-3 complex (O'Shea, Gadina and Schreiber, 2002, Schindler, Levy and Decker, 2007).

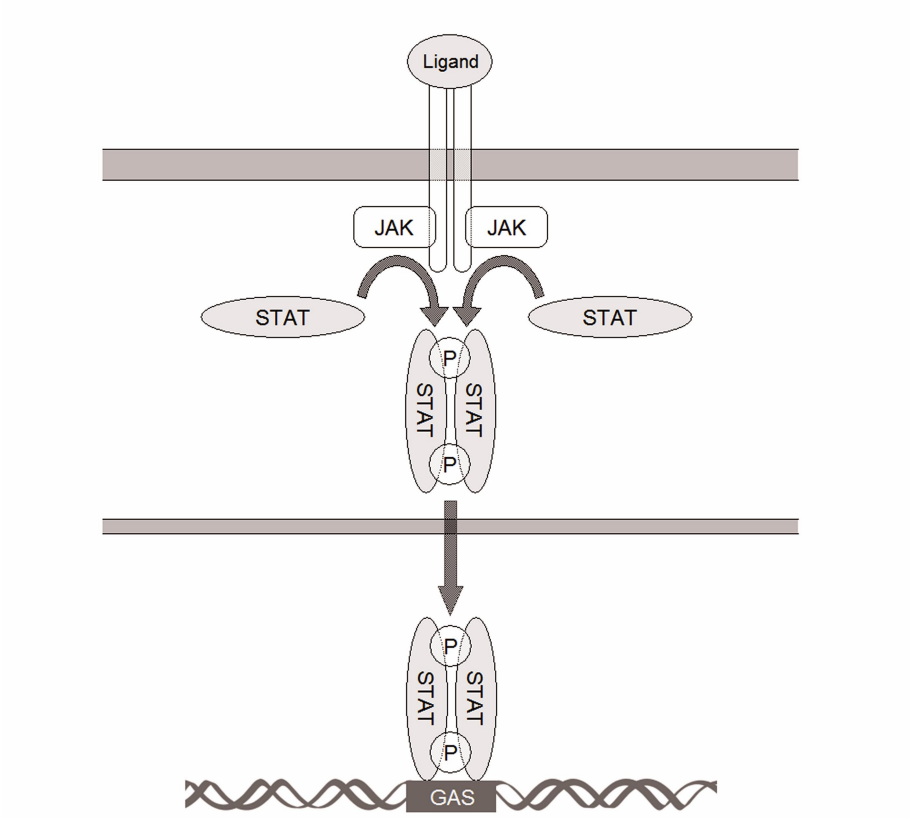

Figure 1. Schematic representation of the JAK-STAT pathway.

2.3. Signal Transducer and Activator of Transcription (STAT) family of proteins

2.3.1. Overview of STATs

The *STAT* gene family has been well conserved throughout the evolution, and in addition to mammals, *STAT* genes have been identified for example in *Drosophila*, *Anopheles*, *Caenorhabditis elegans* and *Dictyostelium*. Seven different *STAT* genes (*STAT1*, *STAT2*, *STAT3*, *STAT4*, *STAT5a*, *STAT5b* and *STAT6*) exist in mammals. Mammalian STAT genes have been born as a result of gene duplication, translocation and divergence, the genes are clustered to three chromosomal positions. *STAT* homologues of lower eukaryotes are most closely related to mammalian *STAT3* and *STAT5* (Kisseleva et al., 2002, Schindler, 2002).

2.3.2. Domain organization of the STATs

The STAT proteins vary in size between 734-851 amino acids. Approximately 700 N-terminal amino acids of all the STATs are relatively homologous (Ihle et

al., 1995). The principal domain organization among STATs is identical, and STATs can be divided into six structurally and functionally conserved domains (Figure 2.).

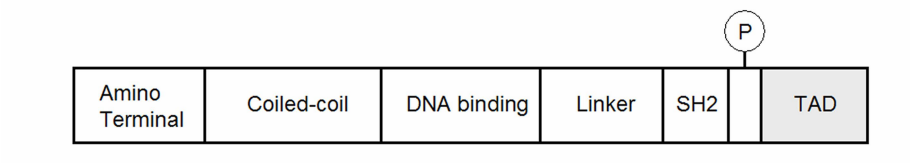

Figure 2. Domain organization of STATs.

The N-terminal domain of STATs consist of a conserved region of ~125 amino acids, and is rich in α-helices (Schindler, 2002, Vinkemeier et al., 1998). The N-domain enables the cooperative binding of polymerized STAT dimers to DNA. This enables cooperative DNA binding on the promoters containing multiple STAT recognition sites (Vinkemeier et al., 1998, Xu, Sun and Hoey, 1996). The N-terminal domain regulates the nuclear translocation of STATs (Strehlow and Schindler, 1998), as well as the dimerization of inactive STATs, when they dissociate from the enhancer elements (Mertens et al., 2006). The transcriptional coactivator CBP/p300 (Horvath, 2000) and the proteins of the PIAS family (Shuai, 2000) have been reported to interact with the N-terminal domains, and the N-terminus may also be involved in receptor recognition, phosphorylation and dephosphorylation (Leung, Li and Stark, 1996, Murphy et al., 2000).

The coiled-coil domain (amino acids ~135 to ~315) forms a four-helix bundle that protrudes about 80 Å laterally from the core structure (Becker, Groner and Muller, 1998, Chen et al., 1998). This domain is an important protein interaction domain, and may also be involved in receptor binding, tyrosine phosphorylation and nuclear export (Begitt et al., 2000, Bhattacharya and Schindler, 2003, Kisseleva et al., 2002, Zhang et al., 2000)

The DNA binding domain (DBD)(amino acids ~320 to ~480) consists of a β-barrel with an immunoglobulin fold (Chen et al., 1998). DBD permits DNA binding when STATs are present as dimers. The crystal structures of STAT1 and STAT3a bound to DNA reveal that the DBDs of the STAT dimer bind to the proximal regions of the GAS element (Becker, Groner and Muller, 1998, Chen et al., 1998) (Figure 3.). The structure of DBD may be affected by the changes in SH2 (Src-homology 2) domain structure that are resulted by the phosphate binding. The linker domain (amino acids ~480 to ~575) located in between the DBD and SH2 domains may mediate the effects (Chen et al., 1998, Kisseleva et al., 2002). The linker domain may also regulate the nuclear export of STATs (Bhattacharya and Schindler, 2003).

SH2 domain (amino acids ~575 to ~680) is the most highly conserved motif and is essential for STAT activation and function. It consists of an anti-parallel β-sheet flanked by two α-helices forming a pocket. At the base of this pocket lies a conserved arginine, which mediates the interaction with the phosphorylated tyrosine residues in the cytokine receptor. SH2 domain also mediates the dimerization of active STATs by the reciprocal interaction of the SH2 domains

with the phosphorylated tyrosines (Kisseleva et al., 2002). The phosphorylated STAT dimer bound to DNA via the DBDs forms a nutcracker-like structure where the SH2 domains form the hinge (Chen et al., 1998).

The carboxy-terminal domain is poorly conserved among the STATs and varies considerably in both length and sequence between different STAT family members. However, a single conserved tyrosine residue, which is subjected to phosphorylation by JAKs is located in the C-terminal domain. Tyrosine phosphorylation of this residue is essential for the function of the STATs (Kisseleva et al., 2002). The C-terminal domain also constitutes a transactivation domain (TAD). Variability in TAD sequences contributes to the specificity in target gene selection. This is largely due to the differential recruitment of transcriptional coregulatory proteins (Kisseleva et al., 2002, Schindler, 2002). STAT TADs are essential for the function of the proteins, and the removal of the TAD generates a dominant-negative mutant (Lu et al., 1998, Moriggl et al., 1997). The TAD is relatively acidic and proline-rich in all STATs, which results in high structural flexibility (Darnell, 1997, Hoey and Schindler, 1998). TADs have not been structurally characterized in detail, because the obtained crystal structures of STATs lack these domains (Becker, Groner and Muller, 1998, Chen et al., 1998).

In addition to tyrosine phosphorylation, several STATs undergo serine phosphorylation. Most of the STATs are phosphorylated also on serine residues located within the C-terminus. Various signaling pathways and phosphorylating kinases are involved in serine phosphorylation of different STATs. Serine phosphorylation modulates the transcriptional activity of STATs, but the mechanisms by which this occurs, as well as the biological impact it causes, are mostly not known (Decker and Kovarik, 2000).

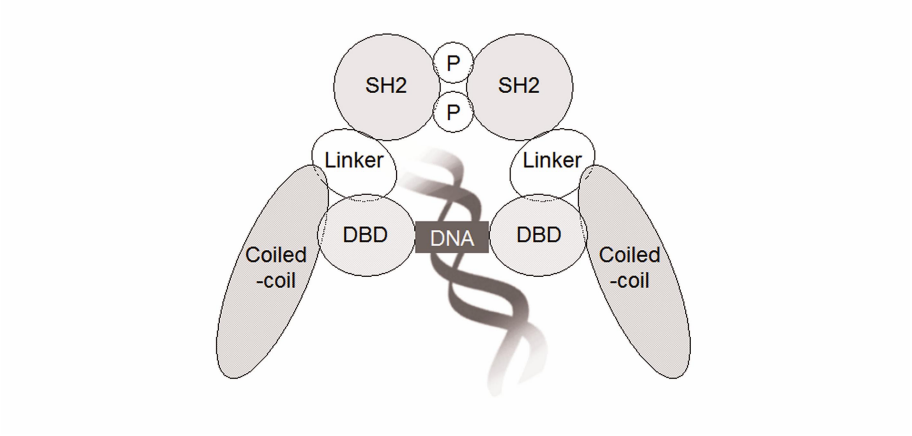

Figure 3. Schematic representation of the STAT dimer bound to DNA.

2.3.3. Specific functions of different STATs

STATs are differentially expressed in different tissues, and mediate biologically distinct and specific functions. STATs are also activated in response to different extracellular signaling proteins. Cell type- and promoter-specific functions of STATs are most likely modulated by the interaction with other transcription factors (Akira, 1999, Ihle, 2001).

STAT1 mediates the biological responses to type I and II interferons, and has both pro-inflammatory and anti-proliferative functions. STAT2 mediates responses to type I interferons by heterodimerizing with STAT1. STAT3 and STAT5 transduce signals from a wide variety of cytokines. STAT3 has an important role in development, it regulates the expression of anti-apoptotic and pro-survival genes, and it is also associated with cancer. In addition, STAT3 promotes anti-inflammatory activities (Kisseleva et al., 2002, Levy and Darnell, 2002, Schindler, Levy and Decker, 2007). STAT5a and b are functionally nearly redundant, and have important roles especially in erythropoiesis and lymphopoiesis. STAT4 is an important mediator of biological responses to IL-12, including the polarization of naive CD4+ lymphocytes into Th1 type cells (Kisseleva et al., 2002, Levy and Darnell, 2002, O'Shea, Gadina and Schreiber, 2002). STAT6 mediates the resposes to IL-4 and IL-13, and has an opposing role to STAT4 directing the polarization of CD4+ lymphocyte into Th2 direction. STAT6 also promotes the proliferation and maturation of B cells (Schindler, Levy and Decker, 2007).

3. STAT6-mediated transcriptional activation

3.1. Upstream signalling events leading to STAT6 activation

STAT6 was originally purified from cell extracts as an IL-4 stimulated, GAS binding protein (Hou et al., 1994). Later, STAT6 has been shown to be activated by IL-13, which shares a receptor chain with IL-4 (Lin et al., 1995).

Interleukin-4 (IL-4) is a haematopoietic cytokine originally identified as B cell growth factor 1 (BCGF1). IL-4 is secreted by T helper type 2 (Th2) cells, mast cells and basophiles, NKT cells and eosinophils. In addition to being a growth and differentiation factor for B cells, IL-4 is an important regulator of many other cell types, including T cells, haematopoietic precursor cells, monocytes, macrophages, dendritic cells, fibroblasts, endothelial cells, hepatocytes and keratinocytes (Paul, 1991).

