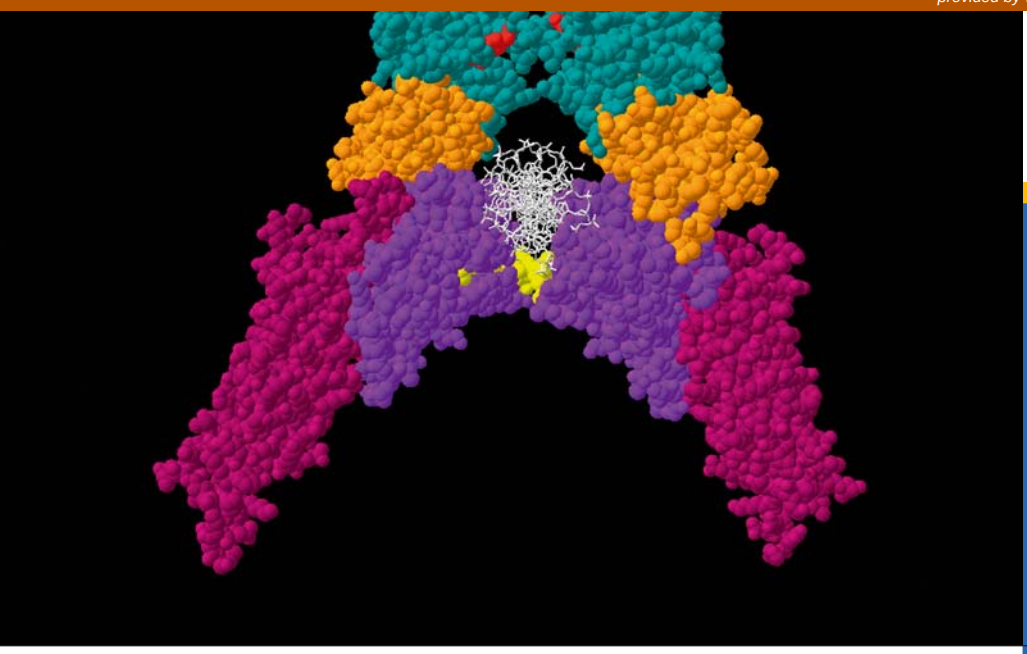

Riku Fagerlund

Nuclear Import Mechanisms of STAT and NF-κB Transcription Factors

Publications of the National Public Health Institute A 2/2008

Department of Viral Diseases and Immunology
National Public Health Institute
and
Department of Biological and Environmental Sciences
Division of General Microbiology
University of Helsinki, Finland

Helsinki 2008

Riku Fagerlund

NUCLEAR IMPORT MECHANISMS OF STAT AND NF-κB TRANSCRIPTION FACTORS

A C A D E M I C D I S S E R T A T I O N

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in the small hall, University Main Building, on 8th of February, at 12 o´clock noon.

Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki, Finland

and

Department of Biological and Environmental Sciences, Division of General Microbiology, University of Helsinki, Finland

Helsinki 2008

Publications of the National Public Health Institute
KTL A2 / 2008

Julkaisija-Utgivare-Publisher

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ISBN 978-951-740-754-0
ISSN 0359-3584
ISBN 978-951-740-755-7 (pdf)
ISSN 1458-6290 (pdf)

Kannen kuva - cover graphic: STAT1 homodimer bound to DNA

Yliopistopaino
Helsinki 2008

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To living things… and viruses

Riku Fagerlund, Nuclear import mechanisms of STAT and NF-κB transcription factors

Publications of the National Public Health Institute, A2/2008, 89 Pages
ISBN 978-951-740-754-0; 978-951-740-755-7 (pdf-version)
ISSN 0359-3584; 1458-6290 (pdf-version)
http://www.ktl.fi/portal/4043

ABSTRACT

The eukaryotic cell nucleoplasm is separated from the cytoplasm by the nuclear envelope. This compartmentation of eukaryotic cells requires that all nuclear proteins must be transported from the cytoplasm into the nucleus. Transport of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs), large protein complexes that penetrate and fuse the nuclear envelope. NPCs are freely permeable to small molecules (such as metabolites and water) but they restrict the movement of larger molecules to those containing specific transport signals. Proteins to be targeted into the nucleus by the classical nuclear import system contain arginine/lysine-rich nuclear localization signals (NLSs), which are recognized by importin α, the NLS receptor. Importin α binds to importin β, which docks the importin-cargo complex on the cytoplasmic side of the NPC and mediates the movement of the complex into the nucleus. Presently six human importin α isoforms have been identified: importin α1, α3, α4, α5, α6 and α7. Many intracellular signal transduction pathways are regulated by controlling the nuclear localization of specific proteins.

Transcription factors are among the most important regulators of gene expression in eukaryotic organisms. Transcription factors bind to specific DNA sequences on target genes. These sequences are usually located in the promoter regions preceding the gene, and the binding of transcription factors to these sites modulates the activity of the target gene. Many transcription factors, including signal transducers and activators of transcription (STAT) and nuclear factor κB (NF-κB), reside in the cytoplasm in an inactive form, and upon activation they are rapidly transported into the nucleus. In the nucleus STATs and NF-κB regulate the activity of genes whose products are critical in controlling numerous cellular and organismal processes, such as inflammatory and immune responses, cell growth, differentiation and survival.

The aim of this study was to investigate the nuclear import mechanisms of STAT and NF-κB transcription factors. This work shows that STAT1 homodimers and STAT1/STAT2 heterodimers bind specifically and directly to importin α5 molecule

via unconventional dimer-specific NLSs. The results of this work further suggest that two of these NLS elements, one in each monomer, have to be intact for STAT1 homodimers and STAT1/STAT2 heterodimers to be recognizable by importin α5 and subsequent nuclear import to take place. Moreover, we found that importin α5-STAT complex is composed of one STAT dimer and two importin α5 molecules. Importin α molecules have two regions, which have been shown to directly interact with the amino acids in the NLS of the cargo molecule. The Arm repeats 2-4 comprise the N-terminal NLS binding site and Arm repeats 7-8 the C-terminal NLS binding site. In this work it is shown that the binding site for STAT1 homodimers and STAT1/STAT2 heterodimers is composed of Arm repeats 8 and 9 of importin α5 molecule.

The results in this work are the first to demonstrate interactions between importin α molecules and NF-κB proteins. This work demonstrates that all NF-κB proteins are transported into the nucleus by importin α molecules. In addition, NLS was identified in RelB protein. The interactions between NF-κB proteins and importin α molecules were found to be directly mediated by the NLSs of NF-κB proteins. Furthermore, this study shows that the classical and the alternative NF-κB pathway components have somewhat different specificities for importin α isoforms. Moreover, we found that p50 binds to the N-terminal and p65 to the C-terminal NLS binding site of importin α3. The results in this work show that the nuclear import of p52/RelB dimers is exclusively mediated by the NLS of RelB protein, whereas the nuclear import of p52/p65 dimers is solely regulated by the NLS of p52 protein.

The results from this thesis work identify previously uncharacterized mechanisms in nuclear import of STAT and NF-κB. These findings provide new insights into the molecular mechanisms regulating the signalling cascades of these important transcription factors from the cytoplasm into the nucleus to the target genes.

Keywords: transcription factor, nuclear transport, nuclear localization signal, NLS, importin, STAT, NF-κB

Riku Fagerlund, Nuclear import mechanisms of STAT and NF-κB transcription factors

Kansanterveyslaitoksen julkaisuja, A2/2008, 89 sivua
ISBN 978-951-740-754-0; 978-951-740-755-7 (pdf-versio)
ISSN 0359-3584; 1458-6290 (pdf-versio)
http://www.ktl.fi/portal/4043

TIIVISTELMÄ

Tumakalvo erottaa eukaryoottisolun nukleoplasman sytoplasmasta. Tämän vuoksi kaikki sytoplasmassa tuotetut tumaproteiinit kuljetetaan tumakalvolla sijaitsevien tumahuokosten kautta sytoplasmasta tumaan. Tumakuljetus on aktiivinen tapahtuma, johon osallistuvat tumahuokosten proteiinit sekä erityiset kuljettajaproteiinit. Tumalokalisaatiosignaalit (nuclear localization signals; NLSs) välittävät tumaan kuljetettavien proteiinien tunnistusta. Yleisimmin NLS-signaalit ovat lyhyitä, emäksisiä aminohappoja sisältäviä jaksoja tumaan kuljetettavissa proteiineissa. NLS-signaalin tunnistaa α-importiini, NLS-reseptori, joka puolestaan sitoutuu β-importiiniin. β-importiini on kuljetusproteiini, joka välittää tumaan kuljetettavan proteiinin ja α-importiinin muodostaman kompleksin sitoutumisen tumahuokoseen ja siirtymisen tumaan. Ihmisellä α-importiini-molekyylejä on tunnistettu kuusi erilaista alatyyppiä: α1-, α3-, α4-, α5-, α6- ja α7-importiinit. Useissa solunsisäisissä signaalinvälitysreiteissä tiettyjen proteiinien tumakuljetuksen säätely on hyvin keskeistä.

Transkriptiotekijät ovat tärkeimpiä geenien ilmentymiseen vaikuttavia molekyylejä. Transkriptiotekijät sitoutuvat tarkoin määrättyihin säätelyjaksoihin DNA-molekyylissä ja vaikuttavat geenien ilmentymiseen. Kyseiset säätelyjaksot sijaitsevat yleisimmin geeniä edeltävillä promoottorialueilla. Useat transkriptiotekijät, kuten sytokiinien indusoimat signal transducers and activators of transcription (STAT) ja nuclear factor κB (NF-κB), esiintyvät sytoplasmassa inaktiivisina ja aktivoiduttuaan kulkeutuvat nopeasti tumaan saaden aikaan muutoksia geenien ilmentymisessä. STAT- ja NF-κB-transkriptiotekijät ovat hyvin tärkeitä geenien toiminnan säätelijöitä mm. elimistön puolustusjärjestelmässä viruksia ja mikrobeja vastaan, solujen kehittymisessä ja erilaistumisessa sekä solukuoleman säätelyssä.

Tässä työssä selvitimme STAT- ja NF-κB-transkriptiotekijöiden tumakuljetusta. Ensimmäisessä ja toisessa osatyössä tutkimme STAT1- ja STAT2-proteiinien sitoutumista α5-importiiniin. Ensimmäisessä osatyössä osoitimme STAT1-

homodimeerien ja STAT1/STAT2-heterodimeerien sitoutuvan suoraan α5-importiiniin epätavallisten, rakenteellisten NLS-signaalien välityksellä. Lisäksi havaitsimme STAT/α-importiini-kompleksin koostuuvan yhdestä STAT-dimeeristä ja kahdesta α5-importiinista. Mutaatioanalyysillä selvitimme lisäksi, että aktiivisessa STAT-dimeerissä molempien STAT-proteiinien NLS-signaalien on oltava toiminnallisia, jotta dimeeri kykenee sitoutumaan α5-importiiniin ja siirtymään tumaan.

Toisessa osatyössä selvitimme STAT1- ja STAT2-proteiinien sitoutumiskohtia α5-importiinissa. α-importiinissa on kaksi aluetta, joiden on aiemmin osoitettu suoraan sitoutuvan tumaan kuljetettavien proteiinien NLS-signaalien aminohappoihin. Nämä alueet koostuvat α-importiinin N-terminaalisista Arm 2-4 -toistojaksoista, sekä C-terminaalisista Arm 7-8 -toistojaksoista. Selvitimme STAT1-homodimeerien ja STAT1/STAT2-heterodimeerien sitoutuvan α5-importiinin Arm 8 ja 9 -toistojaksoihin.

Kolmannessa ja neljännessä osatyössä tutkimme NF-κB-proteiiniperheen tumakuljetusta. NF-κB-proteiinien ja α-importiinien välisiä interaktioita ei aikaisemmin ole osoitettu. NF-κB-perheen proteiineista p50:n, p52:n, p65:n ja c-Rel:n on osoitettu sisältävän yksiosaisen NLS-signaalin. Sitä vastoin RelB-proteiinin NLS-signaalia ei ole tunnistettu. Tutkimuksemme osoitti kaikkien NF-κB-proteiinien sitoutuvan suoraan α-importiineihin NF-κB-proteiineissa olevien NLS-signaalien välityksellä. Mutaatioanalyysi osoitti p50-proteiinin sitoutuvan α3-importiinin N-terminaaliseen Arm 3 -toistojaksoon ja p65-proteiinin C-terminaalisiin Arm 7 - ja Arm 8 -toistojaksoihin. Lisäksi tunnistimme RelB-proteiinista NLS-signaalin. Tutkimuksemme osoitti myös, että RelB/p52-dimeerin tumakuljetus välittyy ainoastaan RelB-proteiinin NLS-signaalin kautta, kun taas p52/p65-dimeerin tumakuljetus on riippuvainen ainoastaan p52-proteiinin NLS-signaalista.

Tässä väitöskirjatyössä identifioimme aiemmin tuntemattomia STAT- ja NF-κB-transkriptiotekijöiden tumakuljetustapahtumia. Tämä väitöskirjatyö syventää tietämystä molekylaarisista mekanismeista signaalinvälitysreiteissä, jotka välittävät STAT- ja NF-κB-transkriptiotekijöiden siirtymistä sytoplasmasta tumaan kohdegeenien säätelyalueille.

Avainsanat: transkriptiotekijä, tumakuljetus, tumalokalisaatiosignaali, NLS, importiini, STAT, NF-κB

CONTENTS

ABBREVIATIONS

AMP	adenosine monophosphate
AMPK	AMP-activating kinase
ARD	ankyrin repeat domain
Arm	armadillo repeat domain
BAFF	B cell-activating factor
CD	cluster of differentiation
cDNA	complementary DNA
CIS	cytokine induced SH2 protein
CK	casein kinase
ER	endoplasmic reticulum
FCS	fetal calf serum
FPLC	fast protein liquid chromatography
GAS	gamma activated sequence
GAF	gamma activated factor
GDP	guanidine diphosphate
GRR	glycine rich region
GST	glutathione S-transferase
GTP	guanidine triphosphate
HIV	human immuno-deficiency virus
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IKK	inhibitory κB kinase
IL	interleukin
INM	inner nuclear membrane

IRF	interferon regulatory factor
ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
IU	international unit
Jak	Janus tyrosine kinase
LZ	leucine zipper
LPS	lipopolysaccharide
mRNA	messenger RNA
NE	nuclear envelope
NES	nuclear export signal
NF-κB	nuclear factor kappa-B
NIK	NF-κB-inducing kinase
NK	natural killer
NLS	nuclear localization signal
NPC	nuclear pore complex
NTF2	nuclear transport factor 2
ONM	outer nuclear membrane
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIAS	protein inhibitor of activated STAT
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RanBP	Ran-binding protein
RHD	Rel homology domain
SDS	sodium dodecyl sulphate

SH2	Src homology region 2
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TAD	transcriptional activation domain
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
tRNA	transfer RNA

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Fagerlund R, Melén K, Kinnunen L, Julkunen I. Arginine/lysine-rich nuclear localization signals mediate interactions between dimeric STATs and importin α5. 2002. *J Biol Chem.* 277(33):30072-8.

II Melén K, Fagerlund R, Franke J, Köhler M, Kinnunen L, Julkunen I. Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. 2003. *J Biol Chem.* 287(30):28193-200.

III Fagerlund R, Kinnunen L, Köhler M, Julkunen I, Melén K. NF-κB is transported into the nucleus by importin α3 and importin α4. 2005. *J Biol Chem.* 280(16):15942-51

IV Fagerlund R, Melén K, Cao X, Julkunen I. NF-κB p52, RelB and c-Rel are transported into the nucleus via a subset of importin α molecules. Submitted for publication.

1 INTRODUCTION

The overall process, by which a cell converts one kind of signal or stimulus into cellular responses, as well as the individual steps in this process, is termed signal transduction. The sensing and processing of stimuli are mediated by signal transduction cascades. These molecular networks are constructed from receptors, channels, enzymes and regulatory proteins. Cells are highly responsive to signals from their environment. Extracellular signalling molecules produce a specific response only in target cells, which have receptors for the signalling molecules. Binding of a ligand onto its receptor on the cell surface usually causes a conformational change in the cytoplasmic domain of the receptor that ultimately leads to induction of specific cellular responses. In eukaryotic cells, the response to extracellular stimulus often involves the activation of signal transducers by phosphorylation cascades via receptor coupled enzymes. In some signalling pathways, the receptor itself possesses intrinsic kinase or phosphatase activity, whereas in other pathways, the receptor interacts with membrane associated kinases or recruits cytosolic kinases to the receptor.

Signal transduction cascades often result in the control of gene expression. Transcription factors are proteins, which bind to DNA and modulate the transcriptional activity of the target gene. Human genome encodes more than 2000 transcription factors. In contrast to general transcription factors that are present in all cells and contribute to the synthesis of every mRNA molecule, many transcription factors function by specifically identifying promoter-proximal regulatory elements in a limited number of genes. Many of these site-specific transcription factors are signal-dependent and sequestered inactive prior to receptor stimulation. Signal transducers and activators of transcription (STATs) and nuclear factor κB (NF-κB) are site-specific transcription factors, which, under unstimulated cellular conditions, are sequestered in the cytoplasm as an inactive, latent state. After activation by various extracellular signals, these transcription factors rapidly translocate into the nucleus, where they specifically modulate the expression of the target genes.

In eukaryotic cells, the extracellular signal has to be transmitted from the cell surface into the nucleus in order to modulate gene expression. The eukaryotic cell nucleus is separated from the cytoplasm by the nuclear envelope. Nuclear envelope consists of double lipid bilayer membrane that has nuclear pore complexes (NPCs) embedded. NPCs are channels that provide the only route of movement of

macromolecules into and out of the nucleus. Active nucleocytoplasmic transport of macromolecules is mediated by specific import and export systems. The classical nuclear import machinery includes importin α (presently 6 human isoforms identified) and importin β. Importin α recognizes a mono- or bipartite nuclear localization signal (NLS) in the protein to be targeted into the nucleus. Importin α binds to importin β, which is responsible for the transport of the importin α-cargo complex through the NPC into the nucleus.

Prior to this thesis study, observations in our laboratory had demonstrated that STAT1 and STAT2 proteins have unconventional, dimer-specific NLSs. A few years before that, the nuclear import of STAT1 had been proposed to involve the function of importin α5 molecule. In NF-κB proteins, monopartite NLSs have been identified in p50, p52, p65 and c-rel, whereas the NLS of RelB has remained uncharacterized. Prior to this thesis work, there were no published data on the possible interactions of NF-κB proteins with importins.

In order to elucidate the nuclear import mechanisms of STAT and NF-κB transcription factors, this thesis work focused on the analysis of the possible interactions of STAT and NF-κB with importin α molecules.

2 REVIEW OF THE LITERATURE

2.1 Nuclear transport

2.1.1 Nuclear envelope

In eukaryotic cells the transcription machinery of the nucleus is separated from the cytosol by nuclear envelope (NE). Since the translation of proteins occurs in the cytoplasm, this compartmentation of eukaryotic cells requires that all nuclear proteins must be transported from the cytoplasm into the nucleus. In contrast, transfer RNAs (tRNA), and messenger RNAs (mRNA) are synthesized in the cell nucleus and need to be exported from the nucleus to the cytoplasm for protein synthesis. The biogenesis of some molecules, e.g. ribosomes involves multiple crossings over the NE. Ribosomal proteins are first synthesized in the cytoplasm and then transported into the nucleus where they are assembled with ribosomal RNAs. After that the ribonucleoproteins are exported back to the cytoplasm as ribosomal subunits. In addition, a great number of molecules shuttle continuously between the cell nucleus and the cytoplasm (91).

The NE is composed of two membrane bilayers named as the outer nuclear membrane (ONM) and the inner nuclear membrane (INM). The ONM is continuous with endoplasmic reticulum (ER), has functional ribosomes attached and provides anchoring sites for structural elements of the cytoplasm. The space enclosed between the two nuclear membranes, termed the perinuclear space (10 – 40 nm), is also continuous with the lumen of ER and, thus, with the secretory system of the cell, but may also have unique functions. The INM has unique characteristics. It contains a distinct set of integral and membrane associated proteins that provide binding sites for nuclear lamina and chromatin. The nuclear lamina underlies the INM. It is a dense network composed of intermediate filament-like proteins called lamins and several integral and peripheral lamin-associated proteins. Nuclear lamina is essential for nuclear integrity and it also functions in organization of chromatin, replication of DNA and regulation of transcription (53, 107, 284). Higher eukaryotes have an open mitosis. In animalian cells, NE breaks down during cell division and the membrane fragments containing nucleoporins and integral membrane proteins disperse to the cytoplasm and nuclear and cytoplasmic compartments become mixed (82, 147). At the end of mitosis, NE rapidly reassembles around the decondensing chromatin (158). In dividing cells, the compartmentalization of nuclear components happens within minutes, whereas the subsequent expansion and maturation of the NE takes at least 1h (69). During interphase, however, all macromolecular transport

between the cell cytoplasm and the nucleus occurs through nuclear pore complexes (NPCs), structures that penetrate and fuse the double bilayer membrane of the NE (277) (Figure 1).

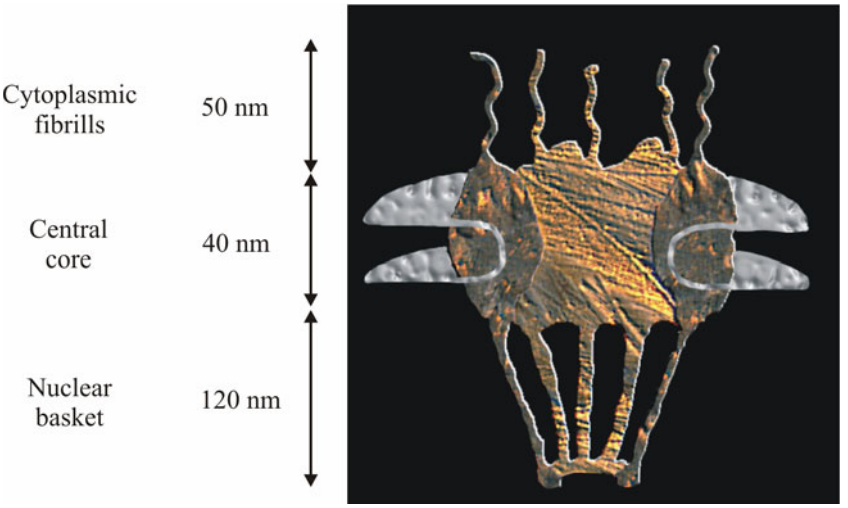

Figure 1. ***A schematic representation of the nuclear pore complex (NPC). The main structural components of the NPC include the central core framework, which is embedded in the double membrane of the nuclear envelope (grey), the cytoplasmic fibrills and the nuclear basket. The cytoplasmic fibrills and the nuclear basket are mostly composed of the naturally unfolded FG-nucleoporins, which make direct contacts with the proteins to be transported through the NPC.***

2.1.2 Nuclear pore complexes

NPCs are large multiprotein complexes that form the only direct route for transport of macromolecules into and out of the nucleus. With the size of ~50 MDa in yeast and up to 125 MDa in vertebrates the NPC is one of the largest assemblies of defined macromolecular structures of the cell. Despite of its gigantic size, the NPC is composed of only about 30 different proteins, called nucleoporins or Nups, many of which are present in multiple copies per NPC (51, 63, 217).

NPCs provide a diffusion channel for small molecules such as ions and metabolites between the nucleus and the cytoplasm but can also mediate the active transport of very large complexes, up to several megadaltons in molecular mass such as

ribosomal subunits or viral capsids and genomes (129, 216, 281). The diameter of the diffusion channel of NPC is ~9 nm, but during an active transport the channel opens to a maximum size of approximately 40 nm (203). Passive diffusion is extremely slow in the case of bovine serum albumin (68 kDa, 7 nm in diameter), relatively inactive for ovalbumin (46 kDa, 6 nm in diameter) and reasonably fast only for proteins smaller than 25 kDa (3, 91). Yet, many proteins or RNAs that are smaller than 25 kDa, such as histones and tRNAs, normally cross the NPC in an active and carrier-mediated fashion (124). The involvment of specific carrier and regulatory proteins allows the transport to occur in a controlled fashion. As a whole, nuclear transport is a highly organized and an efficient process. Transport through NPCs can be supprisingly rapid. For example, it has been presented that a single NPC is capable of translocating ~800 transportin molecules (molecular mass 100 kDa), or up to 2500 nuclear transport factor 2 (NTF2) homodimers (30 kDa) per second (222).

The structure of the NPC can be superficially divided into three basic elements (Figure 1): the nuclear basket, the central core (the transporter), which defines the maximum channel diameter and the cytoplasmic filaments, which make direct contacts with the elements of the cytoskeleton (13). The NPC is crowded with nucleoporins characterized by the presence of phenylalanine-glycine (FG)-rich motifs. These FG-rich domains are unfolded in their native state and constitute nearly half of the nucleoporins. The movement of the cargo across the NPC requires direct contacts with these so-called FG-Nups (205, 249, 251). Nuclear transport is initiated by an interaction of transport cargo with the cytoplasmic fibrils (import) or with the peripheral structures of the nuclear basket (export). The import or export is then completed by a series of interactions between the transported cargo-complex and NPC interactions over a distance of 100-200 nm, required for complete passage from one extreme of the NPC to the other (200, 250). In addition to structural and transport-dependent functions of nucleoporins, some of them also have an active role in mitosis (23). Furthermore, it has been demonstrated that certain Nups associate preferentially with transcriptionally active DNA and interact with the promoters of activated genes (266). In spite of their size and complex structure, NPCs undergo breakdown and reformation during cell division.

A proliferating human cell contains approximately 3000-5000 NPCs. NPCs do not distribute randomly around the NE, and the number of functional NPCs varies depending on the growth state of the cell that in turn affects the overall permeability of the nucleus. Moreover, it has been suggested that the protein composition of NPCs may vary from one tissue to another, between developmental stages and within the NE of a single cell (201, 271). This indicates that the composition of NPC can be varied in a manner that may specifically regulate the transport of certain

molecules between cytoplasm and nucleus. However, the overall NPC architecture is generally conserved across the species from yeast to human (258).