IL-4 functions as an important regulator of humoral immune responses. IL-4 is essential for immunoglobulin class switch, and stimulates the transcription of immunoglobulin heavy-chain germline Igε and Igγ4 genes, which results in the expression of IgE and IgG4 in humans (Warren and Berton, 1995). IL-4 also

induces the expression of major histocompatibility complex class II (MHC II) and the low affinity receptor for IgE (FcεRII or CD23) (Glimcher and Kara, 1992, Rousset et al., 1988). Another main function of IL-4 is to regulate the differentiation of naive T cells. IL-4 promotes naive T cell differentiation into T helper 2 type cells capable of producing a variety of cytokines including IL-4 itself (Kopf et al., 1993). IL-4 also regulates Th2 response-related diseases, such as asthma and allergies (Chatila, 2004, Lukacs, 1996, Renauld, 2001). IL-4 deficient mice do not produce IgE and have reduced levels of IgG1. These mice show impaired Th2 type responses whereas the Th1 type responses are elevated (Kuhn, Rajewsky and Muller, 1991).

IL-4 receptor complex (IL-4R) consists of two subunits. IL-4 signals predominantly through a heterodimeric complex of two cytokine receptor proteins, IL-4 receptor alpha (IL-4Rα) and the common γ-chain. The common γ-chain is also a subunit of the receptors for IL-2, IL-7, IL-9, IL-15 and IL-21. IL-4Rα can also form homodimers or heterodimerize with IL-13 receptor (Fujiwara et al., 1997, Nelms et al., 1999). (Figure 4.)

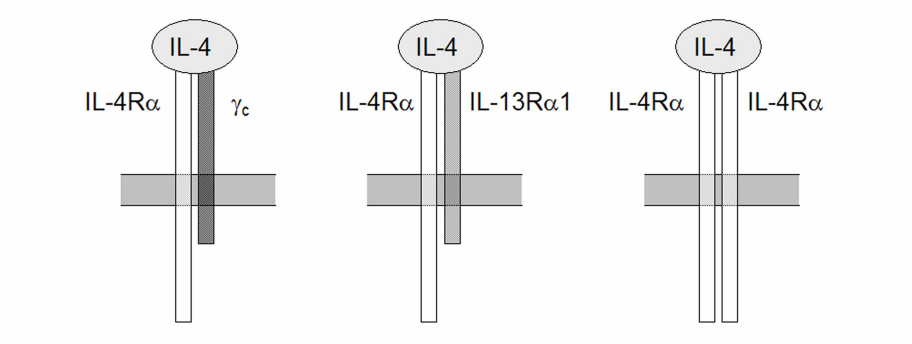

Figure 4. Schematic representation of IL-4R complexes.

As with other hematopoietic cytokines, IL-4 ligand binding promotes IL-4R dimerization. JAK1 and JAK3 associate with the IL-4Rα and the γ-chain receptor subunits respectively (Miyazaki et al., 1994, Russell et al., 1994), although in certain cell lines JAK2 has been shown to associate with the IL-4Rα chain (Murata, Noguchi and Puri, 1996). JAKs phosphorylate certain tyrosine residues on cytoplasmic receptor tails, which are recognized and bound by the SH2 domains of latent cytoplasmic STAT6 monomers. JAKs phosphorylate STAT6 monomers on a tyrosine residue adjacent to the SH2 domain (Y-641). STAT6 phosphorylation catalyzes the dimerization of the phosphorylated monomers through reciprocal interactions between SH2 domain of one monomer and the phosphorylated tyrosine of another monomer (Darnell, 1997, Mikita et al., 1996) Activation and dimerization allow STAT6 dimers to translocate into the nucleus, and activate the transcription of their target genes.

The mechanism of STAT6 nuclear transport has not yet been identified. Phosphorylated STAT6 has been shown to be attached to a detergent sensitive factor in cytoplasm (Daines et al., 2003). Factor bound STAT6 is unable to bind to DNA. This, yet unidentified factor may be functionally related to importin α5,

which has been reported to block the STAT1 DNA binding site and may be involved in STAT1 nuclear transport (McBride et al., 2002). Methylation of STAT6 has also been linked to the nuclear translocation of STAT6. As with STAT1, the methylation of a conserved N-terminal arginine (Arg27) has been reported to regulate STAT6 phosphorylation, nuclear translocation, and DNA-binding activity (Chen, Daines and Hershey, 2004). However, later studies have contradicted these results, showing that STAT1 and STAT3 are not methylated on concerved arginine residue (Komyod et al., 2005, Meissner et al., 2004). Therefore, further experiments are also needed to to clarify the observed effects on STAT6.

3.2. Target genes of STAT6

As STAT6 dimer translocates into the nucleus, it attaches to specific STAT6 binding sites at the promoters or upstream enhancer elements of target genes. STAT family of proteins bind to a consensus sequence called the IFN-γ activating sequence (GAS) (Decker, Kovarik and Meinke, 1997). Although STAT6 binds with a low activity to the N2 or N3 GAS sequence ($TTC(N)_2GAA$ or $TTC(N)_3GAA$ respectively, it has been shown to favor N4 binding sites with a four bp spacer ($TTC(N)_4GAA$) (Mikita et al., 1996). Natural STAT6 responsive promoters usually contain N3 or N4 binding sites with distinct palindromic sequence extensions. *In vivo*, within a cell, binding of STAT factors to the correct response elements is much more specific, than *in vitro*, due to different conditions (Kraus, Borner and Hollt, 2003). Therefore, the existence of different response elements among STATs provides an additional level to the specificity between these factors (Ehret et al., 2001, Seidel et al., 1995).

Dimerization of STAT6 has been commonly considered a requirement for nuclear translocation and gene activation, but in addition to dimeric STAT6, monomeric STAT6 has been under some circumstances shown to translocate into the nucleus and bind to DNA to activate transcription. Among the STATs, this feature seems to be restricted only to STAT6. The functional implications of the STAT6 monomer binding to GAS in the IL-4-induced gene activation are still not clear, but it is assumed, that the protein behavior and DNA-binding properties of STAT6 may differ from other STATs (Lee and Park, 2001).

Numerous gene loci are regulated by IL-4 through STAT6 signaling. These include immunoglobulins (e.g. Igε, Igγ1, Igγ3, Igγ4), cytokines (e.g. IL-4, lymphotoxin-α), chemokines (e.g. eotaxin-1 and -3), adhesion molecules (E- and P-selectin, β(3) integrin), enzymes (e.g. 12/15-lipoxygenase, arginase-1), receptors (e.g. IL-4Rα, CD23, MHC-II, polymeric Ig receptor, μ-opioid receptor), intracellular signaling molecules (e.g. suppressor of cytokine signaling-1 (SOCS-1)) and a group of miscellaneous genes (e.g. β-casein) (Hebenstreit et al., 2006). The number of known STAT6 regulated loci is constantly increasing, and recent microarray screens in IL-4 stimulated B or T lymphocytes have revealed several novel genes that are potential targets for STAT6 regulation (Chen et al., 2003, Schroder et al., 2002).

The immunoglobulin heavy-chain germline epsilon promoter (Igε promoter) is one of the best studied IL-4 responsive promoters. The binding sites for STAT6 at the Igε promoter are atypical N4 binding sites that lack distinct palindromic sequence extensions. Therefore, interactions of N4 binding site bound STAT6 with other transcriptional co-factors are necessary to confer responsiveness to IL-4 (Delphin and Stavnezer, 1995, Mikita et al., 1996). Other *cis*-acting elements identified from human Igε promoter include binding sites for CCAAT/enhancer binding protein β (C/EBPβ), NF-κB, PU.1, B cell specific activator protein BSAP and Bcl-6 (Harris et al., 1999, Stutz and Woisetschlager, 1999, Thienes et al., 1997).

3.3. STAT6 transactivation domain

The TAD of STAT6 is the longest and the most divergent among STATs (Mikita et al., 1996, Moriggl et al., 1997, Shen and Stavnezer, 2001). It has been characterized as a modular region with high structural flexibility (Lu et al., 1997, Moriggl et al., 1997), and it is able to interact with several transcription factors and coregulators. These factors are essential for its function in transcriptional activation. In the experiments in which the TAD of STAT6 was replaced with the TAD from STAT5 or STAT1α, the activation of STAT6 target genes was not observed (Goenka et al., 2003). This indicates that the composition of the transcriptional coregulatory proteins bound by the TAD contribute in part to the specificity of the transcriptional activation among STATs.

In addition to the transactivation function, STAT6TAD has been shown to play a critical role in the IL-4-mediated inhibition of IFN-γ inducible IRF-1 promoter (Goenka et al., 1999). The mechanism of inhibition involves a threshold level of paired STAT6TADs, and is based on induction of a gene product that mediates inhibition of IRF-1 promoter.

3.4. Regulation of STAT6-mediated transcription

3.4.1. Negative regulation

Various effector molecules have been identified to negatively regulate STAT6 activity. These include protein tyrosine phosphatases SHP-1, PTP-BL and TCPTP which dephosphorylate and inactivate STAT6 (Haque et al., 1998, Lu et al., 2007, Nakahira et al., 2007). Tyrosine-phosphorylated STAT6 is also negatively regulated by the action of proteasome mediated protein degradation (Hanson et al., 2003). IFNs inhibit IL-4-induced activation of STAT6 and STAT6-dependent gene expression by inducing expression of SOCS-1. The inhibition was associated with decreased tyrosine phosphorylation and nuclear translocation of STAT6 (Dickensheets et al., 1999). The Pim kinases have been shown to modulate SOCS-1 protein levels, therefore regulating the inhibition by SOCS-1 (Chen et al., 2002).

STATs are subjected to serine phosphorylation (Kirken et al., 1997, Pesu et al., 2000, Visconti et al., 2000, Wen, Zhong and Darnell, 1995, Wick and Berton, 2000, Zhang et al., 1995), and in most cases serine phosphorylation has been shown to enhance STAT mediated transcriptional activity (Visconti et al., 2000, Wen, Zhong and Darnell, 1995, Zhang et al., 1995). STAT6 has also been shown to be subjected to serine phosphorylation (Pesu et al., 2000, Wick and Berton, 2000), but the effect on transcriptional activation has not been as clearly demonstrated. Inhibition of protein phosphatase 2A (PP2A) has been shown to induce serine phosphorylation of STAT6, and negatively control the expression of IL-4-responsive genes by inhibiting DNA-binding of STAT6 (Maiti et al., 2005, Wang et al., 2004, Woetmann et al., 2003).

3.4.2. Regulation by transcription factors

Different transcription factors associate with STAT6 responsive promoters and function in coordination with STAT6 to regulate transcriptional activation. The reported effects of various transcription factors are often contradictory. The regulatory function often seems to depend on cell type, the gene in question and the structure of the promoter. Synergism and antagonism between regulatory factors contributes to the regulatory network that permits appropriate gene activation at a given time and place (Hebenstreit et al., 2006).

C/EBPβ contributes to STAT6-mediated gene activation at murine germline γ1 and ε promoters (Delphin and Stavnezer, 1995, Lundgren et al., 1994). C/EBPβ functions on a mechanism that involves physical interaction with STAT6. Another member of C/EBP family, C/EBPγ can also bind to the consensus site at Igε promoter. Conflictingly, binding of C/EBPβ or C/EBPγ to C/EBP binding site may either enhance or suppress the STAT6-mediated gene activation (Mikita, Kurama and Schindler, 1998, Pan et al., 2000, Shen and Stavnezer, 2001). The positive effect on transcription may relate to the stabilization of the STAT6 DNA binding. Also NF-κB has been implicated in STAT6 promoter binding. At Igε promoter, NF-κB functions in cooperation with STAT6 to activate transcription (Delphin and Stavnezer, 1995). However, at another STAT6 regulated promoter, E-selectin promoter, NF-κB and STAT6 compete for overlapping binding sites (Bennett et al., 1997).