2.1.3 Receptor mediated nuclear import and export

The majority of molecules entering or exiting the nucleus are transported through the NPC by an active and energy-dependent fashion. Active transport of macromolecules between the cytoplasm and the nucleus is carried out by specific soluble transport receptors, which mediate the movement of their cargoes through the NPC. Apart from NTF2 and TAP/p15 heterodimer, which are the transport receptors for RanGTPase and cellular mRNA, respectively, the carriers for actively transported macromolecules are collectively referred to as karyopherins, with those involved in nuclear import termed importins and those involved in nuclear export termed exportins (170). A protein destined for nuclear import or export contains a specific signal, a nuclear localization signal (NLS) or nuclear export signal (NES), which are regognized by importins and exportins, respectively. Transport of large protein complexes through the NPC is fundamentally different from protein import to ER or mitochondria, where proteins cross the membrane singly and in a fully unfolded state. Moreover, the import signals that direct proteins onto the membranes of ER or mitochondria are normally cleaved during the transport event. In contrast, specific signal sequences in cargo molecules mediating nuclear import or export are not removed (170).

Karyopherins are members of the highly conserved importin β protein superfamily. In human cells the importin β superfamily consists of at least 20 members with distinct functions in nuclear transport of specific substrates (186) (Table 1). All members of the importin β family have the ability to bind to RanGTPase, which provides the metabolic energy and controls the directionality (import vs. export) of the transport, to the specific FG-nucleoporins involved in the particular transport process, and to a specific set of cargo molecules (42). Karyopherins can bind directly to their cargo molecules; however, in many cases the interaction between the karyopherin and the cargo is mediated by an adaptor protein.

Table 1. Human importin β family members

Transport factor	Cargo
Import	
Importin β	Cargoes with basic NLSs via importin α, some cargoes with basic NLSs directly, snRNPs via snuportin
Importin 4	Histones, ribosomal proteins
Importin 5	Histones, ribosomal proteins
Importin 7	HIV RTC, Glucocorticoid receptor, ribosomal proteins
Importin 8	SRP19
Importin 9	Histones, ribosomal proteins
Importin 11	UbcM2, rpL12
Transportin 1	hnRNP-A1, histones, ribosomal proteins
Transportin 2	HuR
Transportin SR	SR proteins
Export	
Crm1	Leucine-rich NES cargoes
Exportin-t	tRNA
CAS	Importin α
Exportin 4	eIF-5A
Exportin 5	Pre-miRNA, tRNA, eEF-1A, ILF3
Exportin 6	Profilin, actin
Exportin 7	P50Rho-GAP
Import/Export	
Importin 13	Import: RBM8, UBC9, Pax6; Export: eIF-1A

Adapted from (42, 207)

Importin α-mediated nuclear import

The best characterized system for active transport of macromolecules between the cytoplasm and the nucleus is the classical nuclear import pathway. In this pathway the karyopherin mediating the import process is importin β and the adaptor protein between the cargo and the karyopherin is importin α (91, 170). The NLS used by the classical nuclear import pathway is a short strech of basic amino acids, arginines (R) and lysines (K), that lack strict consensus sequence (monopartite NLS) or two clusters of basic amino acids separated by a 10-12 amino acids long nonspecific linker (bipartite NLS). Original prototypes for the monopartite and bipartite NLSs were Simian virus 40 T antigen NLS (P**KKKRK**V) and the nucleoplasmin NLS (V**KR**PAATKKAGQA**KKKK**LD), respectively (65, 66, 125, 140). However, at present it is known that functional NLSs can be very variable in sequence and structure.

The classical NLS is recognized by importin α. Importin α associates through a separate domain with importin β, which is responsible for the docking of the importin-cargo complex to the cytoplasmic side of the NPC, followed by translocation of the complex through the pore. Passage through the NPC is mediated by transient importin β-nucleoporin interactions (12, 89, 207). After delivering the cargo into the nucleus the cargo-free importin β then translocates rapidly back to the cytoplasm via direct contacts with the NPC. Importin α is recycled back to the cytoplasm for another round of transport by the exportin CAS (139).

Importin α molecules have remained structurally and functionally conserved throughout evolution and can be found in eukaryotes from yeast to human. In humans, only one importin β is known to interact with importin α, while six different importin α molecules have been identified: importin α1, importin α3, importin α4, importin α5, importin α6 and importin α7 (48, 52, 132, 135, 237). Based on their sequence similarity, importin α molecules can be classified into three distinct subfamilies, where importin α1 is the only member of one subgroup, importin α3 and α4 belong to the other, and importin α5, α6 and α7 comprise the third subfamily. All importin α isoforms have the ability to mediate the import of NLS-containing substrates into the nucleus. However, these distinct importin α molecules are functionally divergent. Although importin α isoforms display differencies in their cell- and tissue-specific expression patterns all isoforms exept importin α6 are expressed within the same tissues. Importin α6 has been found to be expressed only in testis (106, 132, 133, 135, 268). While some substrates can be transported into the nucleus by all importin α isoforms, there is a growing number of experimental evidence that several substrates are recogniced specifically only by a particular, or by a subset of importin α molecules (4, 118, 134, 135, 156, 172, 176, 196, 214, 238, 270).

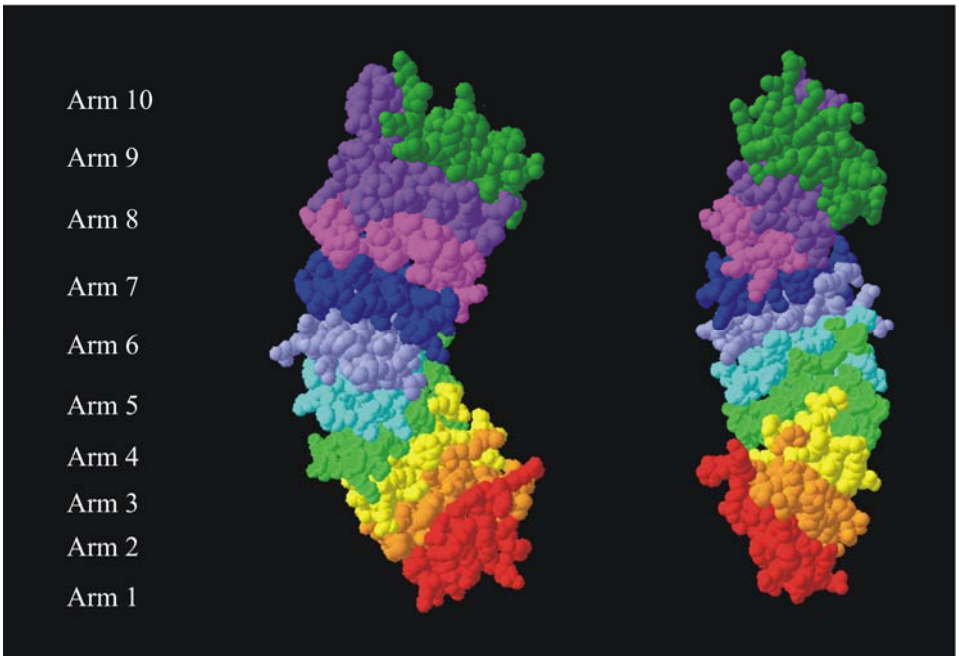

Figure 2. ***Three dimensional structure of importin α armadillo (Arm) domains. Importin α molecule contains ten Arm repeats, which are shown in different colors. The "side view" (left) and the view facing the central groove (right) are related by 90° rotations. The molecule was compiled with program DeepView and it is based on yeast karyopherin α (Protein Data Bank accession number 1bk5).***

The crystal structure of two importin α molecules, yeast importin α and mouse importin α2, have been determined (47, 75) (Figure 2). The structure of importin α can be divided into three structural units: a central NLS binding domain build by the regular stacking of 10 armadillo (Arm) repeats (so named because they were first discovered in the Drosophila Arm protein (206)), a small hydrophobic C-terminal region, which binds to the exportin CAS, and a positively charged N-terminal autoinhibitory domain, which can bind either to the Arm domain (blocking the interaction with the NLS of the cargo molecule) or to importin β. Importin α molecules have two NLS binding sites, which directly interact with the NLS of the cargo. The Arm repeats 2-4 comprise the N-terminal ("the major") NLS binding site and Arm repeats 7-8 the C-terminal ("the minor") NLS binding site. Each Arm repeat consists of approximately 40 amino acids that fold into three α helices. These

α helices contain the highly conserved tryptophan and asparagine residues that directly interact with the basic amino acids on the NLS of the cargo molecule (47, 74, 75, 142) (Figure 2).

CRM1-mediated nuclear export

Nuclear export of proteins is mediated mainly by NESs. NESs are short stretches of amino acids containing 5-6 hydrofobic residues (typically leucines), first described in the protein kinase A inhibitor, PKI (276). The hydrophobic, leucine-rich NES is utilized in all eukaryotes and it is recogniced by the karyopherin Crm1 (also called exportin-1) (138). A common example of NES is the NES found in the Rev protein of HIV: **L**QL**L**PP**L**ER**L**T**L** (73). This type of leucine-rich NES mediates the binding to and export by Crm1, and functions in a great number of proteins. In the nucleus Crm1 binds to RanGTP. This interaction induces conformational changes in Crm1 and activates it for binding to NES-containing cargo. Crm1 docks the complex to the FG-Nups on the nuclear side of the NPC, and the complex is transported through the NPC by Crm1-nucleoporin interactions analogous to the importin β-mediated nuclear import. On the cytoplasmic side of the pore, Ran-bound GTP is hydrolyzed, the complex is dissassembled and the cargo is released to the cytoplasm (91, 186) (Figure 3B). Like importin β, Crm1 can also mediate the export of some cargoes via adapter proteins (207). Crm1 and other exportins interact with their cargoes effectively only in the presence of RanGTP.

The RanGTPase system

The energy for active nuclear transport is provided by the Ras family GTPase Ran (215). The RanGTPase is critical in generating the directionality of the transport process. The loading and unloading of importins and exportins with their cargo molecules is controlled by a concentration gradient of RanGTP across the NE. Like all small GTPases, Ran can switch between GTP and GDP-bound state. The GTP-bound form of Ran is predominantly nuclear, whereas RanGDP is mainly localized in the cytoplasm. Directionality of nuclear transport relies on the specific interactions between karyopherins and RanGTP. Importins bind effectively to their cargoes only in the absence of RanGTP, that is in the cytoplasm. Once in the nucleus, importins have a reatively high affinity for the GTP-bound form of Ran. The binding of importin to RanGTP leads to the dissociation of the importin-cargo complex, and the cargo is released into the nucleus. Ran gradient is critical, because cytoplasmic RanGTP would inhibit nuclear import as it would cause the importin-cargo complexes to dissassemble before they could reach the NPCs. Exportins, on

the other hand, form a trimeric complex with RanGTP and their cargo molecules in the nucleus. Exportins have a relatively low affinity for Ran in the absence of cargo. Exportins are activated for cargo binding by association with RanGTP in the nucleus and the complex dissociates upon GTP hydrolysis in the cytoplasm (207, 275) (Figure 3).