Glucocorticoid receptor (GR) is a ligand-activated transcription factor, which mediates the responses to glucocorticoids. Also GR has been shown to inhibit STAT6-mediated gene activation, and function in co-operation with STAT6 (Biola et al., 2000, Moriggl et al., 1997). Common transcriptional repressor Bcl-6 can attach to STAT6 binding site at Igε promoter, and can inhibit STAT6 binding to DNA (Harris et al., 1999, Harris, Mostecki and Rothman, 2005). However, Bcl-6 mediated block on DNA binding occurs only on a subset of genes regulated by STAT6. This may be due to differences between STAT6 binding sites on different promoters. Another repressor protein related to Bcl-6, BAZF, may also inhibit STAT6 regulated gene activation via GAS binding (Hartatik et al., 2001).

Other transcription factors that have been suggested to regulate STAT6 function include interferon regulatory factor-4 (IRF-4)(Gupta et al., 1999), activator protein-1 (AP-1) (Shen and Stavnezer, 2001), Myb(Monticelli et al., 2002), PU.1(Pauleau et al., 2004, Stutz and Woisetschlager, 1999) and Ets-1(Travagli et al., 2004). IL-4 induced IRF-4 directly interacts with STAT6 and enhances the STAT6-mediated transcriptional activation. IRF-4 is expressed mainly in the cells of lymphoid lineage. AP-1 and Myb have either positive or negative effect on STAT6-mediated gene activation, and the mechanism of their function may relate to the adjacent binding sites on promoters. Ets-1 and PU.1 are members of Ets gene family, which recognize and bind to a consensus sequence 5'-GGAA/T-3'on promoters. Ets-1 has been shown to repress STAT6 regulated SOCS-1 gene, whereas PU.1 has been shown to upregulate some STAT6 activated genes.

3.5. Coregulators of STAT6

In addition to transcription factors, transcriptional coregulators are essential for transcriptional activation. Coregulatory proteins are needed to mediate the interaction of STAT6 with the basal transcription machinery. Transcriptional coregulators mainly interact with STAT6 through TAD domain. The TAD of STAT6 is the longest and the most divergent among STATs (Mikita et al., 1996, Moriggl et al., 1997, Shen and Stavnezer, 2001). In the experiments in which the TAD of STAT6 was replaced with the TAD from STAT5 or STAT1α, the activation of STAT6 target genes was not accomplished (Goenka et al., 2003). This indicates that the composition of the transcriptional coregulatory proteins bound by the TAD contribute in part to the specificity of the transcriptional activation among STATs.

Transcriptional coregulatory proteins can be broadly divided into two classes. The first group consists of coregulators involved in chromatin remodelling and modification. These coregulators assist gene specific regulators, such as STAT6, and the basal transcription machinery to access the transcription initiation site. Another group consists of coregulators functioning as adaptors. Adaptors function as scaffolds or bridges connecting gene specific transcription factors to the basal transcription machinery or to other coregulators (Naar, Lemon and Tjian, 2001, Spiegelman and Heinrich, 2004). Transcriptional coregulatory proteins, general transcription factors, gene specific regulators and basal transcription machinery of a given promoter form a complex called the enhanceosome (transcriptosome) (Merika and Thanos, 2001).

3.5.1. CBP/p300

The most important chromatin modifying coactivator is CBP, the binding protein for cAMP response binding protein (CREB) and p300, collectively addressed as CBP/p300. Both of these proteins are ubiquitously expressed nuclear proteins, structurally highly homologous, and in most cases functionally interchangeable. CBP/p300 are transcriptional coactivators that enhance the activity of several

transcription factors including CREB, E1A, c-Jun, ATF, c-Fos, c-Myb, Sap-1, tax, Myo D, P/CAF, SRC-1, GR and NF-κB by interacting with their activated forms (Chakravarti et al., 1996, Eckner,1996, Janknecht and Hunter, 1996, Kamei et al., 1996). CBP/p300 also function as coactivators for all STAT family members (Bhattacharya et al., 1996, Gingras et al., 1999, Horvai et al., 1997, McDonald and Reich, 1999, Nakashima et al., 1999, Ohmori and Hamilton, 1998, Paulson et al., 1999, Pfitzner et al., 1998, Zhang et al., 1996). CBP/p300 possesses acetyltransferase activity. It has been shown to acetylate a number of proteins including histones and STAT6 itself. Histone acetylation opens the chromatin structure allowing the transcription factors, coregulators and the basal transcription machinery to enter the promoter and the transcription start site. Shankaranarayanan *et al.* (2001) have reported that acetylation of STAT6 is required for the transcriptional activation of 15-LOX-1 (Shankaranarayanan et al., 2001). To date, it is still not known, whether acetylation of STAT6 is also necessary for other STAT6 activated genes.

3.5.2. p100

The human p100 protein (also referred as Tudor-SN) was originally identified as EBNA2 acidic domain binding nuclear factor (Tong et al., 1995). EBNA2 is a viral protein expressed in EBV infected B-lymphocytes, and activates the transcription of several viral and cellular genes. Subsequently, p100 has also been shown to interact with a number of other transcription factors including c-Myb (Dash, Orrico and Ness, 1996), a transcription factor known to function in growth and differentiation and Pim-1 (Leverson et al., 1998), and signal transducer and activator of transcription family members 5 and 6 (Paukku, Yang and Silvennoinen, 2003, Yang et al., 2002).

p100 functions as a transcriptional coactivator; its effect on transactivation has been shown to be due to p100 being a mediator between sequence specific transcriptional activators and the basal transcription machinery. p100 interacts with TFIIE, a general transcription factor that in the transcription initiation complex binds to RNA Pol II, recruits TFIIH, modulates TFIIH helicase, kinase and ATPase activities and assists TFIIH in promoter melting (Tong et al., 1995).

Human p100 is encoded by *SND1* and has orthologues in *Arabidopsis* (Ponting, 1997), Drosophila (Caudy et al., 2003), *C. elegans* (Callebaut and Mornon, 1997, Ponting, 1997), *Histoplasma capsulatum* (Porta et al., 1999), mammals (Callebaut and Mornon, 1997, Tong et al., 1995) and *Schizosaccharomyces pombe*, but not in *Saccharomyces cerevisiae* (Caudy et al., 2003).

Structurally human p100 is composed of a repeat of five domains structurally related to staphylococcal nucleases (SNs), small calcium-dependent enzymes which hydrolyse both DNA and RNA. Four complete, uninterrupted SN-like domains in the N-terminus have conserved SN folds, and share 19,5 to 20,8% sequence identity with SNs (Callebaut and Mornon, 1997). The fifth domain of human p100, TSN, is a modified SN-like domain, and contrary to an earlier

report, consists of a complete SN-like domain interdigitated with a tudor domain (Figure 5.) (Shaw et al., 2007). The tudor domain of p100 bears close resemblance to the Survival of Motor Neuron (SMN) tudor domain.

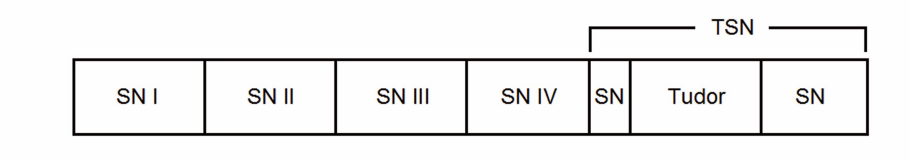

Figure 5. Domain organization of p100.

SNs possess a conserved domain which includes an active site involved in the catalytic mechanism of the enzyme. The catalytic residues located in the active site and required for nuclease activity are absent from SN-like domains of p100, indicating that these domains may not function in nucleic acid degradation (Callebaut and Mornon, 1997). However, more recently Drosophila p100 homologue Tudor-SN was shown to possess non-specific nuclease activity (Caudy et al., 2003) indicating that p100 may function in nucleic acid degradation at least under some circumstances.

The first subdomain of p100 SN-like domains resembles the members of oligonucleotide/oligosaccharide-binding (OB)-fold superfamily. These proteins bind oligonucleotides or oligosaccharides, without necessarily having enzymatic activity (Callebaut and Mornon, 1997). Repeated SN-like domains of p100 may also have evolved mainly into interaction domains. Accordingly, most of the known interacting proteins, including c-Myb, Pim-1, STAT5, STAT6 and RNA Pol II, attach to p100 via the SN-like domains (Dash, Orrico and Ness, 1996, Leverson et al., 1998, Paukku, Yang and Silvennoinen, 2003, Tong et al., 1995, Yang et al., 2002).

SN-like domains of p100 alone are sufficient to enhance STAT6-mediated transcriptional activation, whereas TSN domain does not affect transcriptional activity (Yang et al., 2002), but the hydrophobic core of TSN domains has been suggested to bind to dimethylated arginine/glycine rich sequences (Shaw et al., 2007). The differing functions among the SN-like and TSN domains may indicate that p100 mediates distinct functions through different domains.

p100 has been reported to localize both in the nuclei and to cytoplasmic membrane organelles in several types of mammalian cells (Broadhurst and Wheeler, 2001, Low et al., 2006, Tong et al., 1995). However, Caudy *et al.* (Caudy et al., 2003) demonstrated that the localization of p100 was predominantly cytoplasmic in both mammalian and *D. melanogaster* cells. Similarly, mainly cytoplasmic localization was observed in the cytoplasm of *Xenopus* oocytes (Scadden, 2005). p100 has also been identified as a component of cytosolic lipid droplets and endoplasmic reticulum of milk secreting cells (Keenan et al., 2000).

RNA interference (RNAi) is a mechanism in which dsRNA triggers the degradation of a homologous mRNA (Fire et al., 1998, Montgomery and Fire, 1998). RNAi has been observed in a variety of eukaryotes (Bernstein et al., 2001, Elbashir et al., 2001, Elbashir, Lendeckel and Tuschl, 2001, Moss, 2001), and the RNAi pathway is thought to have evolved to protect the host against unwanted foreign genes, such as viruses and transposones (Haasnoot, Cupac and Berkhout, 2003). RNA silencing plays a role not only in mRNA and dsRNA stability/degradation, but also in regulation of transcription (Baulcombe, 1996).

In RNA silencing pathways, dsRNA is processed to short interfering RNAs (siRNA) with duplexes of 21–22 nt RNAs with symmetric 2-nt 3' overhangs being the most predominant products (Elbashir et al., 2001, Elbashir, Lendeckel and Tuschl, 2001). The siRNAs are assembled into an RNA-induced silencing complex (RISC) that cleaves the homologous target mRNA (Hammond, Caudy and Hannon, 2001). The siRNA is thought to provide target specificity to RISC through base pairing of the guide strand with the target mRNA (Hammond et al., 2000).

Caudy et al. (Caudy et al., 2003) demonstrated that p100 is a component of RISC in *Drosophila*, *Caenorhabditis elegans* and mammals. RISC has been suggested to function as an endonuclease, which cleaves phosphodiester bonds on mRNA targets. Although p100 was originally found to lack the catalytically important amino acids needed for nuclease activity, p100 was shown to exhibit nuclease activity similar to other staphylococcal nucleases. Being the only identified RISC component with ssRNA specific ribonuclease activity, p100 was suggested to mediate siRNA-directed endonucleolytic cleavage of target mRNA observed in RNA interference (Caudy et al., 2003). However, later on it was found that Mg^{2+} is necessary for the catalytical activity of RISC, and the mechanism of endonucleolytic cleavage differs from the nucleolytic mechanism of p100, suggesting that endonucleolytic activity is mediated by some other enzyme of the complex (Schwarz, Tomari and Zamore, 2004). A recent report has identified only three components in human RISC, this result might suggest that p100 could be a component of an alternate RISC (Gregory et al., 2005).

In addition to RNAi mediated mRNA degradation, long dsRNAs can trigger other cellular processes, such as various posttranscriptional mechanisms that alter the nucleotide sequence of RNA. Adenosine deaminases that act on RNA (ADARs) are RNA editing enzymes that create covalent modifications to dsRNA, converting adenosine (A) residues to inosines (I) by hydrolytic deamination (Bass and Weintraub, 1988, Wagner et al., 1989). This editing alters the protein coding genes and the structure of dsRNA. The function of ADARs has been suggested to be antagonistic to RNAi. RNA editing by ADARs may protect dsRNA from being recognized by Dicer. It has also been suggested that introduction of inosine residues by ADARs could sequester siRNA function, by preventing the binding of siRNA to the target sequence, and therefore protecting target from RISC mediated cleavage (Nishikura, 2006).

p100 has been shown to bind and cleave inosine containing dsRNA (I-dsRNA), however, additional factors are needed for cleavage (Scadden, 2005). These factors may stabilize p100-I-dsRNA complex, or regulate its activity.

Alternatively, p100 itself may be needed to activate other nucleases that cleave I-dsRNA independently or together with p100. ADARs have also been reported to have a role in editing some micro-RNAs (pri-miRNAs). These inosine containing pri-miRNAs are rapidly degraded by p100 (Yang et al., 2006). ADAR-TudorSN pathway may be used as a way to dispose unwanted dsRNAs. As p100 has been shown to be a component of both RISC and ADAR pathways, it may have an important function in combining these antagonistic pathways.

3.5.3. CoaSt6

Collaborator of STAT6 (CoaSt6) has been recently identified as a coactivator for STAT6 (Goenka and Boothby, 2006). CoaSt6 enhances the STAT6-mediated transcriptional activation of IL-4 responsive genes, and directly interacts with STAT6 via its macrodomains. CoaSt6 has been suggested to have intrinsic poly(ADP-ribosyl)polymerase (PARP) enzymatic activity, and to ADP-ribosylate itself and p100 protein. PARP activity of CoaSt6 has been suggested to be necessary for STAT6-mediated gene responses, but the actual mechanism of CoaSt6 still remains to be solved (Goenka, Cho and Boothby, 2007).

3.5.4. SRC1 and p/CIP

The steroid receptor coactivator-1 (SRC-1, nuclear receptor coactivator-1 (NCoA-1)), and the p300/CBP co-integrating protein (p/CIP, nuclear receptor coactivator-3 (NCoA-3)), have been reported to associate with STAT6. SRC-1 directly interacts with STAT6 TAD and CBP (Litterst and Pfitzner, 2001, Yao et al., 1996), and has also been shown to interact with general transcription factors TBP and TFIIB (Ikeda et al., 1999, Takeshita et al., 1996). In addition to functioning as a coactivator for STAT6, SRC-1 has been shown to enhance NF-κB, SMAD3 and AP-1 mediated transcriptional activation (Lee et al., 1998b, Na et al., 1998, Yanagisawa et al., 1999). p/CIP in turn interacts with STAT6 indirectly via CBP, and presumably enhances STAT6-mediated transcription on a mechanism that rely on the recruitment of CBP to STAT6 (Arimura et al., 2004). Although SRC-1 and p/CIP possess moderate levels of intrinsic histone acetyltransferase activity (Leo and Chen, 2000), they are assumed to enhance STAT6-mediated interaction on a mechanism that is mainly based on the interaction with CBP (Arimura et al., 2004, Litterst and Pfitzner, 2001).

Aims of the study

The general aim of this study is to investigate the molecular mechanisms of IL-4 induced gene activation. Our studies have focused on investigating the underlying mechanisms of cell type-specific IL-4 responses, and analyzing the composition of STAT6 enhanceosome by characterizing novel coregulators of STAT6. Identification and characterization of transcriptional coregulators will help in understanding both the mechanisms of IL-4 regulated gene activation and general gene regulation in cells.

The specific aims were:

1. To analyse the basis of cell-type specific IL-4 response by characterizing the effect of PU.1 on the transcriptional activation of the STAT6 response element in the Igε promoter
2. To study the interaction and cooperation of transcriptional coactivators p100 and CBP in STAT6-mediated transcriptional activation
3. To evaluate the role of RHA as a transcriptional regulator in STAT6-mediated transcription
4. To investigate the cellular functions of p100 based on the functional interactions of C-terminal TSN-domain

Materials and methods

1. Materials and methods used in the individual studies

Cell culture	I-IV
Transfections	I-IV
Preparation of cell lysates	I-IV
Antibodies, co-immunoprecipitation and Western blotting	I-IV
GST pulldown	I-IV
Mass spectrometry	III, IV
Plasmids and cloning	I-IV
Luciferase reporter assay	I-III
RNA interference	II, III
Chromatin immunoprecipitation	II, III
Histone acetylation assay	II
Confocal microscopy	II
Northern hybridization	IV

1.1. Cell culture (I-IV)

HepG2 (human hepatocellular carcinoma cell line, ATCC HB-8065) and COS7 (SV40-transformed green monkey kidney cell line, ATCC CRL-1651) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker) supplemented with 10% FCS (Gibco-BRL), 2 mM glutamine (BioWhittaker) and antibiotics. HeLa cells (human cervix adenocarcinoma cell line, ATCC CCL-2) and HeLa-p100-Flag stable cell line (Yang et al., 2002) were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 1% non-essential amino acid solution (Sigma) and antibiotics.

1.2. Transfections (I-IV)

Transfections of HepG2 and HeLa cells were performed with the calcium phosphate co-precipitation method. Cells were seeded on plates 24 hours before transfection. 2 M $CaCl_2$ was added to the water diluted plasmid DNA, and 2 × HEPES solution (280 mM NaCl, 1.5 mM Na_2HPO_4, 55 mM N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid, pH 7.0) was mixed with the solution. The mixture was added to the semiconfluent cells. Cells were either left untreated or treated with human IL-4 (PeproTech, London, UK) for different time periods, and analyzed for the expression of target proteins after 24-72 hours.

COS7 cells were transfected by electroporation with a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA, USA). COS7 cells were electroporated at 260 V/960 μF, using 0.5 cm cuvettes. Plasmid DNA was mixed with herring sperm DNA added up to 40 μg, and mixed with the cells in 250 μl of DMEM (3-5 x 10^6 cells) prior to electroporation. After electroporation COS-7 cells were plated on 10 cm plates. Cells were either left untreated or treated with IL-4 (PeproTech, London, UK) for different time periods. The cells were analyzed for the expression of target proteins after 48 hours.

1.3. In vitro translation and preparation of cell lysates (I-IV)

^{35}S-labeled proteins were prepared by *in vitro* translation using TNT T7/T3 Coupled Reticulosyte Lysate System (Promega) according to manufacturer's instructions. To prepare total cell extracts the cells were harvested and lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 50 mM sodium fluoride) supplemented with aprotinin and PMSF. For the preparation of nuclear lysates the cells were lysed in buffer A (50 mM HEPES, pH 7.5, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium molybdate, 2 mM EDTA, 0,2% NP-40, 10 mM magnesium chloride, 2 mM sodium orthovanadate, supplemented with aprotinin and PMSF) and centrifuged. The nuclei were then lysed to buffer C (50 mM HEPES, pH 7.5, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM sodium molybdate, 2 mM EDTA, 0,1% NP-40, 10% glycerol, 0,3 mM NaCl, 2 mM sodium orthovanadate, supplemented with aprotinin and PMSF). Protein concentrations of the lysates were measured using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

1.4. Antibodies, co-immunoprecipitation and Western blotting (I-IV)

1.4.1. Antibodies

Rabbit polyclonal anti-CBP, rabbit polyclonal anti-IgG, goat polyclonal anti-Prp8 and rabbit polyclonal anti-STAT6 were purchased from Santa Cruz Biotechnology, mouse monoclonal anti-FLAG M2 antibody and anti-Flag M2 agarose from Sigma, mouse monoclonal anti-HA (clone 16B 12) from BabCO, mouse monoclonal anti-c-Myc from Roche, mouse monoclonal anti-STAT1 from Transduction Laboratories (BD Biosciences), anti-TMG agarose from Calbiochem and anti-acetyl histone H4 from Upstate Biotech. Rabbit polyclonal anti-p100 was a gift from Dr. Elliot Kieff (Tong et al., 1995). Anti-p100-TD antibody was raised against TSN domain of p100 protein.

1.4.2. Co-immunoprecipitation

The cells were harvested and cytoplasmic extract was prepared as described in *4.1.3.* The extracts were immunoprecipitated with appropriate antibodies and collected on protein A/G beads. The beads were washed with NP-40 lysis buffer supplemented with PMSF and aprotinin. Immunoprecipitated proteins were separated by SDS-PAGE and detected from the gels by Western blotting.

1.4.3. Western blotting

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Micron Separation Inc., Westborough, MA, USA). Immunodetection was performed using specific primary antibodies, biotinylated anti-mouse or anti-rabbit secondary antibodies (Dako A/S, Denmark), a conjugate of streptavidin-biotin horseradish peroxidase and ECL detection (Amersham Biosciences).

1.5. Glutathione S-transferase (GST) pulldown assay (I-IV)

GST fusion proteins were produced in BL21 *Escherichia coli* and purified with Glutathione Sepharose 4B (Pharmacia Biotech) affinity beads. For binding assays, the bead bound fusion proteins were incubated with *in vitro* translated ^{35}S-labeled proteins, total cell or nuclear lysates. After washing, the beads were extensively washed with lysis buffer. To analyze protein binding, the bound proteins were eluted by reducing Laemmli sample buffer. The proteins were separated by SDS-PAGE and visualized by autoradiography, silver staining, Coomassie Brilliant Blue staining or Western blotting with specific antibodies. To continue with Northern blot analysis, the washed beads were incubated with Proteinase K buffer (100 mM Tris pH 8, 150 mM NaCl, 10 mM EDTA, 1% SDS. 0.2 mg/ml proteinase K), RNA was phenol-chloroform extracted, ethanol precipitated and resolved to water.

For identification of interacting proteins by mass spectrometric analysis, the bead bound GST fusion proteins were incubated with total cell or nuclear lysates. After washes the bound proteins were dissociated from bead-bound fusion proteins by incubating in the elution buffer containing 2M NaCl. The eluted proteins were concentrated and desalted with Microcon columns (cut-off 30 kDa, Millipore Corporation, Bedford, MA, USA). The proteins were separated by SDS–PAGE and visualized by silver staining or Coomassie Brilliant Blue staining.

1.6. Mass spectrometry (III, IV)

The bands to be identified by mass spectrometric analysis were cut out from Coomassie blue-stained SDS-PAGE gel and subjected to in-gel trypsin digestion. The molecular masses of the peptide mixtures were determined by matrix-

assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The molecular masses of the tryptic peptides were used to search the OWL protein sequence database for candidate proteins using the ProFound program.

1.7. Plasmids and cloning (I-IV)

IL-4R-reporter plasmid (IL-4R-STAT6x3-luc) contains three copies of STAT6 response element from IL-4-receptor gene promoter (Kotanides and Reich, 1996). It was constructed by cloning annealed oligonucleotides, sense, 5′-p-AGCTTAGCTTCTTCATCTGAAAAGAGCTTCTTCATCTGAAAAGAGCTTCTTCATCTGAAAAGG-3′ and antisense 5′-p-TCGACCTTTTCAGATGAAGAAGCTCTTTTCAGATGAAGAAGCTCTTTTCAGATGAAGAAGCTA-3′, into HindIII and SalI sites of pfLUC plasmid containing c-fos minimal promoter in front of the *Photinus pyralis* luciferase gene.

Stat6REx3 was created by cloning annealed oligonucleotides sense, 5'-p-AGCTTGACGACTTCCCAAGAACAGCGACTTCCCAAGAACAGCGACTTCCCAAGAAG-3' and antisense, 5'-p-TCGACTTCTTGGGAAGTCGCTGTTCTTGGGAAGTCGCTGTTCTTGGGAAGTCGTCA-3' into HindII and SalI sites of pfLUC-plasmid. Underlined bases in the Stat6REx3 oligonucleotide sequences were mutated (A → T or T → A) and were used to construct mStat6REx3 reporter.