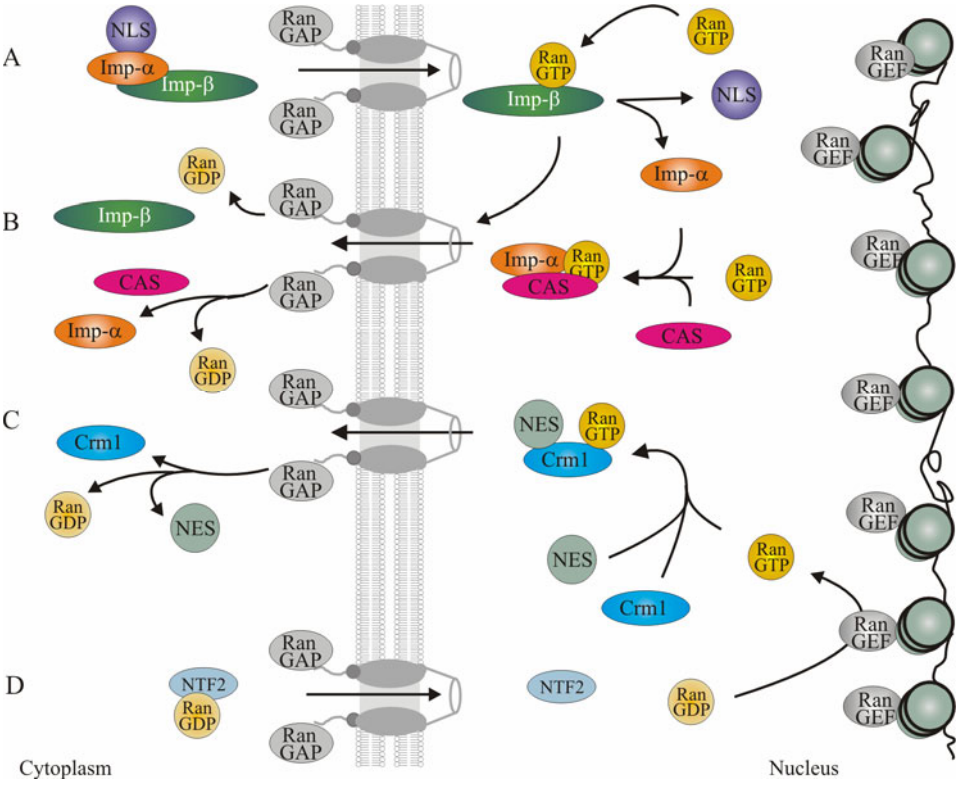

Figure 3. ***Overview of nuclear localization signal (NLS)- and nuclear export signal (NES)-mediated nuclear transport pathways. (A) Nuclear import of NLS cargo mediated via importin α/β. (B) Nuclear export pathways mediating the recycling of importin β and importin α back to the cytoplasm. (C) Nuclear export of NES cargo mediated by Crm1. (D) Nuclear import of RanGDP mediated by nuclear transport factor 2 (NTF2). See text for details.***

Ran has a low intrinsic activity and it has to interact with additional factors to effectively complete the GDP-GTP cycle. Ran GTPase activating protein (RanGAP) catalyzes the conversion of RanGTP to RanGDP (21). This GTPase activation is further stimulated by Ran-binding protein 1 (RanBP1). RanBP1 and RanGAP are excluded from the nucleus and co-operate in the cytoplasm, where RanBP1 binds tightly to the GTP-bound form of Ran. RanGAP is situated on the cytoplasmic side of the NPC, where it catalyzes the conversion of RanGTP to RanGDP. This leads to the dissociation of the exportin-cargo complexes to the cytoplasm. Ran has a high efflux rate from the nucleus, more than 10^5/s/nucleus, due to the constantly exiting exportins (246). However, Ran is predominantly distributed in the nucleus, and the nuclear RanGTP levels exceed the cytoplasmic concentration about 100-fold (92). The import of RanGDP back into the nucleus is mediated by NTF2, which binds only to the GDP-bound form of Ran (245). NTF2 mediates the rapid translocation of RanGDP from the cytoplasm into the nucleus by direct contacts with FG-repeat containing nucleoporins. Ran can also diffuse passively across the NPC, and RanGTP (which cannot bind NTF2) traverses NPCs at the same rate as RanGDP in the absence of NTF2, that is, very slowly (92). Once in the nucleus, RanGDP is converted into RanGTP through the action of guanine nucleotide exchange factor RanGEF (also called RCC1). RanGEF is a nuclear protein, which is stably bound to chromatin through an interaction with the core histones H2A and H2B (195). RanGEF is a very abundant protein, which has been estimated to be present in up to one copy per nucleosome (56). After mitosis and the mixing of nuclear and cytoplasmic contents, the NE accumulates around the decondensing chromatin. The association of RanGEF with chromatin, thus, after the formation of NE at the end of the mitosis provides the primary gradient of RanGTP in the nucleus (91, 170). This provides the primary prerequisites for the control of the directionality of nuclear transport processes.

Regulation of nuclear transport

The proper function of many molecules requires that their nuclear or cytoplasmic concentration and activity is tightly regulated. Therefore, mechanisms must exist to turn the transport of a given cargo on and off. Since distinct karyopherins posses different cargo-recognition mechanisms, one mechanism of regulating a particular transport pathway is through controlling the protein expression levels of karyopherins themselves. The resulting selection of karyopherins will determine what kind of proteins will be targeted into or out of the nucleus (122).

Cargo molecues can be anchored to an insoluble cellular component. In this form of regulated transport, a receptor-cargo complex can form, but cannot be targeted to the

NPC. This kind of regulation has been shown to take place with some molecules, such as sterol regulatory element binding protein, which is attached to the NE and ER in unstimulated cells (124). Upon activation the membrane bound protein undergoes proteolytic cleavage to produce a soluble fragment that enters the nucleus. This type of regulation is irreversible since the active form cannot be anchored back to the membranes by the same mechanism. A recent report suggests that also transport receptors can be tethered inactive by immobile components (204). It has been suggested that significant proportions of importin β, importin α, NTF2 and Ran are not freely diffusing and available for transport functions, but are associated with immobile components such as microtubules in the cell cytoplasm (204).

The transport properties of the NPC can be regulated. It has been suggested that the protein composition of the NPC can be varied throughout the cell cycle. This indicates that the transport receptor-NPC interactions can be reversibly regulated by molecular rearrangements of the NPC (124, 201). It has been suggested that modification of RanGAP with SUMOylation at the NPC serves as a mechanism to control nuclear transport (159, 209, 210).

The cargo molecules can be modified in a way that it affects their ability to bind the import or export receptor. Prevention of target sequence recognition by intramolecular or intermolecular masking of the NLS or NES is one of the most common strategies to regulate the efficiency of nuclear transport.

Controlling of NLS or NES recognition by phosphorylation is a key mechanism of regulating nuclear transport processes. Post-translational modification of signalling molecules by phosphorylation and dephosphorylation is propably the most common and the best understood mechanism known to regulate nuclear transport. Kinases and phosphatases can themselves be regulated by many different intracellular and extracellular signals. Thus, signal-mediated phosphorylation and dephosphorylation to regulate the subcellular localization of molecules provides a direct connection from extracellular signals through intracellular signals to the subsequent response in terms of nuclear import or export of specific signalling molecules such as transcription factors. Many of these transcription factors contain both NLS and NES. As a result, their nuclear concentration and activity can be tightly regulated in response to a plethora of different types of signals (120, 121, 124).

2.2 Transcription factors

Transcription factors are proteins that bind to specific DNA sequences on target genes. These DNA sequences are normally located in the promoter regions preceeding the targe gene, and the binding of transcription factor to these sites

modulates the transcriptional activity of the gene. Transcriptional control of gene expression often involves series of signal transduction cascades along the way from the cell surface through cell cytoplasm into the nucleus to the target genes. Binding of extracellular proteins to cell-surface receptors can change nuclear gene expression in minutes. In unstimulated cells many transcription factors, like signal transducer and activator of transcription (STAT) and nuclear factor κB (NF-κB), are sequestered in the cytoplasm as inactive, latent forms. After stimulation these factors are activated and rapidly translocate into the nucleus where they modulate the activity of the target gene. Control of activation by phosphorylation is a common mechanism in controlling and regulating the transcriptional activity of many transcription factors.

2.2.1 STATs

STATs are latent cytoplasmic transcription factors that are activated by a large number (>50) of extracellular signalling molecules including cytokines, hormones and growth factors (254). STAT proteins have fundamental roles in highly diverse biological processes such as mediating host antimicrobial responses and cell development, differentiation, proliferation and survival. In addition, constitutively-activated STAT signalling is associated with malignant progression of many human cancers (1, 26). STAT proteins are well conserved in eukaryotic evolution, and STAT homologues have been identified in slime mold *Dictyostelium*, nematode *C. Elegans* and *Drosophila* (108, 150). Seven STAT proteins have been identified in humans: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (78, 110, 151, 233, 289, 298).

Overview of STAT functions

STAT1, STAT3, STAT5a and STAT5b are activated by many distinct and sometimes overlapping ligands, whereas only a few cytokines activate STAT2, STAT4 and STAT6. STAT1 is involved in interferon (IFN) signalling and mediates antiviral and antibacterial responses, growth inhibition and tumor supression. STAT2 is also activated by IFNs and it is essential for antiviral responses of IFN-α/β. STAT3 is activated by many cytokines and growth factors including interleukin-6 (IL-6) family members and epidermal growth factor. STAT3 is the only STAT protein shown to be required for early development as STAT3 null mice are embryonically lethal. STAT4 is activated by IL-12 and IL-23 and it is involved in regulation of Th1 cell differentiation. STAT5a and STA5b are closely related STAT proteins (sharing ~96% sequence identity) that have overlapping but also

distinct functions, and these proteins are activated by many IL-2 family cytokines. STAT5a is involved in prolactin and STAT5b in growth hormone signalling. STAT6 is activated by IL-4 and IL-13 and it participates in the regulation of Th2 development (117, 148, 199, 234, 286).

STATs have the ability to mediate signals from cell membrane into the nucleus to activate gene transcription, thus bypassing the involment of any additional signalling cascades between cell-surface receptors and the target genes. STAT proteins form active homodimers in response to ligand stimulation. In some cases STATs prefer heterodimer formation, e.g. STAT1/STAT2 in response to IFN-α/β activity, STAT1/STAT3 in response to IL-6 activity and STAT5a/STAT5b in response to growth hormone activity (108). STAT2 is unique in that it has not been found to bind DNA as a homodimer. Cytokine stimulation leads to a rapid accumulation of active STAT dimers into the nucleus. Once in the nucleus, STAT dimers can directly bind to DNA sequences known as γ-activated sequences (GAS) in the promoter regions of cytokine-responsive genes, resulting in modulations of gene activity. STAT activation is normally rapid and transient in nature, lasting from several minutes to a few hours. The activation is usually quickly down-regulated to keep cytokine responses under control. However, it has become apparent that some STATs are also involved in controlling constitutive and ligand independent gene expression (31). Moreover, it has been shown that STAT1 can also negatively regulate certain promoters in response to IFN-γ (220).

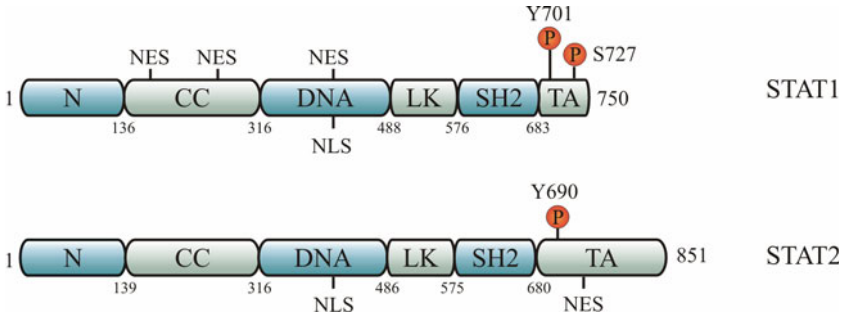

Figure 4. ***Schematic representation of STAT1 and STAT2 structure. The N-terminal end and the coiled coil domain (CC) mediate interactions with various proteins. The DNA binding domain (DNA) is located in the middle of the molecule followed by a linker domain. SH2 domain interacts with phosphotyrosine residue of the other molecule in STAT dimers. Transactivation domain (TAD) is located at the C-terminal end and contains the conserved tyrosine residue critical for dimer formation.***

Structure of STAT proteins

Full-length STAT proteins are between 750 (STAT1) and 851 (STAT2) amino acids long and share several functional domains (Figure 4). N-terminal domain (residues 1-~140) is involved in formation of dimers, tetramers and other higher order complexes between STAT family members. This domain also mediates other protein-protein interactions e.g. STAT1 with CBP/p300 transcriptional coactivators (296). Next from the N-terminus is a coiled coil region (amino acids ~130-315), which mediate many protein-protein interactions in solution or on target promoters. The coiled coil region of STAT3 has been shown to contain amino acids essential for interaction with importin α molecules (156). Two functional NESs have been identified in the coiled coil domain of STAT1 (16, 188). Furthermore, coiled coil domain of STAT1 and STAT2 have been shown to interact with interferon regulatory factor (IRF) 9 (109, 168). The DNA-binding domain at amino acids ~300-500 contains an immunoglobulin (Ig)-like fold and it resembles that of NF-κB DNA-binding domain. DNA-binding domains of STAT1, STAT2 and STAT3 contain amino acids essential for interaction with importin α molecules and subsequent nuclear import of activated dimers (156, 172, 177). In addition, yet another functional NES of STAT1 has been mapped to the DNA binding domain of STAT1 (173). The short linker domain connects the DNA-binding domain to the src homology-2 (SH2) domain. SH2 domains are found in a large number of proteins involved in signal transduction and these domains function to specifically recognize phosphorylated tyrosine residues (185). In all STAT molecules the conserved activating tyrosine residue (Tyr^{701} of STAT1 and Tyr^{690} of STAT2) closely follows the SH2 domain. This conserved tyrosine residue undergoes phosphorylation upon activation and forms intermolecular interaction with the SH2 domain of the other STAT molecule in the active dimer. Dimerization via tyrosine phosphorylation is critical in activation of all STAT dimers. However, STATs are the only transcription factors known to be activated by tyrosine phosphorylation. The region from the activating tyrosine residue to the C-terminal end comprises the transcriptional activation domain (TAD). This is the most diverse domain among STAT proteins. In the promoters of target genes the TADs of STATs interact with other proteins involved in regulation of gene transcription. NES has been identified in the TAD of STAT2 (9). The TAD of STAT1 has been shown to interact with CBB/p300 (296). The TADs of STAT1, STAT3, STAT4, STAT5a and STAT5b can be modulated by serine phosphorylation to further regulate the transcriptional activities of the proteins (57, 150). STAT proteins have been described to undergo proteolytic processing and alternative splicing to produce shorter isoforms. The full-length STATs are called as α isoforms and the shorter products as β, γ or δ isoforms (14, 37, 150, 283). The crystal structures of several STAT dimers have been determined (14, 37, 163, 194, 283).