Igε-promoter-reporter plasmid (pfNεN4-luc) contains four copies of IL-4 responsive elements from the human Igε heavy chain constant region promoter placed upstream of c-fos minimal promoter (Pesu et al., 2000).

pCIneo-N-termHA-STAT6 and pCIneo-C-termHA-STAT6 (Yang et al., 2002) were generated by PCR using primers containing the HA-epitope sequence inserted into the N- or C-terminus of STAT6 respectively.

GST-St6-TAD was generated by cloning PCR products corresponding to amino acids 642–847 of human STAT6 into pGEX-4T-1 vector (Pharmacia) (Yang et al., 2002). GST-St6-TAD-6 was constructed by cloning PCR products corresponding to amino acids 794–847 of human STAT6 into pGEX-4T-1 vector. GST-St1-TAD was constructed by cloning PCR products corresponding to amino acids 711–750 of human STAT1 into pGEX-4T-1 vector (Amersham Biosciences).

To generate pSG5-p100-Flag, full-length open reading frame from pBp100 was amplified by PCR and cloned into pSG5-Flag vector (Yang et al., 2002). pSG5-p100-SN-Flag contains the four SN-like domains of p100 (aa 1-639), and pSG5-p100-TD-Flag contains the TSN domain of p100 (aa 640-885). They were generated by PCR using primers containing C-terminal Flag-sequence (Yang et al., 2002). For construction of GST-p100-SN and GST-p100-TD, the four SN-

like domains of p100 (aa 1-639) or the TSN domain of p100 (aa 640-885) were cloned into pGEX-4T-1 vector. The pSG5 expression plasmids containing first and second SN-like domain (SN1 + 2, amino acids 1–319), or third and fourth SN-like domain (SN3 + 4, amino acids 320–639) of human p100 protein were generated by PCR using primers containing C-terminal Flag sequence.

GST-CBP-D1 (amino acids 1–452), D2 (amino acids 452–1099), D3 (amino acids 1099–1620), D4 (amino acids 1620–1877), D5 (amino acids 1877–2441), and HAT (amino acids 1099–1758) were generated by PCR amplification of the relevant domain of CBP and inserted into pGEX-4T-1 vector. pBlue-CBP-D1 (amino acids 1–452), D2 (amino acids 452–1099), D3 (amino acids 1099–1758), and D4 (amino acids 1758–2441) were constructed by PCR amplification of the related domains of CBP and inserted into BamHI and XbaI sites.

GST-SMN-Tudor was generated by cloning PCR products corresponding to amino acids 92–156 of SMN and inserted into EcoRI and NotI sites of pGEXT-4T-1.

pBluescript plasmids containing different domains of U5-220 were constructed by cloning PCR products corresponding to amino acids 1–209 (domain 1), 303–668 (domain 2), 673–907 (domain 3), 914–1170 (domain 4), 1167–1698 (domain 5), 1697–2121 (domain 7), 2040–2332 (domain 7).

Plasmid encoding AdML pre-mRNA was provided by Dr R. Reed (Zhou et al., 2002). pGEX-RHA1 (amino acids 1–262), -2 (amino acids 255–664), -3 (amino acids 649–1077) and -4 (amino acids 1064–1270), pcDNA3-HA/RHA and pcDNA3-HA/RHAmATP plasmids were kindly provided by Dr T. Nakajima (Aratani et al., 2001, Nakajima et al., 1997).

1.8. Luciferase Reporter gene assays (III)

HeLa or HepG2 cells were transfected on six-well tissue-culture plates with reporter plasmid, pCMV-β-galactosidase plasmid, and indicated expression plasmids. One day after transfection, cells were starved overnight in DMEM containing 1% FBS and treated or left untreated with 10 ng/ml of recombinant human IL-4. Cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI) and luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) according to manufacturer's instructions. The luciferase values were normalized against β-galactosidase activity of the lysates.

1.9. RNA interference (II, III)

siRNAs were generated using the Ambion Silencer^{TM} siRNA Construction Kit according to the manufacturer's protocol. Alternatively, siRNAs were purchased from Dharmacon. The following target sequences were used: p100 I 5'-AAGGCATGAGAGCTAATAATC-3' (corresponding to nucleotides 602–622 of human p100 mRNA), p100 II 5'-AAGGAGCGATCTGCTAGCTAC-

3’(corresponding to nucleotides 2218–2238 of human p100 mRNA), RHA I 5’-AACCACACAGGTTCCCCAGTT-3’ (corresponding to nucleotides 1331–1351 of human RHA mRNA), RHA II 5’-AAGGAAAGCAACTGGGCTATA-3’ (corresponding to nucleotides 2764–2784 of human RHA mRNA) and STAT6 5’-AAGTCCTGAGAACCCTCGTCA-3’ (corresponding to nucleotides 1059-1079 of human STAT6 mRNA). The scrambled siRNA sequences were used as negative controls.

Transfection of cells was performed as follows: HeLa cells were transfected on 24-well tissue-culture plates. Cotransfection of reporter plasmid, β-galactosidase and siRNA was performed with Oligofectamine (Invitrogen). Twenty-four hours after transfection cells were either stimulated with 10 ng/ml of IL-4 or left untreated. Cells were lysed in Reporter Lysis Buffer (Promega, Madison, WI) 48 h after transfection. Luciferase expression was determined using the Luciferase Assay System (Promega, Madison, WI) and the results were normalized against β-galactosidase activity of the lysates.

1.10. Chromatin Immunoprecipitation (II, III)

HeLa cells were transfected with the indicated plasmids. The cells were starved and stimulated with IL-4 (10 ng/ml) for 4 h. Formaldehyde was added to the IL-4 or mock-treated cells at a final concentration of 1%, and the cross-linking was quenched by the addition of glycine.

The soluble chromatin was prepared essentially according to the protocol by Nissen and Yamamoto (Nissen and Yamamoto, 2000): The cells were washed with PBS and harvested from the plates with ChIP lysis buffer (50 mM HEPES-KOH pH 8, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) supplemented with PMSF, pepstatin A and aprotinin. Nuclei were collected by centrifugation and washed with ChIP wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl) supplemented with PMSF, pepstatin A and 5 aprotinin. The nuclei were resuspended in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100) supplemented PMSF, pepstatin A and aprotinin. The samples were sonicated to an average length of 200–1000 bp using VibraCell 500 watt sonicator (Sonics & Materials, Inc., Newtown, CT) with microtip.

After centrifugation, samples were precleared with protein A beads and immunoprecipitated with appropriate antibodies, and the immunocomplexes were collected onto protein A beads. Beads were washed with 10 mM Tris-HCl, pH 8, 140 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 and once with TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The chromatin fragments were eluted from beads with 62.5 mM Tris-HCl, pH 6.8, 200 mM NaCl, 2% SDS, 10 mM dithiothreitol, and cross-links were reverted by heating at 65 °C overnight.

DNA was phenol/chloroform-extracted, ethanol precipitated, and analyzed for human Igε promoter by PCR or quantitative RT–PCR using the following

primers: 5′-TGGGCCTGAGAGAGAAGAGA-3′ and 5′-AGCTCTGCCTCAGTGCTTTC-3′. The PCR reactions were performed in a 25-µl volume with 10 µl of immunoprecipitated material, using AmpliTaq Gold DNA polymerase (PerkinElmer Life Sciences) and 35 PCR cycles. The extract aliquoted before the immunoprecipitation step (total input chromatin) was also used for PCR analysis. Quantitative RT–PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. The results were normalized against the total input DNA.

1.11. Histone acetylation assays (II)

The total cell lysates of transfected COS-7 cells were prepared as described in *4.1.3.* The extracts were immunoprecipitated with appropriate antibodies and collected on protein A/G beads. The beads were washed with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) and HAT buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA) supplemented with PMSF and aprotinin. The beads were incubated with H4 substrate (synthetic peptide containing the first 23 amino acids of histone H4 coupled through a linker sequence to a biotin molecule) and [3H]acetyl-CoA (Amersham Biosciences). The acetylated substrate was collected with immobilized streptavidin (Sigma) and washed with RIPA buffer. Following the washing, the resin was resuspended to HAT buffer. Scintillation fluid (Optiphase Supermix liquid scintillation mixture; PerkinElmer Life Sciences, Wallac) was added, and the associated radioactivity was counted with 1450 MicroBeta Plus liquid scintillation counter (PerkinElmer Life Sciences, Wallac).

1.12. Confocal Microscopy (II)

HeLa cells were starved in serum-free medium for 4 h and treated with IL-4 (100 ng/ml) for 20 min or left untreated. Cells were fixed and permeabilized with 4% formaldehyde, 0.2% Triton X-100 in PEM buffer (100 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8) for 10 min at room temperature and postfixed with methanol for 5 min at -20 °C. Cells were stained with the mixture of appropriate antibodies, followed by fluorescent secondary antibodies (Molecular Probes). Confocal images were collected using LSM510 program and Zeiss confocal microscope, equipped with an Argon laser (488 nm) and HeNe laser (543 nm) and a X63 objective. Green emission was detected using a 505-nm low pass filter and red emission using a 630-nm low pass filter.

1.13. Northern hybridization (IV)

Radiolabeled probes of U1 snRNA, U2 snRNA, U4 snRNA, U5 snRNA and U6 snRNA were made by *in vitro* transcription of the linearized snRNA plasmids

using Maxiscript SP6 kit (Ambion). U7 and 7SK templates were prepared by PCR and *in vitro* transcribed with Maxiscript T7 kit (Ambion).

RNAs were separated by denaturing 6% (w/v) urea PAGE, transferred to a nylon filter with semi-dry blotter in 0.5× TBE buffer using a constant 3 mA cm^{-2} current for 1 h, and cross-linked with a Kodak UV X-linker. Prehybridization was carried out at 60°C for 1 h in 6× SSC, 25 mM sodium phosphate, pH 6.5, 0.5% SDS, 5× Denhardt's solution, 50% formamide, and 0,15 mg/ml carrier tRNA. RNA probes were added and the hybridization performed at 42°C over night. The membranes were washed at room temperature for 15 min in 2× SSC plus 0,1% SDS, 15 min in 0,5× SSC plus 0,1% SDS, 15 min in 0,1× SSC plus 0,1% SDS, and at 60°C for 15 min in 0,1× SSC plus 0,1% SDS. The signals were visualized by autoradiography. Stripping of the membranes was performed at 100°C with 0,01× SSC plus 0,5% SDS.

Results

1. PU.1 regulates the transcriptional activation of the Stat6 response element in the Igε promoter (I)

1.1. Identification of a PU.1 binding sequence within the STAT6 response element of Igε gene promoter

Transcription factor PU.1, a member of the Ets gene family, has been previously shown to bind to a binding site that overlaps with the distal NF-κB1 binding sequence in the immunoglobulin heavy-chain germline Igε gene promoter (Stutz and Woisetschlager, 1999). Functional studies suggested that loss of PU.1 DNA binding has no adverse effect on Igε promoter activation, but defective binding of both PU.1 and NF-κB simultaneously led to abrogation of cytokine inducibility.

In this study, we have investigated the effect of PU.1 on transcriptional activation of the STAT6 response element in the Igε gene promoter. We compared the STAT6 response elements of several IL-4 responsive genes, and identified a minimal low-affinity PU.1 binding sequence (5'-AGAA-3') (Himmelmann et al., 1997) within the STAT6 binding sequence in the Igε gene promoter. On the contrary, the STAT6 binding sequence of IL-4/STAT6 responsive IL-4Rα gene promoter did not contain PU.1 binding site.

1.2. Expression of PU.1 confers the IL-4 inducibility of STAT6 response element in HepG2

Tissue-specific gene expression is the result of several interacting levels of gene regulation. Promoters, such as IL-4/STAT6 responsive promoters, contain *cis*-acting elements that mediate the DNA binding of several transcription factors. The composition of these elements and transcription factors vary between different promoters. IL-4 responsive genes are expressed in a tissue-dependent manner, but the *cis*-acting elements and transcription factors that contribute to the specificity are largely not known.

In an earlier study, STAT6 response element of Igε promoter was found to be activated in response to IL-4 stimulation in B cells, but not in non-hematopoietic HepG2 cell line (Pesu et al., 2002). This suggests that a B cell-specific transcription factors or transcription factors are needed for STAT6-response element mediated transactivation. PU.1 is expressed in a wide variety of cells of haematopoietic lineage, and modulates the proliferation and differentiation of

several haematopoietic precursors (Lloberas, Soler and Celada, 1999). In Study I we coexpressed PU.1 and a reporter construct containing repeats of STAT6 response element of Igε gene promoter, and found out that expression of PU.1 restored the IL-4 inducibility of STAT6 response element in HepG2. Inducibility of IL-4Rα gene promoter STAT6 response element was not affected by expression of PU.1.

HepG2 cell line stably expressing PU.1 was generated, and IL-4 inducibility analyzed with several different reporter constructs containing binding sites for STAT6, NF-κB, C/EBPβ and PU.1. The presence of STAT6 response element in the reporter resulted in an enhanced response to IL-4 stimulation in all the constructs studied. We also investigated the effect of PU.1 on the STAT6TAD, which is capable of functioning as an independent transactivator, and is not regulated by IL-4. According to our results, the IL-4 inducibility of STAT6TAD fused to DNA-binding domain of GAL4 was not affected by expression of PU.1, although the basal activity of GAL4-STAT6TAD was slightly higher in PU.1 expressing cells.

1.3. PU.1 interacts with STAT6 and enhances STAT6-mediated transcription

To further analyze the mechanism of PU.1 mediated enhancement of transcription, we performed GST pulldown assays and EMSAs to detect the potential interaction between STAT6 and PU.1. STAT6 and PU.1 were found to interact in GST pulldown experiments, but the complex did not cause mobility shift in EMSAs, indicating the *in vivo* interaction may be weak.

To analyze the importance of an intact PU.1 core binding sequence, we designed a reporter construct, where PU.1 binding sequence had been mutated (A→T). As a result, IL-4 inducibility of the reporter was lost both in Daudi B cells and HepG2 cells expressing PU.1. EMSA experiments pointed out, that mutation did not affect DNA binding of STAT6. We were not able to detect the binding of PU.1 to wild-type or mutated STAT6 response element in EMSAs, which may be due to the low affinity of binding.

Serine phosphorylation mutant and different deletion mutants of PU.1 were created to analyze the structural requirements of PU.1 for STAT6-mediated transactivation. According to our results, serine phosphorylation of PU.1 was not found to be required for its function on the STAT6 response element. Deletion of the N-terminal acidic transactivation domain completely prevented PU.1 from activating STAT6-mediated transcription. In addition, DNA binding domain of PU.1 was essential for PU.1 function in STAT6-mediated transactivation.

2. STAT6 recruits CBP and chromatin modifying activities to the promoter via p100 (II)

2.1. p100 functions as an adapter bridging STAT6 to CBP

Several lines of evidence indicate that coactivators CBP/p300 critically modulate the functions of STATs. CBP/p300 are suggested to stimulate transcription by their intrinsic histone acetyltransferase activity and by association with p/CAF, another histone acetyltransferase (Bannister and Kouzarides, 1996, Ogryzko et al., 1996, Yang et al., 1996) but also by functioning as a bridging factor connecting transcription factors and members of basal transcription machinery (Chan and La Thangue, 2001).

It has been previously shown, that CBP is required for STAT6-mediated gene activation (Gingras et al., 1999, Litterst and Pfitzner, 2001, McDonald and Reich, 1999, Ohmori and Hamilton, 1998). However, the precise mechanisms and functions of CBP in STAT responses are not known. The interaction between CBP and STAT proteins has most often been shown to occur via TAD domains, but a common amino acid motif involved in the interaction has not been identified. Indeed, the TADs are the most variable domains among the STATs, and each STAT appears to make contact with CBP in a unique fashion.

An earlier study has suggested a TAD-mediated direct interaction for STAT6 and CBP (Gingras et al., 1999). In our studies, we failed to detect this direct interaction, which led us to search for an alternative explanation. p100 was earlier identified as a STAT6 interacting protein (Yang et al., 2002). p100 is a transcriptional coactivator protein, which mediates transcriptional responses by bridging sequence specific activators to the basal transcription machinery. Our previous results suggested that p100 functions as a coactivator for STAT6. This led us to investigate, whether p100 functions as an adapter between CBP and STAT6.

Our results indicate that while STAT1-TAD interacted directly with CBP, the interaction between STAT6-TAD and CBP was found to be indirect. p100 was found to interact with STAT6 and CBP both *in vitro* and *in vivo*, and to mediate the interaction between these proteins. When the intracellular localization of STAT6, CBP and p100 was investigated in HeLa cells by confocal microscopy, IL-4 stimulation induced nuclear colocalization of STAT6 and p100, and to lesser extent, STAT6 and CBP, indicating that only a small fraction of nuclear CBP associates with STAT6.

Chromatin immunoprecipitation studies further confirmed that p100 and CBP are recruited to the Igε promoter by STAT6 *in vivo*. In response to IL-4 stimulation, the promoter binding of STAT6 is increased, and ectopic expression of p100 further enhances the STAT6 promoter binding. IL-4 stimulation also results in the formation of a ternary complex of STAT6, p100 and CBP at the natural Igε promoter, as indicated by immunoprecipitation of endogenous CBP.

2.2. p100 mediates the histone acetyltransferase activity of CBP to STAT6

According to our results, the direct interaction between p100 and CBP was mapped to the staphylococcal nuclease-like (SN)-domains of p100 and amino acids 1099-1758 of CBP. These amino acids comprise the HAT domain of CBP, a domain responsible for the histone acetyltransferase activity of CBP. On the contrary, p100 did not associate with SRC-1, another coactivator of STAT6 known to possess some intrinsic HAT activity (Litterst and Pfitzner, 2001). Since the targeting and the regulation of chromatin modifying activities at STAT6 responsive promoters have remained elusive, the interaction results tempted us to further study the role of p100 in this context.

To analyze the histone acetyltransferase activity, we performed acetylation assays with transfected COS-7 cell lysates. The lysates were immunoprecipitated with specific antibodies and acetylation assays were performed with histone H4 as a substrate. Our results indicated that on the contrary to STAT1, coexpression of STAT6 with CBP in COS-7 cells did not result in a marked increase in associated HAT activity. However, coexpression of STAT6 and CBP together with p100 elicited a clear enhancement in associated HAT activity, indicating that p100 recruits histone acetyltransferase (HAT) activity to STAT6 *in vivo*. Chromatin immunoprecipitation studies further demonstrated that p100 regulates histone acetylation. As expected, IL-4 stimulation resulted in an enhancement in histone H4 acetylation at Igε promoter, but in addition, ectopic expression of p100 increased the amount of acetylated histone H4 at the promoter.

3. RNA helicase A (RHA) functions as a coactivator in STAT6 enhanceosome (III)

3.1. Identification of RHA as STAT6-TAD associating protein

In study III we aimed at identificating and characterizing novel STAT6-TAD interacting nuclear proteins. These studies led to the identification of RNA helicase A (RHA) as a component of STAT6 enhanceosome.

To identify possible co-regulators of STAT6, the TAD of STAT6 was expressed as a GST fusion protein and used to purify interacting nuclear proteins. GST fusion proteins were bound to glutathione-coupled beads and incubated with nuclear extracts from [^{35}S]methionine-labeled or non-labeled Ramos B cells. GST–STAT6TAD was found to interact specifically with several different proteins. Initially, we carried out Western blot analysis using antibodies against some potential candidate proteins to identify STAT6 interacting proteins. Other STAT6-TAD interacting proteins were identified by mass spectrometry or by N-terminal peptide sequencing.

The band corresponding to a 140 kDa protein was recovered and subjected to in-gel trypsin digestion. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to measure the molecular masses of the digested peptides. The mass maps obtained were compared against theoretical tryptic peptide mass maps in the OWL protein sequence database using the ProFound program. As a result, the 140 kDa protein was identified as human ATP-dependent RNA helicase A, RHA (Nuclear DNA helicase II, NDH II, DDX9, DEAH box protein 9).

Human RHA was originally isolated and characterized by Lee and Hurwitz (1992) (Lee and Hurwitz, 1992) as an abundant 130-kD nuclear helicase. RHA belongs to the DExD/H family of proteins, which share several conserved motifs. One of these motifs is the characteristic 'DExD/H' sequence, where x can be any amino acid. DExD/H proteins exhibit ATP-dependent RNA helicase activity, but may also be acting as RNA 'chaperones', promoting local RNA unwinding, or as RNPases by mediating RNA–protein association/dissociation. DExD/H proteins play an important role in RNA synthesis and function, including replication, transcription, RNA processing and translation (Lee and Hurwitz, 1993, Zhang, Maacke and Grosse, 1995).

RHA shares sequence homology with the Drosophila 'maleless' (Mle) protein, the amino acid sequences of Mle and RHA are 49% identical (Lee and Hurwitz, 1993). RHA homologues have also been found in *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Danio rerio* and in mammals. For example, the mouse and human RHA proteins share 87% identity (Lee et al., 1998a). The protein structure is highly conserved among these species. Functional domains of RHA include two copies of a dsRNA-binding domain at its N terminus, a helicase core with an ATP binding site in the central region of the molecule, and a C-terminal RGG box nucleic acid-binding domain. (Figure 6.) The catalytic activity is mediated by the helicase motifs, but the RNA-binding domains and RGG box influence and regulate RNA helicase A activity (Zhang and Grosse, 1997).

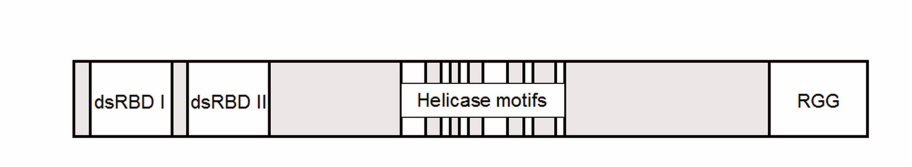

Figure 6. Domain structure of RHA.

Drosophila Mle functions as a regulator of X-linked gene expression. It is required for the equalization of the X-linked gene dosage between the two sexes. Male Mle mutants die at embryonic stage indicating that Mle is an essential factor in Drosophila development (Kuroda et al., 1991). Early embryonic lethality was also observed with RHA knock-out mice, indicating that RHA has an important role in mammalian development (Lee et al., 1998a).

RHA is able to bind both RNA and DNA, but it has been suggested to be mainly pre-mRNA and RNA binding protein. RHA also has a strong affinity towards single stranded DNA. It has been suggested, that RHA might have a transient

role in DNA binding during transcript formation and it might be able to unwind promoter DNA, therefore promoting the engagement of the transcription apparatus on signal responsive promoters. RHA has also been suggested to participate in unwinding of DNA:RNA hybrids during transcription (Zhang and Grosse, 1994, Zhang, Herrmann and Grosse, 1999).

RHA has multiple functions in transcription and in mRNA processing and transport. The main function of RHA is to catalyze the unwinding of both dsRNA and DNA from 3' to 5' direction. The unwinding function is dependent on the ATPase activity (Lee and Hurwitz, 1993).

RHA associates with several different transcriptional regulators, including PolII, CBP, BRCA1, the rat mineralocorticoid receptor (MR), MBD2a and SMN (Anderson et al., 1998, Fujita et al., 2003, Kitagawa et al., 2002, Nakajima et al., 1997, Pellizzoni et al., 2001). In transcriptional complexes RHA functions both as a helicase and a mediator transmitting signals to PolII.

3.2. Interaction of RHA with STAT6 is mediated by p100

To verify the original identification of RHA as STAT6-TAD interacting protein, we performed GST pulldown and coimmunoprecipitation assays. We did not detect a direct interaction between STAT6 and RHA either *in vivo* or *in vitro*, suggesting that the originally found interaction was indirect.