STAT1 and STAT2 mediated interferon signalling

The first STATs identified 15 years ago were STAT1 and STAT2. They were discovered as targets of IFN activation (78, 233). IFNs are a family of cytokines that have the ability to interfere with viral replication and infection. IFNs can be divided in three distinct groups: Type I interferons (predominantly IFN-α and -β) are produced by many cell types as a first line of defence against viral infections (239). IFN-γ is the only member of type II IFNs. IFN-γ is produced by natural killer cells and T cells and it has multiple effects in the regulation of immune responses involved in inflammation, antibody production and viral infection (239). The recently described type III interferons consist of IFN-λ1, IFN-λ2 and IFN-λ3 (136, 241). Although type I and type III IFNs signal through distinct receptor complexes, both types induce STAT1, STAT2 and STAT3, and as a result type III interferons have antiviral functions similar to those of type I interferons (299). Binding of interferons to their cell surface receptors activates antiviral etc. responses via Janus tyrosine kinase (Jak)-STAT pathway (55). Jaks are receptor associated tyrosine kinases that posess auto- and transphosphorylation functions (148). Presently, four members of Jaks have been described in humans: Jak1, Jak2, Jak3 and Tyk2 (290). Jak-STAT pathway is the most common and best characterized signalling pathway in response to a wide variety of cytokines (292). The intracellular activation of Jak-STAT pathway occurs when ligand binding induces the multimerization of receptor subunits.

Type I and type II interferons signal through distinct but related pathways (Figure 5). IFN-α/β receptor is composed of two transmembrane receptor chains: IFNα/βR1 and IFNα/βR2. The cytoplasmic domains of the receptor subunits are specifically and constitutively associated with Tyk2 (IFNα/βR1) and Jak1 (IFNα/βR2) tyrosine kinases. During unstimulated state the receptor subunits are not preassociated with one another strongly. Binding of IFN-α/β to its receptor causes dimerization of the subunits and brings the receptor associated Jaks into close proximity. This induces sequential activation of Tyk2 and Jak1 by auto- and transphosphorylation. Activated Tyk2 and Jak1 in turn phosphorylate IFNα/βR1 at specific tyrosine residues, constructing a docking site for the SH2-domain of STAT2. Next, Tyk2 and Jak1 phosphorylate receptor-bound STAT2 at Tyr^{690}. This enables the binding of STAT1 to the receptor. Receptor associated STAT1 is phosphorylated by Jaks at Tyr^{701}, which is critical for the formation of the active STAT1/STAT2 heterodimer. Once activated, the dimer dissociates from the receptor. The receptor-dissociated STAT1/STAT2 heterodimer, together with the dimer associated IRF9, generates the active interferon-stimulated gene factor 3 (ISGF3) complex characteristic of IFN-α/β stimulation. In the nucleus ISGF3 complex binds to the interferon-stimulated response element (ISRE) sites on the promoter regions of IFN-α/β responsive genes.

The products of these genes induce the primary IFN-α/β induced response for viral infections (239, 248).

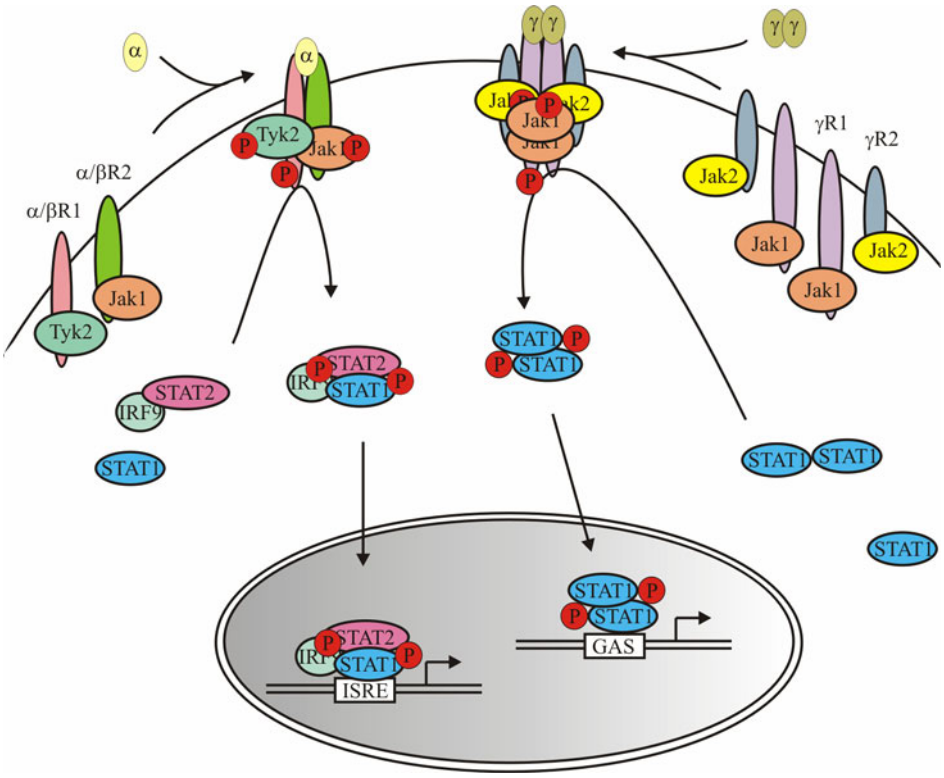

Figure 5. ***Type I (left) and type II (right) interferon (IFN) signaling. IFN binding to its receptor results in heteromerization of the receptor subunits. This induces phosphorylation of receptor-associated Jaks and receptor chains. STATs bind to the receptor via their SH2 domains. Receptor-bound STATs are phosphorylated by Jaks. Phosphorylated STATs form dimers, dissociate from the receptor, enter into the nucleus and bind to the promoter regions of IFN-responsive genes.***

The subunits of the IFN-γ receptor, IFNγR1 and IFNγR2, are preassociated with Jak1 and Jak2, respectively (Figure 5). The active form of IFN-γ is a homodimer. Ligand binding to the IFNγR1 subunit leads to the oligomerization of the receptor and activation of the receptor-associated kinases by auto- and transphosphorylation. Jak2 is activated first and it is needed for the activation of Jak1 (28). Activated Jaks in turn phosphorylate the IFNγR1 subunit providing an attachment site for STAT1 via its SH2 domain. Receptor-associated STAT1 molecules are phosphorylated by Jaks at Tyr^{701}, which enables the formation of an active STAT1 homodimer, the γ-activated factor (GAF). It has been proposed that Jak1 is mainly responsible for the receptor phosphorylation, and once STAT1 is bound to the receptor, the phosphorylation of STAT1 is mediated by Jak2 (28). Phosphorylated STAT1 homodimer dissociates from the receptor and the dimer translocates into the nucleus. In the nucleus STAT1 homodimers bind GAS sites on interferon γ-activated genes. The transcriptional function of STAT1 homodimers and ISGF3 complexes can be further regulated by serine phosphorylation of STAT1 (211). IFN-α/β and IFN-γ have partly overlapping functions because to a certain extent STAT1 homodimers are formed upon IFN-α/β stimulation, and also because STAT1 homodimers induced by IFN-γ can also be associated with IRF9 and bind to the ISRE sequences (248). In addition, STAT1-independent gene regulation has been described in respone to IFN-α/β and IFN-γ (86, 218, 219). Although a great variety of cytokines and cytokine receptor combinations signal through the four Jak molecules and the seven STAT proteins, other STAT family members are activated by various cytokines similary to STAT1 and STAT2 through receptor oligomerization and receptor-associated Jaks, making the IFN signal transduction pathway presented above a generic model for STAT-mediated cytokine signalling.

Originally, the standard model postulated that latent STATs exist as a monomeric pool prior to cytokine stimulation (242). However, it has become evident that unphosphorylated STATs most likely exist as dimers and probably as tetramers and higher order complexes in unstimulated cells (236). First, it was presented that STAT3 homodimers and STAT1/STAT3 heterodimers can be coimmunoprecipitated from unstimulated cells (94, 198). In addition, large multiprotein complexes (up to 1-2 MDa) of STAT3, STAT1, STAT5a and STAT5b were identified from Hep3B cells and rat liver cytoplasm prior to cytokine stimulation (193). Furthermore, crystallographic analyses of unphosphorylated STAT1 revealed a tetrameric conformation (163). However, by further biochemical analyses the authors concluded that STAT1 exists predominantly as a dimer prior to cytokine stimulation. Moreover, the authors presented two different conformations for unphosphorylated STAT1, designated as “parallel” and “antiparallel” models

(163, 297). The parallel model was shown to resemble the structure of the tyrosine-phosphorylated STAT1 homodimer, whereas the antiparallel dimer had a unique conformation. The authors further speculated that the antiparallel form is the predominant structure in the latent state, whereas the parallel conformation was proposed to be the receptor bound form of STAT1 prior to tyrosine phosphorylation. Tyrosine phosphorylation upon ligand binding most likely induces a conformational change in STAT dimer that causes dissociation of the dimer from the receptor and induces a conformation that is recognizable by importin α molecules.

STAT inactivation

STATs are inactivated in the cytoplasm by several proteins. Supressor of cytokine signalling (SOCS) proteins prevent further activation from the receptors by interacting with phosphorylated receptor subunits or Jaks (70). SOCS can also induce STAT degradation by the ubiquitin-proteasome pathway (137). Cytokine-induced SH2 protein (CIS) competes with STATs for the same docking sites on the receptor (169). In the nucleus, protein inhibitors of activated STATs (PIAS) inhibit the DNA binding of active STATs (2), while certain phosphatases have been described to dephosphorylate active STATs in the nucleus (263, 285). Phosphorylated STATs are not transported back to the cell cytoplasm. Previous models of STAT inactivation presented that dephosphorylation releases STATs from DNA (97, 248). However, at present it seems that it is the other way round: DNA binding protects STATs from dephosphorylation (182, 297). Only upon dissociation from the promoter regions STAT dimers are subjected to dephosphorylation. It has been proposed that phosphorylated STAT1 dimer undergoes conformational rearrangements to the antiparallel form when not bound to DNA. This rearrangement of the dimer is most likely required for the efficient presentation of phosphorylated STAT1 dimer to the tyrosine phosphatases prior to their nuclear export (297).

STAT nucleocytoplasmic transport

Classical NLSs have not been identified in STATs. The first report of the existence of NLS signals in STAT molecules emerged when an unconventional, structural and dimer-specific NLS was identified in STAT1 and STAT2 (177). This structural NLS is situated in the DNA-binding domain of the molecule, and two of these elements, one in each monomer, were shown to be required for nuclear import of STAT1 and STAT2. The amino acids required for nuclear import were mapped to lysines 410

and 413 of STAT1 and to arginine 409 and lysine 415 of STAT2. Another study has described leucine 407 to be essential for STAT1 nuclear import (172). Previously, the mechanism that regulates STAT1 nuclear import was shown to involve the function of importin α5 (238).

STAT3 has been shown to contain two distinct arginine-rich sequence elements, which are required for its nuclear import (157). These elements are situated in the coiled coil (arginines 214 and 215) and DNA-binding (arginines 414 and 417) domains of STAT3. The NLSs of STAT1, STAT2 and STAT3 differ from the classical NLS signals to some extent. They do not resemble the consensus sequences of classical mono- or bipartite NLSs, and they become active only in dimers. Recently, it was presented that the nuclear import of STAT3 is mediated by importin α5 and importin α7 in a cytokine-stimulation dependent fashion (156). However, it has also been suggested that signal-dependent nuclear import of STAT3 is mediated by other importin α isoforms (270). In addition, it has been suggested that nuclear import of STAT3 is independent of tyrosine phosphorylation and mediated by importin α3 (155). Moreover, a valine-rich sequence consisting of amino acids 466-469 of STAT5b has been proposed to be required for growth hormone-induced nuclear accumulation of STAT5b (102). At present, there is no detailed information describing the molecular mechanisms of nuclear import of STAT4, STAT5a, STAT5b or STAT6.

It has been suggested that unphosphorylated STATs shuttle continuously between the nucleus and cytoplasm. It has also been proposed that the nuclear import of unphosphorylated STATs and tyrosine phosphorylated STAT dimers are mechanistically distinct from each other (9, 180, 181, 212, 295). These observations were confirmed by import assays with digitonin-permeabilized cells, which retain an intact NE but are devoid of cytoplasmic proteins, and thus, importins. It was revealed that only unphosphorylated STAT1 could enter the nucleus in the absence of cytosolic proteins, whereas the nuclear import of tyrosine phosphorylated STAT1 dimers required metabolic energy and added cytosol (164). Similar results were obtained also for unphosphorylated mouse STAT3 and sheep STAT5 (164). In fact, the authors further demonstrated that the constitutive nucleocytoplasmic shuttling of STAT1 is mediated by direct interactions of STAT1 with the FG-rich nucleoporins of the NPC. Thus, it is apparent that STATs use two distinct pathways. Before cytokine stimulation, unphosphorylated STATs shuttle in and out of the nucleus in a karyopherin-independent mechanism that involves direct contacts with nucleoporins. Active nuclear import of tyrosine phosphorylated STATs, instead, is dependent on importins and metabolic energy. These distinct pathways are most likely the consequence of the conformational differences between the unphosphorylated dimers and the phosphorylation-activated STAT dimers.