We have previously identified p100 as a STAT6 interacting protein that connects STAT6 to the basal transcription machinery (Yang et al., 2002). p100 functions as a coactivator for STAT6 enhancing STAT6-mediated transcription. In study II p100 was also found to mediate the interaction between STAT6 and CBP. Because p100 had been shown to function as an adapter protein in STAT6 enhanceosome and also other transcriptional complexes, we wanted to investigate, whether p100 can link STAT6 to RHA.

According to our results, RHA interacted directly with p100 both *in vitro* and *in vivo*. The interaction between RHA and p100 was mediated by the helicase motif containing core domain of RHA and the SN-like domains of p100. The wild-type and mutant form of RHA lacking ATPase and helicase activities were able to bind p100 equally well, indicating that the interaction does not depend on the helicase/ATPase activities of RHA.

Chromatin immunoprecipitation experiments were performed, to investigate the function of RHA in the natural Igε promoter. As indicated by the interaction assays, RHA alone had no effect for STAT6 promoter binding. However, coexpression of p100 and RHA resulted in an enhanced promoter binding of endogenous STAT6 after IL-4 stimulation. These results suggest that RHA contributes to the assembly of STAT6 enhanceosome, and p100 connects RHA to STAT6 both *in vitro* and *in vivo.*

3.3. RHA enhances STAT6 regulated transcription

RHA cooperates with CBP and acts as a positive regulator in the transcriptional activation via the cAMP responsive factor CREB (Nakajima et al., 1997). The helicase activity of RHA, which is dependent on the ATP binding activity, is required for mediating the target gene activation. We have demonstrated by a reporter assay method that RHA enhances STAT6-mediated transcription in HeLa cells. Increased amounts of RHA resulted in enhanced transcription of Igε- or IL-4R-promoter containing reporter genes after IL-4 stimulation, whereas the mutant form of RHA lacking ATPase and helicase activities inhibited transcription. This suggests that the function of RHA as a coactivator for STAT6 is dependent on the ATP-binding and helicase activities of RHA.

4. Delineating the STAT6 enhanceosome (II, III)

We have previously identified p100 as a coactivator for STAT6 (Yang et al., 2002). In study III we identified another novel component of STAT6-dependent enhanceosome, RHA. To analyze the functional significance of these coregulators in STAT6 enhanceosome, we performed siRNA knockdown experiments utilizing Igε-reporter constructs. In our experiments, siRNAs directed against STAT6 completely abrogated transcriptional activation of Igε-reporter gene. Also downregulation of p100 and RHA by siRNAs was found to effectively inhibit Igε-reporter gene expression. Our results suggest that p100 and RHA have an important role in the assembly of STAT6 dependent enhanceosome and STAT6-mediated transcription activation. Based on previous reports by others (Chen et al., 1997, Litterst and Pfitzner, 2001, Nakajima et al., 1997, Torchia et al., 1997), and our findings, we are able to delineate a present view of the STAT6 enhanceosome at Igε-promoter. (Figure 7.)

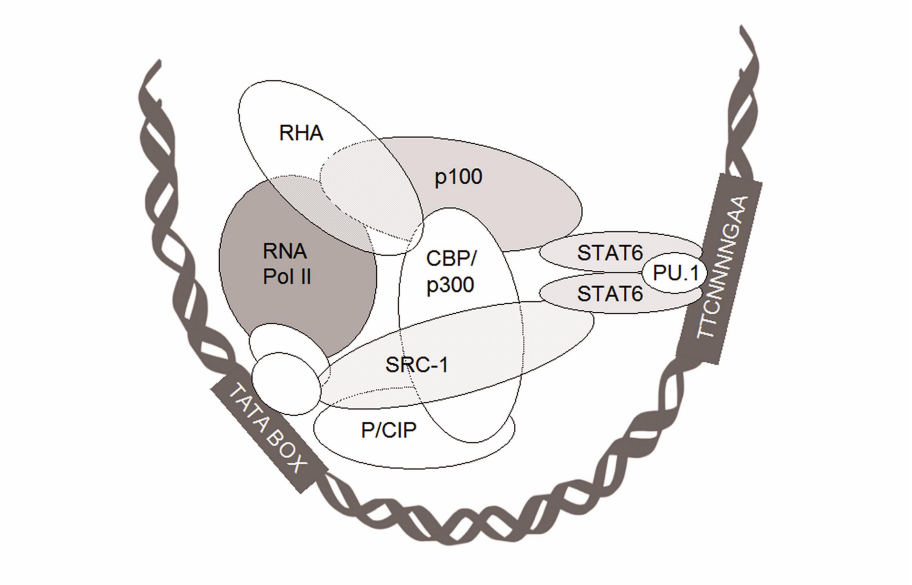

Figure 7. Schematic presentation of STAT6 enhanceosome.

5. p100 interacts with components of spliceosome and regulates pre-mRNA splicing (IV)

5.1. TSN domain of p100 interacts with snRNP complexes

Human p100 protein has been shown to function as a transcriptional coactivator for several transcription factors, including STAT6 (Yang et al., 2002). Most of these factors have been found to associate with SN-like domains of p100, and according to the interactions, it is apparent, that the SN-like domains mediate the transcriptional coactivator function of p100. Consistently, in our previous reporter gene experiments, overexpression of the SN-like domain of p100 alone was sufficient to enhance STAT6-mediated reporter gene activity in response to IL-4 stimulation, whereas the expression of the C-terminal TSN domain alone did not affect the activity of the reporter gene (Yang et al., 2002). TSN domain has not been implicated in protein interactions, and the function of the domain has remained elusive.

In this project, we have performed an analysis of the TSN interacting proteins. We identified p220 as U5-220 (splicing factor Prp8 in yeast), p200 as U5-200 (DExH family RNA helicase, Brr2 in yeast) and p116 as U5-116 (a putative GTPase that is homologous to ribosomal elongation factor EF-2, Snu114 in yeast). Our results from immunoprecipitation assay confirmed the *in vivo* association of p100 protein and hPrp8. To further delineate the interaction, we performed GST pulldown experiments, in which domain 2 (aa 303–668) of U5-220 specifically interacted with TSN domain of p100.

To investigate, whether TSN domain of p100 also associates with other snRNPs, we performed Northern blot analysis using snRNA-probes specific for the major U2-dependent spliceosome (U1, U2, U4, U5 and U6 snRNAs), and as controls U7 snRNA functioning in histone mRNA processing, or 7SK snRNA, a component of the transcription elongation factor P-TEFb. The spliceosomal snRNA-specific probes and U7-specific probe were recognized by the p100-TSN, whereas no interaction was observed with the 7SK. To further analyze the association of p100 with snRNPs, nuclear extracts of stable HeLa-p100 cells were immunoprecipitated with anti-trimethylguanosine (TMG) cap antibody, and the precipitates were subjected to Western or Northern blot analyses. Western blot analysis revealed that p100 was co-immunoprecipitated with anti-TMG-cap antibody. In Northern blot analysis, U1, U2, U4, U5 and U6 snRNAs were all present in the anti-TMG immunoprecipitates. Together, these results suggest that U5-specific proteins and Sm-proteins may associate with p100, whereas TMG-cap structure is not likely to be involved in the interaction.

5.2. p100 protein functions in spliceosome assembly and pre-mRNA splicing

The interaction of p100 with U5-specific proteins and Sm-proteins suggested that p100 could participate in the splicing process. *In vitro* splicing assays using

pre-mRNA splicing substrate were performed to analyse, whether p100 is involved in splicing. According to the results, addition of p100 to the splicing reaction dose-dependently fastens the appearance of splicing intermediates, which suggests that p100 has a stimulatory effect on the splicing reaction. Our results indicate that especially the first steps of splicing before the catalysis are accelerated due to p100. On the other hand, the amount of spliced product was not increased in response to p100.

To analyze the effect of p100 on spliceosome assembly, the formation of different splicing complexes were investigated. The formation of pre-spliceosomal complex A was enhanced in response to p100. We also observed a dose-dependent acceleration in the formation of A, B and C spliceosomal complexes, as well as increased turnover of the complexes. In the splicing assays, the separated TSN domain of p100 produced similar results as the full length p100, and proved to be sufficient to enhance the splicing kinetics. Recombinant SN-like domains completely blocked the splicing reaction and formation of specific splicing complexes.

Discussion

1. Transcriptional regulation of Igε promoter

Gene specific activators bind to specific binding sites on the promoters of their target genes, and on promoters contact multiprotein machinery that directs transcription. STATs are gene specific activators that are activated by distinct cytokine signals, have separate physiological roles and activate unique set of genes. In addition to STATs, the regulatory elements of the STAT regulated genes bind other transcription factors and coregulators to form enhanceosome complexes. The composition of the enhanceosomes may be similar or vary depending on the STAT and the specific gene in question. The enhanceosomes are assembled in a modular fashion, and their composition varies. Combinatorial assembly of individual components may allow differential responses to the activation of an individual STAT or a target gene.

The immunoglubulin heavy-chain germline Igε gene promoter is one of the best characterized IL-4/STAT6 responsive promoters thus far. It has been reported to contain *cis*-acting binding sites for STAT6, BSAP, PU.1, NF-κB, C/EBPβ and Bcl-6 (De Monte et al., 1997, Fenghao et al., 1995, Harris et al., 1999, Iciek, Delphin and Stavnezer, 1997, Mikita, Kurama and Schindler, 1998, Stutz and Woisetschlager, 1999). These transcription factors interact with STAT6 either directly or indirectly, and modulate the DNA-binding or transactivation activity of STAT6 (Oettgen, 2000). Constitutively expressed transcription factors PU.1, C/EBPβ and BSAP are unresponsive to IL-4 stimulus, whereas STAT6 is recruited to the promoter in response to the activation of the IL-4 signaling cascade.

Transcription factor PU.1 is expressed solely in cells of haematopoietic origin (Lloberas, Soler and Celada, 1999) and functional PU.1 binding sites have been identified in most myeloid-specific promoters and in a number of B cell-restricted regulatory elements. For example, myeloid specific IFN-γ-stimulated activation of the FcγRI gene has been shown to be strictly dependent on binding of both STAT1 and PU.1 to the promoter. At FcγRI promoter PU.1 mediates contacts with basal transcription machinery components TBP and PolII (Aittomaki et al., 2002)

STAT6 binding site within germline Igε gene promoter is a modified GAS binding sequence with a four base pair spacer. Study I demonstrated a minimal PU.1 core binding sequence within the STAT6 binding sequence. PU.1 has been previously shown to enhance STAT6-mediated transcription (Stutz and Woisetschlager, 1999) on a mechanism that involves binding of PU.1 to a

different binding site which partly colocalizes with the distal NF-κB site. According to our results, B cell-specific Igε gene promoter was activated in response to IL-4 in B cells, whereas in non-hematopoietic cell line the activation was not observed. Ectopic expression of myeloid and lymphoid cell specific PU.1 was able to restore the IL-4 inducibility of Igε gene promoter in non-hematopoietic cells. PU.1 was found to enhance STAT6-mediated transcription from the STAT6 response element reporter construct that was deficient for the distal NF-κB site, indicating the presence of another binding site within the STAT6 consensus sequence. In IL-4R reporter construct lacking the low affinity binding site for PU.1, or Igε reporter construct with mutated PU.1 binding site, PU.1 was not found to affect transcriptional activation of STAT6. The results suggest that PU.1 enhances STAT6-mediated transcription from germline Igε gene promoter by binding to a low activity binding site within the STAT6 binding site. The effect of PU.1 on STAT6-mediated transcription activation may represent a fine-tuning mechanism that restricts the expression of certain B cell-specific genes to haematopoietic cells.

In addition to the transcription factors, transcriptional coregulators are necessary for the full transcriptional activation of many genes. The genes are wrapped around by histones, and one of the main functions for transcription coactivators is to help the DNA binding activators and the transcription machinery to overcome the inhibitory barrier effect of packed chromatin. Another main function for coactivators is to function as adapters, bridging sequence specific activators to the basal transcription machinery.