A few years ago, three groups identified a leucine-rich NES in STAT1 (16, 173, 188). All of these functional export signals were mapped to distinct locations of the molecule. Later, multiple functional NES motifs have been identified in STAT3 and STAT5b (19, 295). In addition, the carboxyl terminus of STAT2 has been shown to contain leucine rich NES, which mediates the nuclear export of STAT2 and STAT2/IRF9 complexes (9). The carrier-dependent nuclear export of STATs is mediated by the exportin Crm1. But, as for nuclear import, there is evidence for carrier-independent nuclear export that proceeds through contacts with the nucleoporins (164). The existence of these distinct transport pathways allows the controlled nuclear transport to occur during cytokine induction, as well as the carrier- and energy-free nucleocytoplasmic cycling to proceed without metabolic energy under unstimulated cellular conditions. However, apart from that model, it has been proposed that STAT2 is constitutively associated with IRF9, and the nuclear shuttling of unphosphorylated STAT2 has been proposed to be dependent on the NLS of IRF9 and on the C-terminal NES of STAT2 (9, 144, 168). However, no karyopherins have been associated to the nuclear import of IRF9 so far.

2.2.2 NF-κB

NF-κB describes a structurally and evolutionary conserved family of ubiquitously expressed transcription factors that have important roles in the regulation of a great variety of normal cellular and organismal processes, such as immune and inflammatory responses, developmental processes, cell proliferation, differentation and apoptosis (7, 85, 100). In addition, dysregulation of NF-κB has been associated in a number of common diseases including cancer, chronic inflammation, neurodegenerative diseases and diabetes (8, 49, 178, 221, 235). NF-κB was initially identified more than 20 years ago as a DNA-binding factor for the enhancer of the Ig κ light-chain in activated B cells (240). However, as it is known today, NF-κB is neither a critical regulator of κB light gene nor it is B cell specific. NF-κB is a stimulus-responsive pleiotropic regulator of gene control present in all cell types.

NF-κB is activated by a great variety of physiological and nonphysiolocical stimuli, including bacterial, viral and fungal products as well as inflammatory cytokines, oxidative stress, ultraviolet and ionizing radiation and genotoxic drugs (202). Moreover, nearly 800 inhibitors of NF-κB signalling have been identified so far, including a large variety of natural and synthetic molecules (87). In most unstimulated and untransformed cells, NF-κB is sequestered in the cytoplasm in an inactive, latent state by association with inhibitor of NF-κB (IκB) molecules (6, 280). The activation of NF-κB is controlled mainly via its nuclear localization. Upon stimulation, NF-κB is activated in most cases through the IκB kinase (IKK)

complex-dependent phosphorylation of IκB proteins and subsequent degradation of IκBs from the IκB/NF-κB complexes by the ubiquitin-proteasome system (40, 128, 232). The IKK complex contains two kinase subunits, IKKα and IKKβ, and a noncatalytic regulatory subunit IKKγ (64, 179, 226, 291, 294). Once liberated from the inhibitors, NF-κB transcription factors translocate into the nucleus. In the nucleus NF-κB binds to functional κB sites in the promoter regions of target genes and modulates the expression of several hundred target genes, including genes, which produce cytokines and chemokines, growth factors, pro- and antiapoptotic proteins, cell surface receptors, cell adhesion molecules, immunoreceptors and transcription factors (104, 105) (an extensive list can be found at: www.nf-kb.org). Since the activation of NF-κB does not require de novo protein synthesis, some genes can be transcriptionally upregulated within minutes after the stimulation.

NF-κB and IκB proteins

NF-κB transcription factors are dimers of polypeptides belonging to the Rel family of proteins (85). All of these proteins share an N-terminal 300 amino acids long highly conserved domain called the Rel homology domain (RHD). RHD was first recogniced in the transforming gene of the avian reticuloendotheliosis virus (35, 93). In mammals NF-κB family of transcription factors is comprised of five members: p50, p52, p65 (RelA), c-Rel and RelB (85) (Figure 6). Mature NF-κB proteins are between 433 (p50) and 619 (c-Rel) amino acids in lenght. In contrast to p65, c-Rel and RelB, which are synthesized as mature proteins, p50 and p52 are first synthesized as large precursor molecules of 105 (p105) and 100 kDa (p100), respectively. Removal of the C-terminal halves of the precursors by the proteasome results in the formation of active p50 and p52 subunits (18, 71, 185).

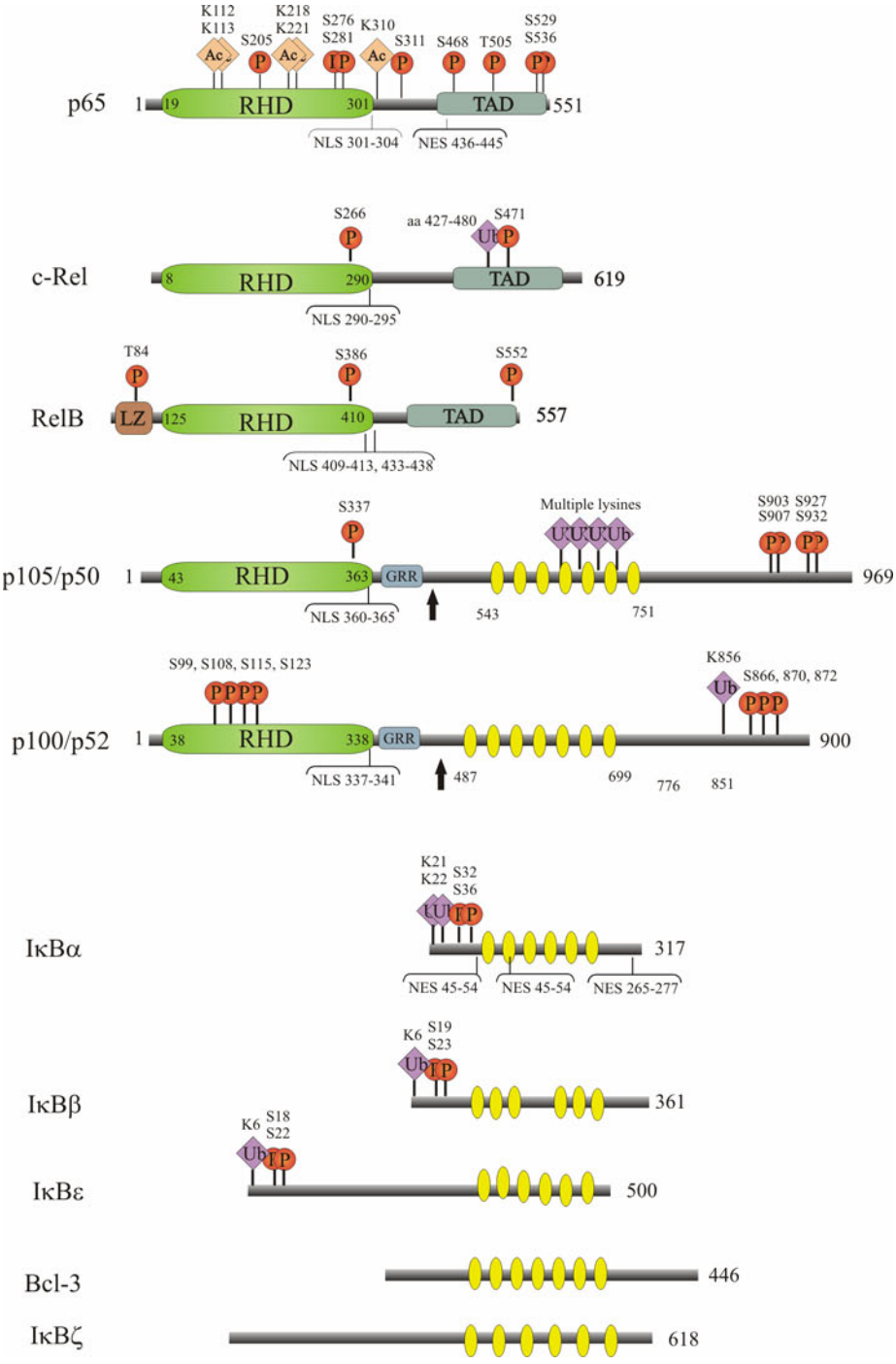
p65
K112
K113
S205
K218
K221
S276
S281
K310
S311
S468
T505
S529
S536
RHD
TAD
NLS 301-304
NES 436-445
c-Rel
S266
aa 427-480
S471
RHD
TAD
NLS 290-295
RelB
T84
S386
S552
LZ
RHD
TAD
NLS 409-413, 433-438
p105/p50
S337
Multiple lysines
S903
S907
S927
S932
RHD
GRR
NLS 360-365
543
751
p100/p52
S99, S108, S115, S123
K856
S866, 870, 872
RHD
GRR
NLS 337-341
487
699
776
851
IκBα
K21
K22
S32
S36
NES 45-54
NES 45-54
NES 265-277
IκBβ
K6
S19
S23
IκBε
K6
S18
S22
Bcl-3
IκBζ

Figure 6. ***Schematic representation of NF-κB and IκB proteins. The Rel homology domain (RHD) mediates dimerization between the subunits and makes specific contacts with the target DNA. Transactivation domains mediate the activation of transcription. The glycine-rich domains (GRR) of p105 and p100 are involved in proteolytic processing of the precursors to the mature p50 and p52 subunits. The arrows point to the proteolytic processing sites for p50 and p52. Ankyrin domains (yellow) mediate the binding of IκBs to the RHDs of NF-κB proteins. For post-translational modifications see text for details.***

The RHD of NF-κB proteins can be further divided into two distinct motifs, which are connected with a flexible linker and present a β sheet Ig-like fold. The N-terminal Ig-like motif within the RHD makes specific contacts with target DNA, and the C-terminal motif of RHD is exclusively responsible for dimer formation between the NF-κB subunits. The C-terminal motif within the RHD also forms binding site for IκB molecules and makes non-specific contacts with DNA (33). NLSs of NF-κB proteins are located at the very C-terminal end of the RHDs. RelB has an additional leuzine zipper (LZ) motif in its N-terminus preceeding the RHD, which is required for transcriptional activation by RelB. The glycine-rich regions (GRR) next to RHDs of p105 and p100 are required for efficient processing of the precursors to p50 and p52. Active, DNA-binding form of NF-κB is a dimer. The ubiquitously expressed p50/p65 dimer is the most abundant form of NF-κB encountered in most cells, but all members of the family can associate to form homodimers or heterodimers exept for RelB, which can only form heterodimers *in vivo* (34, 50, 67, 105, 113, 166, 228, 278). It has been proposed that the LZ domain of RelB may contribute to the instability of RelB homodimers *in vivo* (113). p65, c-Rel and RelB proteins share a carboxy-terminal TAD, which strongly activates transcription and allows them to form transcription activating dimers with each other and with p50 and p52. However, p65/RelB dimers are considered inhibitory since they cannot bind to any tested κB sites indicating that p65/RelB heterodimers are unable to bind DNA (166). p50 and p52 lack TADs and can therefore activate transcription only upon heterodimerization with p65, c-Rel or RelB. Homodimers of p50 and p52 function as transcriptional repressors, but can activate transcription when bound to predominantly nuclear IκB protein Bcl3 (25, 79). The crystal structures of several NF-κB dimers have been described, including those bound to DNA or complexed with IκB (34, 50, 83, 112, 113, 116, 119, 162, 189).

NF-κB proteins undergo a variety of post-translational modifications to achieve full biological activity. Of the NF-κB proteins, the major target for post-translational modifications described is p65. Acetylation plays a significant role in regulating the nuclear activity of p65 containing dimers. p65 is acetylated at multiple lysine

residues, and the acetylation of each of these lysines appears to regulate different biological properties. Acetylation of lysine 310 stimulates transcription, whereas acetylation of lysines 122 and 123 has an inhibitory effect (36, 131). Acetylation of lysines 218 and 221 inhibits p65 binding to nuclear IκBα and thus, enhances the duration of NF-κB activation. This also links the acetylation to nuclear localization of NF-κB since deacetylation of lysine 221 promotes IκBα binding and leads to rapid nuclear export of p65 containing NF-κB dimers (36).

Phosphorylation of p65 at various locations, including serines 205, 276, 281, 311, 468 and 529, has been shown to be important in the initiation of transcription (208, 282). It has been demonstrated that a part of p65 pool is constitutively phosphorylated at serine 536 (29, 171, 231). In nuclear translocation of p65, the phosphorylation at serine 536 seems to have an interesting role by defining an IκBα-independent NF-κB pathway (231). Serine 536 phosphorylated p65 was not associated with IκBα or p50 and chemical inhibition of the proteasome reduced the nuclear translocation of total p65 but not that of serine 536 phosphorylated p65 (29, 171, 231). Furthermore, a dominant negative IκBα mutant did not suppress the serine 536 phosphorylated p65 transcriptional activity. These findings indicate that nuclear localization of p65 phosphorylated on serine 536 is not regulated by IκBα. In response to TNF-α stimulation p65 is phosphorylated at threonine 254 (227). This induces the binding of p65 to peptidyl-prolyl isomerase Pin-1, which in turn, disrupts the interaction of p65 with IκBα and induces the nuclear translocation of p65 containing NF-κB dimers (227). In the nucleus phosphorylation at threonine 505 within the p65 TAD inhibits p65 mediated transcription (224).

Other NF-κB proteins are also targets for post-translational modifications. It has been shown that IKKε phosphorylates c-Rel, a modification that induces its nuclear translocation due to the dissociation of IκBα/c-Rel complexes, however the phosphorylation sites were not determined (96). Serine 266 phosphorylation mediated by PKA has also been suggested to induce the nuclear localization of c-Rel (187). Serine 471 phosphorylation of c-Rel seems to be required for TNF-α-mediated activation (167). In addition, c-Rel has been shown to undergo proteolytic processing upon ubiquitination by a mechanism similar to the inducible degradation of IκB proteins (32). Phosphorylation of p50 at serine 337 has reported to enhance its binding to DNA (111). RelB has been shown to be phosphorylated at two serine and one threonine residues: serine 386 phosphorylation induces RelB dimerization with p100 and inhibits its processing to p52 and phosphorylations of threonine 84 and serine 552 lead to inducible degradation of RelB in T cells in a fashion similar to IκBs (160, 165).

The majority of p50 is generated by constitutive co-translational processing of the precursor p105. This generates the basal pool of inhibitory p50/p50 homodimers

bound to κB sites under unstimulated cellular conditions. Rel homology domain of p105 can undergo co-translational dimerization (152). While required for efficient p50 production, the coupling of co-translational processing and dimerization uniquely generates p50/p105 heterodimers (152). p105 can also be degraded by stimulus specific manner (46, 141). This involves the phosphorylation of p105 at C-terminal serine residues 927 and 932 by IKKβ and leads to the ubiquitination of p105 at multiple lysines and complete degradation of p105 without generation of p50. IKKβ can also mediate the inducible processing of p105 to p50, a process which is poorly understood mechanistically. In this process, glycine-rich region of p105 at amino acids 376-404 functions as processing stop signal for the proteasome (153). This inducible processing involves C-terminal phosphorylation of p105, and probably ubiquitination at single lysine residue similar to the inducible processing of p100 (45, 46). In resting cells, p105 can also be phosphorylated by glycogen synthase kinase-3 β at serine residues 903 and 907. This stabilizes p105 under resting conditions, but is required for TNF-α stimulation mediated inducible processing of p105 (61).

In contrast to p105, p100 is not constitutively processed to generate p52 under normal cellular conditions. Inducible processing of p100 involves the activation of NF-κB inducing kinase (NIK) and IKKα and leads to the phosphorylation of p100 at its N-terminal serine residues 99, 108, 115 and 123 and C-terminal serine residues 866, 870 and 872 (288). Phosphorylation of p100 at these sites leads to the ubiquitination of p100 at lysine 856 and subsequent degradation of the C-terminal half of p100, and activation of p52 containing (mostly p52/RelB) NF-κB dimers (58, 208, 288). The glycine-rich region at amino acids 346-377 inhibits the complete degradation of p100 (103). The N-terminal serine phosphorylation may also play a role in the dimerization functions of p100 with NF-κB subunits (213). Constitutive processing of p100 has been observed in leukemic T cells and in various lymphomas associated with *nfκb2* (p100 producing) gene rearrangements (257, 287).

The activity of NF-κB is tightly regulated by its association with IκB proteins, a subfamily of the large ankyrin repeat domain (ARD) containing protein superfamily (7, 145, 183, 244) (Figure 6). IκB proteins contain six or seven ARDs that mediate the binding of IκBs to the RHDs of the NF-κB proteins, preventing their nuclear translocation and DNA binding (98, 99). IκB proteins found in humans include IκBα, IκBβ, IκBε, IκBγ (C-terminal half of p105), IκBδ (C-terminal half of p100), Bcl3 and recently described IκBζ (154, 197, 265, 279, 280, 293). The classical IκB proteins, IκBα, IκBβ and IκBε, are preferentially localized in the cytoplasm, where they bind to NF-κB dimers masking one or both NLSs on the dimer (15, 154, 161, 162, 279, 293). The C-terminal regions of p105 and p100 contain seven ARDs and function as autoinhibitory IκB domains to retain p105 and

p100 containing NF-κB dimers in the cytoplasm (191, 192, 223). In addition, p100 can form trimeric complexes with p50/p65 or p50/RelB dimers, but the function of these complexes has remained uncharacterized (59, 62, 127). In contrast to other IκB proteins, Bcl3 and IκBζ are not degraded upon stimulation of NF-κB activating pathways. The predominantly nuclear Bcl3 and IκBζ proteins regulate the nuclear NF-κB activity rather than its translocation from the cytoplasm into the nucleus. Bcl3 associates specifically with the inhibitory p50/p50 and p52/p52 homodimers in the nucleus, and in contrast to the classical IκB proteins, this association with Bcl3 leads to an increased transcriptional activity (25, 76, 77); Bcl3 can induce the p50/p50 homodimers to dissociate from the κB site providing space for the trans-activating NF-κB dimers containing p65, c-rel or RelB, or it can directly activate transcription by forming a trimeric complex with DNA-bound p50/p50 or p52/p52 homodimers (25). The recently described IκBζ stably accumulates in the nucleus where it associates with p50 and p65 and, opposite to the function of Bcl3, inhibits the NF-κB activity (265).

All the major cytoplasmic IκBs, IκBα, IκBβ and IκBε, have a conserved motif in their N-terminal end, which contains two serine residues that become phosphorylated by IKKβ upon activation of the IKK complex. Phosphorylation leads to the ubiquitination of the nearby lysine residues, and subsequent degradation of the inhibitors from the IκB/NF-κB complexes (208, 232). Once ubiquitinated, IκBα is degraded very rapidly, whereas the activation of IκBβ and IκBε containing NF-κB complexes occurs more slowly. Altough IκBα and IκBβ bind preferentially to p65 and c-Rel containing NF-κB dimers they posses functional differences *in vivo*. The activation of NF-κB by IκBα degradation is rapid but transient, whereas IκBβ degradation leads to more persistent NF-κB activation in a cell type and stimulus specific manner (264, 267). This functional difference can be explained by the fact that activation of NF-κB rapidly upregulates the expression of its inhibitor IκBα but not IκBβ (267). Moreover, the newly sythesized IκBα rapidly enters the nucleus where it can cause the dissociaton of NF-κB dimers from DNA (267).

In response to ultraviolet light treatment or certain oncogenes, IKKβ-independent phosphorylation of IκBα has been reported (225). IKKβ-independent phosphorylation occurs to the C-terminal end of IκBα and it is mediated by casein kinase II (CK2)-dependent pathway. C-terminal phosphorylation leads to degradation of IκBα, but the mechanism is independent of the proteasome and poorly characterized. C-terminal end of IκBβ can also be phosphorylated by CK2 leading to a loss of IκBβ mediated NF-κB inhibition (43).

Two NF-κB pathways

NF-κB is activated through two main signalling pathways: the classical pathway and the alternative pathway (Figure 7). These two pathways have distinct regulatory functions: the classical pathway is mostly involved in innate immunity and the alternative pathway in adaptive immunity (17, 24, 100). The classical pathway mostly targets p50/p65 and p50/c-Rel dimers maintained in the cytoplasm by IκB proteins. The classical pathway is triggered by pro-inflammatory cytokines, such as TNF-α and IL-1, as well as by microbial products. Toll-like receptors (TLRs) recognice diverse microbial products, collectively known as pathogen-associated molecular patterns (PAMPs). Ten different TLRs have been found in human cells, and despite the diversity of the TLRs, stimulation of all TLRs lead to the activation of NF-κB (68, 174). Upon activation by PAMPs, a TLR triggers a signalling cascade, culminating in the eventual activation of NF-κB, which binds to the promoter regions of genes that produce inflammatory cytokines such as TNF-α, IL-1 and IL-2 (68). The release of these cytokines and cytokines such as IFN-γ is the characteristic feature of the cellular response to the activation of the innate immune system (17, 24, 100).

The classical pathway signals to NF-κB by activating the IKK complex. IKK complex is activated through lysine 63-linked ubiquitination of the regulatory subunit IKKγ (39, 126). Lysine 63-linked ubiquitination, in contrast to lysine 48-linked, does not lead to proteasomal degradation, but instead, is mostly involved in protein targeting (273). Ubiquitination of IKKγ is required for the activation of IKKβ. Activated IKKβ, in turn, phosphorylates IκBα, IκBβ and IκBε at two N-terminal serine residues leading to their lysine 48-linked polyubiquitination and subsequent proteosomal degradation (128). The IKKα subunit of the IKK complex is dispensable for the activation of the classical pathway (232).

The alternative pathway is activated by a more limited number of stimuli and functions mainly in response to stimulation of a subset of the TNF-receptor subfamily, including receptors for lymphotoxin-β, B cell-activating factor (BAFF) and CD40 ligand (44, 58, 60, 130). Stimulation of these receptors leads to the activation of NIK. NIK selectively phosphorylates IKKα/IKKα homodimers, essential for the activation of the alternative pathway (288). The activation by NIK and IKKα (not requiring IKKβ or IKKγ) leads to the N- and C-terminal phosphorylation of p100, ubiquitination of p100 at lysine 856 and subsequent proteasomal degradation of the C-terminal half of p100 (58, 208, 213, 288). As p100 is most commonly associated with RelB, activation of the alternative pathway mostly results in nuclear translocation of p52/RelB dimers. The products of the genes activated by the alternative NF-κB dimers are mainly involved in the development and the maintenance of secondary lymphoid organs (58).

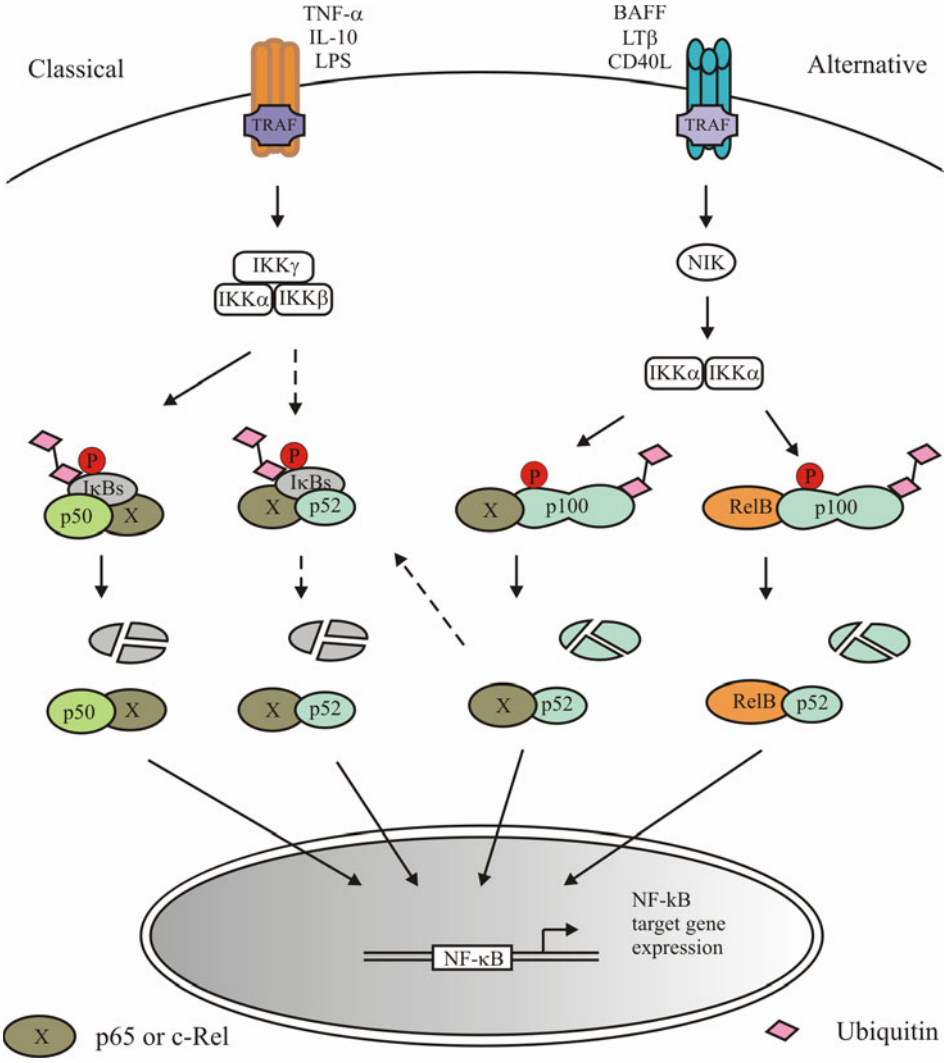
Classical
TNF-α
IL-10
LPS
TRAF
BAFF
LTβ
CD40L
TRAF
Alternative
IKKγ
IKKα
IKKβ
NIK
IKKα
IKKα
P
IκBs
p50
X
P
IκBs
X
p52
P
X
p100
P
RelB
p100
p50
X
X
p52
X
p52
RelB
p52
NF-κB
NF-kB
target gene
expression
X
p65 or c-Rel
Ubiquitin