STAT6 is not able to activate transcription *per se*, and additional proteins, transcription factors and coregulatory proteins are needed for the process. STAT6 is expected to interact either directly or indirectly with several coregulatory proteins. One of the major aims of the present study was to identify novel coregulatory proteins of STAT6 and characterize their functions in STAT6 regulated enhanceosome.

Previous reports have demonstrated that coactivator CBP/p300 is involved in the transcriptional activation of all the STATs (Bhattacharya et al., 1996, Gingras et al., 1999, Horvai et al., 1997, McDonald and Reich, 1999, Nakashima et al., 1999, Ohmori and Hamilton, 1998, Paulson et al., 1999, Pfitzner et al., 1998, Zhang et al., 1996). The interaction of CBP with STAT6 had been linked to the TAD domain of STAT6, which markedly diverges from the TADs of other STATs. This suggests that STAT6 may also apply a different mechanism to recruit chromatin modifying activities of CBP to the promoters. In study II, the interaction of CBP/p300 with STAT1TAD and STAT6TAD was compared. Interaction between CBP/p300 and STAT1TAD was readily detected by different methods, whereas the previously detected interaction between CBP/p300 and STAT6TAD was identified to be either indirect or very weak. On the contrary, CBP was found to directly interact with p100, a previously identified coactivator of STAT6. Our results suggest that a ternary complex of CBP, p100 and STAT6 is formed at Igε promoter, and the complex formation is necessary for the recruitment of histone acetyltransferase activities of CBP to the promoter.

Study III identified RHA as a novel coregulator of STAT6. RHA is previously known to be a member of several coactivator complexes, and function both as a bridging factor and chromatin modifier. RHA is connected to STAT6TAD by p100, which further underlines a central role of p100 in STAT6 enhanceosome. RHA was identified as a coactivator for STAT6, and the activating function was dependent on the ATP-binding and helicase activities of the protein. Strong interaction of RHA with p100 may also indicate that these factors may collaborate also in other cellular processes. For example, both proteins have been reported to interact with PolII and the RISC complex involved in RNAi (Caudy et al., 2003, Nakajima et al., 1997, Robb and Rana, 2007, Tong et al., 1995).

In the present study we aimed at identifying and characterizing the components of STAT6 enhanceosome. The eukaryotic enhanceosome complexes have been most thoroughly studied in the context of nuclear receptors. So far, relatively little is known about the composition and assembly of other enhanceosomes, such as cytokine regulated STAT enhanceosomes, although the large scale proteomic approaches have identified multiple putative interaction partners and coregulatory proteins for STATs. The detected protein-protein interactions allowed us to draw a schematic view of the STAT6 enhanceosome at Igε promoter summarizing the currently known components and the interacting partners of the enhanceosome (Figure 5.).

In 2003 Lerner *et al.* (Lerner et al., 2003) published the first and so far the only detailed report on the assembly and disassembly of a STAT-dependent enhanceosome. The transcription factor binding sites and components of STAT3 dependent, glucocorticoid supplemented enhanceosome of the α2-macroglobulin (α2-M) gene were studied in detail using mutational analysis, and reporter and chromatin immunoprecipitation assays. The enhanceosome was found to constitutively contain glucocorticoid receptor (GR), c-Jun and OCT-1.

The timing of enhanceosome assembly and disassembly was also investigated, and the authors found out that transcription factors bind to the promoter 5-10 min before the arrival of PolII, and the enhanceosome was dissociated within 30 min from the removal of the stimulus (Lerner et al., 2003). Especially the time course analysis of enhanceosome assembly and disassembly gives important information on the mechanisms of STAT3 mediated gene activation, and allow the authors to investigate the recruitment of the enhanceosome components and their activities in detail. For example, Pol II is recruited to the promoter at least 5-10 minutes after GR and STAT3, which may represent the time needed for the transcription factors or coactivators to recruit PolII to the promoter. The coactivators of this enhanceosome have not been yet identified, but in addition to the Mediator suggested by the authors, it is likely, that CBP/p300 is a member of the complex. Therefore, the timing may represent the approximate time course for the assembly of STAT6 enhanceosomes.

The results of this thesis study mainly concern a single STAT6 responsive promoter, Igε gene promoter. Currently, it is unknown how well the composition of individual enhanceosome complex compare to other enhanceosomes on other

STAT6 regulated promoters. The interactions between the proteins may not be the same within all enhanceosomes. However, in the case of RHA, we observed a similar stimulatory effect on transcriptional activation with both Igε gene promoter and another STAT6 regulated promoter, IL-4R promoter, indicating that RHA may be more commonly present in STAT6 linked enhanceosomes. The strongest evidence on the importance of the identified factors comes from RNAi experiments. Removing either p100 or RHA form the enhanceosome complex resulted in markedly reduced transcriptional activation. This indicates true cooperation of enhanceosomal proteins in the complex.

2. The novel functions of p100 protein

p100 consists of staphylococcal nuclease (SN)-like and Tudor-SN (TSN) domains (Callebaut and Mornon, 1997). The recently modeled three-dimensional structure indicates that the overall structure of full-length p100 resembles a stick with a hook, where the SN-like domains form the stick, and the tudor domain interdigitated with the fifth SN-like domain the makes up the hook (Shaw et al., 2007).

p100 has been shown to function as a transcriptional coactivator for several transcriptional regulators (Dash, Orrico and Ness, 1996, Leverson et al., 1998, Paukku, Yang and Silvennoinen, 2003, Tong et al., 1995, Yang et al., 2002). The transcription coactivator function of p100 is clearly mediated by the SN-like domains of p100, as indicated by the detected interactions with STAT6 (Yang et al., 2002), other coregulators (studies II and III), and the reporter assays with truncated constructs (Yang et al., 2002). Individual SN-like domains seem to contribute to the binding equally, although detailed understanding of the interaction mechanisms of p100 awaits for the structural analysis of the SN-domains. The modular SN-like domains seem to function as protein interaction domains, and a flexible structure may be necessary to permit binding to multiple targets. According to the recent report by Shaw et al. (Shaw et al., 2007), the surface charge in p100 SN-like domains 1-3 is positive, as in SNs, whereas the charge in the fourth SN-like domain is negative. This may contribute to ligand binding, although differences in binding partners between individual SN-like domains have not yet been found.

TSN domain is not necessary for coactivator function of p100, and so far the function of this domain has remained elusive. It has been previously suggested that based on the protein sequence TSN might function as a protein interaction domain (Tong et al., 1995). The tudor domain within TSN domain contains an aromatic cage composed of three tyrosine residues (Tyr721, Tyr738 and Tyr741) and a phenylalanine residue (Phe715) (Shaw et al., 2007). The aromatic cages are found in many proteins known to interact with methyl groups. Therefore, also p100 may trap methylated ligands via this structure. However, no interacting partners for TSN have been identified so far.

In the present study we identified an interaction between p100 and snRNP specific proteins, including human U5-220 (hPrp8, splicing factor Prp8 in yeast), that function in pre-mRNA splicing. hPrp8 binds to 5'SS, the branchpoint (BP) and the 3'SS in the pre-mRNA, and U5 and U6 snRNAs, and is located in the catalytic core of the spliceosome. It has been suggested, that hPrp8 functions in RNA catalysis as a cofactor. hPrp8 associates with pre-mRNA, splicing reaction intermediates and excised introns as well as several large protein complexes. It is a component of the U5 snRNP and U5·U4/U6 tri-snRNP complexes. hPrp8 has been reported to associate with transcriptional cofactors, such as BRG1, which is a component of SWI/SNF remodelling complex (Grainger and Beggs, 2005).

The detected interaction with snRNP specific proteins indicates that p100 may also have a role in splicing. Consistently, SMN (Survival of Motor Neurons) protein, which contains a Tudor domain highly similar to TSN, has been shown to be a component of small nuclear riboproteins invoved in pre-mRNA splicing (Buhler et al., 1999). Interestingly, also RHA, which was identified as a p100 interacting coactivator for STAT6 in study III, interacts with SMN, and has been previously isolated as a component of human prespliceosomes (Hartmuth et al., 2002). RHA has also been shown to act together with ADAR for a coordinated editing and splicing of the glutamate receptor pre-mRNA (Bratt and Ohman, 2003). This may indicate that the interaction between p100 and RHA may also extend to other cellular processes beyond transcription.

In functional studies, p100 and purified TSN domain alone were able to accelerate the kinetics of spliceosome assembly. Also SMN has been shown to function in U snRNP assembly and bind methylated Sm proteins (Paushkin et al., 2002). Thus our results suggest that p100 protein is a novel dual function regulator of gene expression that participates via distinct domains in both transcription and splicing. The mechanism of this dual function is to be determined, but it may involve the need of hydrophobic core amino acids. In SMN Tudor domain they have been shown to mediate interactions with the dimethylarginine modifications of the Sm-proteins (Brahms et al., 2001, Sprangers et al., 2003). Consistently, mutations of the conserved tyrosine residues of the aromatic cage abolished the interactions between TSN domain and snRNAs.

Several factors have previously been identified to function both in transcription and splicing. The two nuclear events may be coordinated by a global complex that tethers both transcription and RNA processing factors to the transcribed gene (Terns and Terns, 2001). The connection between nuclear processes is an example of a communication network that influences the gene expression.

Conclusions and perspectives

The expression of protein coding genes is controlled at multiple levels including transcription and RNA editing. Gene-specific transcriptional regulation is based on *cis*-acting control elements located on promoter-enhancer regions of protein coding genes. These elements specify the location, magnitude and timing of the gene expression. Higher-order multiprotein complexes, enhanceosomes, composed of transcription factors and transcriptional coregulators, assemble around gene specific activators that bind to specific *cis*-acting control elements. Enhanceosomes activate transcription mainly by opening the chromatin structure and connecting sequence-specific activators to the basal transcription machinery. Another important aspect of gene regulation is that RNA editing is strongly linked to transcription. Many splicing factors have also been shown to be involved in transcription and vice versa. PolII is the main connector of nuclear processes.

STAT6 is the main regulator of the IL-4 mediated gene responses. The main focus of this study was to investigate the mechanism of STAT6-mediated transcriptional activation. This study was aimed at investigating the underlying mechanisms of cell-type specific IL-4 responses, identifying novel coregulators of STAT6 and characterizing their functions in gene regulation.

In the present study we examined the effect of PU.1 in transcription activation of the STAT6 response element in Igε promoter (I). PU.1 had previously been found to enhance STAT6 regulated transcription via an unknown mechanism that involves binding to a *cis*-acting element on the STAT6 regulated promoters. Study I identified a novel binding site within the STAT6 response element at the Igε promoter, and the cooperation between STAT6 and PU.1 was found to regulate the tissue-specificity of the gene expression.

STAT6 transcriptional activation can be positively regulated by interaction with coactivators p100 and CBP/p300. p100 has been shown to function as an adapter connecting sequence specific activators to the basal transcription machinery, whereas CBP/p300 have an important function in modifying the chromatin structure by histone acetylation. This study identified p100 as an important mediator of chromatin modifying and histone acetylation activities in STAT6 enhanceosome (II).

To further investigate the composition of STAT6 enhanceosome, we aimed at identifying novel coregulators, and identified RHA as a novel coactivator for STAT6 (III). The composition and spatial arrangement of the STAT6 enhanceosome was evaluated by interaction assays with previously known components of the enhanceosome.

In studies II and III and previous studies p100 was identified as an important regulator of STAT6-mediated gene responses and a central adapter protein in STAT6 enhanceosome at Igε promoter. Besides the role of p100 as a transcription coactivator, very little is known about the other functions of this protein. Study IV investigated the currently unknown functions of p100 in cells according to the functional interactions of C-terminal TSN-domain, which is not involved in transcriptional regulation. p100 was characterized as having a dual regulatory role in mRNA synthesis, it functions both in transcription and splicing via distinct domains. p100 enhances splicing by facilitating spliceosome assembly. Identification of transcriptional regulators in splicing complexes is strong evidence for the cooperativity and functional coordination of these processes. The identification of p100 as a novel splicing regulator may also open up novel insights into the regulation of STAT6-mediated gene expression.

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