Figure 7. ***NF-κB signaling pathways. Upon receptor stimulation, the classical NF-κB pathway (left) activates the inhibitory κB kinase (IKK) complex. IκBs are degraded upon IKKβ-mediated phosphorylation and subsequent ubiquitination. The activation of the alternative NF-κB pathway (right) leads to the actvation of IKKα homodimers via an upstream NF-κB-inducing kinase (NIK). p100 is processed to p52 in response to phosphorylation by IKKα. The dashed lines represent the "hybrid" pathway (see text for details). Liberated NF-κB dimers translocate into the nucleus, where they bind to the promoter regions of NF-κB responsive genes. The classical pathway is activated within minutes, whereas the activation of the alternative pathway can take a few hours.***

In the alternative pathway, p100 can also be dimerized with p65 or c-Rel. Degradation of the C-terminal part of p100 from these dimers generates p52/p65 and p52/c-Rel heterodimers. These dimers can then enter the nucleus or, alternatively, bind to other IκB molecules (RelB containing dimers are not well bound by the classical IκB proteins (247)). After stimulation of the classical NF-κB pathway and phoshorylation via the classical IKK complex, these IκBs are degraded releasing the active p52/p65 or p52/c-Rel heterodimers. This pathway is at the crossroads of the classical and the alternative pathways, and has sometimes been called as the "hybrid" pathway (58). The classical and the alternative NF-κB pathways are further linked, since the inducers of the alternative pathway also stimulate the classical pathway and the activation of the classical pathway drives the expression of p100 and RelB (24, 27, 58). While the classical pathway is activated within minutes, the activation of the alternative pathway can take a few hours.

Nucleocytoplasmic transport

p50, p65, p52 and c-Rel proteins have been shown to contain monopartite NLSs, which mediate the nuclear import of the proteins (22, 30, 81, 143, 149). NLS of RelB has remained uncharacterized. Once in the nucleus, NF-κB induces the expression of its own inhibitor IκBα (and to a lesser extent IκBε) but not IκBβ (41, 256, 261, 265, 267). The newly synthetized IκBα rapidly enters the nucleus, where it binds to NF-κB dimers on the promoters of NF-κB responsive genes and inhibits NF-κB-mediated transcription (293). This provides a negative feedback loop for the rapid activation determined by IκBα degradation. IκBα has been shown to contain an unconventional NLS, but no importins have so far been shown to be involved in the nuclear import of IκBα (230). The nuclear import of IκBα has been proposed to be mediated by additional NLS-containing proteins, or by direct contacts with the proteins of the NPC (229, 269). In addition, IκBα has been shown to contain two

strong NESs, one in its C-terminus and other in its N-terminus (5, 114, 115, 123). IκBα nuclear export is mediated via the Crm1 pathway (5, 115). Free IκBα can continuously shuttle between the nucleus and the cytoplasm by the means of its NLS and NESs. Both NESs, together with the NLS of IκBα, function in postinduction repression of NF-κB by mediating the nuclear export of IκBα/NF-κB complexes. The NLS of IκB is masked when the protein is bound to NF-κB dimers. IκBε has been shown to contain leucine-rich NES and the nuclear export of IκBε is mediated by Crm1 (146). Furthermore, like IκBα, also IκBε can regulate the nuclear export of certain NF-κB dimers (146).

No NLSs have been described in other IκB proteins. However, it has been shown that IκBβ can enter the nucleus when not bound to NF-κB dimers (259, 274). The mechanism of IκBβ nuclear import has not been determined. In the nucleus IκBβ can sustain the transcriptionally active NF-κB complex by binding to p50/p65 dimers, and thus preventing the binding and subsequent postinduction repression by IκBα (11).

Several reports suggest that NF-κB/IκBα complexes shuttle continuously between the nucleus and the cytoplasm in uninduced cells (114, 123, 260). It has been shown that in the IκBα/p50/p65 complexes, IκBα masks only the NLS of p65 protein (161). The shuttling of the IκBα/p50/p65 complex is proposed to be mediated by p50 NLS, which remains accessible in the complex. In contrast to IκBα containing complexes, IκBβ/NF-κB, IκBε/NF-κB or IκBγ/NF-κB complexes remain exclusively in the cytoplasm in unstimulated cells (84, 115, 161, 184, 261). It has been proposed that the cytoplasmic retention of these complexes could involve additional factors that mask their exposed NLSs (38, 184), or that the free NLS in the IκBα regulated complex is more accessible than the IκB contact free NLSs in the complexes regulated by other IκB molecules (261, 262). However, another report proposes a different model for the nucleocytoplasmic shuttling of IκBα/NF-κB complexes (30). In that model the shuttling is suggested to be a consequence of inhibitor dissociation followed by separate nuclear import of the subunits rather than the complex as a whole.

p65 possesses a strong NES, which efficiently mediates the nuclear export of p65 even in the absence of IκBα (95). However, overexpressed p50/p65 complexes efficiently localize into the nucleus, indicating that when bound to p50, the NES of p65 is masked and cannot interact with Crm1. The nuclear export of p50/p65 complexes is proposed to be mediated by IκBα (260). No NESs has been described in p50, p52, RelB or c-Rel proteins.

It has been demonstrated that the cellular localization of RelB is regulated by p100 and not by IκB proteins (247). It was further proposed that RelB/p100 complexes

shuttle in and out of the nucleus, and that the shuttling is mediated by one or more putative NESs present between amino acids 744 and 900 of p100. The possible nuclear import mechanisms of RelB/p100 complexes were not discussed.

In conclusion, the control of NF-κB activity by nucleocytoplasmic shuttling of NF-κB and IκB proteins is a multi-phased and highly regulated process. Furthermore, most likely additional interactions between distinct IκB and NF-κB proteins, as well as additional mechanisms and molecules involved in the regulation of nuclear import and export of IκB molecules and IκB/NFκB complexes still remains to be discovered.

3 AIMS OF THE STUDY

Control of nuclear localization is a key mechanism in regulating the activity of many transcription factors. The principal aim of this stydy was to analyze the nuclear import mechanisms of STAT1, STAT2 and NF-κB proteins.

The specific aims of this study were:

1. To analyze the possible interactions of STAT1 homodimers and STAT1/STAT2 heterodimers with importin α molecules (I, II)
2. To determine the binding sites of importin α molecules for STAT1 and STAT2 proteins (II)
3. To analyze the possible interactions of NF-κB proteins with importin α molecules (III, IV)
4. To analyze the functionality of the NLSs of the individual NF-κB subunits in the importin α interactions and in the nuclear import of different NF-κB dimers (III, IV)

4 MATERIALS AND METHODS

4.1 Cell culture (I-IV)

Human lung carcinoma A549 (ATCC, CCL 185), human larynx carcinoma Hep-2C (ATCC, CCL 23) and human hepatocellular carcinoma Huh7 (190) cell lines were maintained in Eagle-MEM (Sigma) supplemented with antibiotics, glutamine and 10% fetal calf serum (FCS; Integro). Human NK-92 (ATCC, CLR 2407) cell line was maintained in MEM alpha medium (Sigma) supplemented with 0.2 mM i-inositol, 20 mM folic acid, 40 mM 2-ME, antibiotics, glutamine, 100 IU/ml rIL-2, 12% FCS and 12% horse serum (Invitrogen). Monolayers and suspension cultures of *Spodoptera frugiperda (Sf9)* insect cells that were used for baculovirus expression were maintained in TNM-FH medium as described by Summers and Smith (255).

4.2 DNA manipulation (I-IV)

Expression vectors pGEX-2T and pETM-30 were used for producing GST-fusion proteins in *Esterichia coli* (*E. coli*). pcDNA3.1(+) (Invitrogen), FLAG-pcDNA3.1(+) (175) and pcDNA3.1-Myc-his(-) (Invitrogen) expression vectrors were used for *in vitro* translation and transient transfection of cultured cells. The plasmid pAcYM1 (255) was used for producing recombinant baculoviruses.

cDNAs to be cloned into the expression vectors were first modified by PCR to create N- and C-terminal *Bam*HI, *Bcl*I or *Bgl*II sites for further cloning into the BamHI site of pGEX-2T, pcDNA3.1(+), FLAG-pcDNA3.1(+), pcDNA3.1-Myc-his(-) or pAcYM1 vectors or to create N-terminal *Nco*I and C-terminal *Xho*I sites for cloning into pETM-30 vector. To create a GST fusion vector for baculovirus expression system GST-encoding cDNA was first modified by PCR using the pGEX-2T fusion vector as a template. The PCR product was subcloned into the pAcYM1 baculovirus expression vector. Point mutations to genes were done directly to the genes in the expression vectrors using the QuickChange™ site-directed mutagenesis kit (Stratagene). All DNA manipulations were performed according to standard protocols.

4.3 Protein expression and analysis

4.3.1 Production of GST-fusion proteins in *E. coli* cells (I-IV)

Proteins were expressed in *E. coli* BL-21 Star™ (DE3) cells as GST fusion proteins under 0.2 – 1.0 mM IPTG induction at 21 °C for 4 – 24h. Bacteria were lysed in 50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 (lysis buffer) with 5 mg/ml lysozyme (Sigma) and protease inhibitors (Complete, Roche Applied Science) at room temperature for 15 – 30 min. Lysates were briefly sonicated and clarified by Eppendorf centrifugation (13 000 rpm, at 4°C, 10 min).

4.3.2 Baculovirus expression (I-IV)

For protein expression, *Sf*9 cells were infected with protein expressing baculoviruses for 42 h. *Sf*9 cells were collected, and whole cell extracts were prepared by disrupting the cells in lysis buffer on ice for 10 min. The cells were further disrupted by passing them through a syringe. Lysates were clarified by Eppendorf centrifugation (13 000 rpm, at 4°C, 10 min).

4.3.3 Western blotting (I-IV)

Cleared whole cell lysates (10 – 30 μg) or proteins in the samples from a particular experiment were separated by SDS-PAGE using the Laemmli buffer system. The proteins were transferred electrophoretically onto Immobilon-P membranes. Membranes were stained with antibodies in PBS + 0.05% Tween-20 containing 5% non-fat milk for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence system (Amersham Biosciences). Detailed information about the antibodies used can be found in the original publications.

4.3.4 Gel filtration and protein oligomerization analysis (I)

E. coli or baculovirus-infected *Sf*9 cell lysates were gel filtrated in lysis buffer using 24 ml Superose 12 fast protein liquid cromatography (FPLC) gel filtration column (Amersham Biosciences). To study STAT-importin α5 complex formation STAT and GST-importin α5 protein-containing cell extracts were mixed and gel filtrated. The proteins in the gel filtration fractions were separated by SDS-PAGE followed by Western blotting and staining with anti-GST, anti-STAT1, anti-phospho-STAT1 and anti phosphotyrosine antibodies. To estimate the relative amounts of STAT and importin α5 proteins in STAT1/STAT2-importin α5 complexes, the proteins in the

gel filtration fractions were separated by SDS-PAGE followed by Coomassie blue staining. The quantitation of protein bands was performed with the Kodak electrophoresis documentation and analysis system 120.

4.3.5 GST-importin/fusion protein binding assay (I-IV)

For GST pull-down experiments, GST fusion proteins from *E. coli* or *Sf*9 cell extracts were first allowed to bind to 25 µl of glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences) at 4 °C for 1 h in lysis buffer in slow rotation followed by washing three times with the buffer. 25 µl of glutathine-Sepharose-immobilized GST fusion protein was mixed with 0.1 – 1.2 ml of cell lysate and rotated at 4 °C for 2 h followed by washing three times with lysis buffer. Proteins in the samples were dissolved in 50 µl of 2X Laemmli sample buffer and separated by SDS-PAGE. The gels were stained with Coomassie blue or Western blotted and stained with antibodies.

4.4 Protein-DNA interaction studies

4.4.1 Oligonucleotide DNA precipitation (III)

Cleared A549 whole cell lysates from TNF-α-stimulated cells or control cells were allowed to bind to 5'-biotinylated CCL5 or CXCL10 promoter NF-κB oligonucleotides. The complexes were separated by streptavidine-agarose beads, and the oligonucleotide bound proteins were separated on SDS-PAGE and analyzed by Western blotting with anti-p65 and anti-p50 antibodies.

4.5 Indirect immunofluoresence and confocal laser microscopy (I, III, IV)

For confocal laser microscopy transiently transfected or untrasfected HuH7 cells were grown on glass coverslips. Cells were left untreated or stimulated with human leucosyte IFN-α (100 IU/ml) or TNF-α (100 ng/ml). The cells were fixed and stained with antibodies. The antibody-stained proteins were visualized and photographed on a Leica TCS NT confocal laser microscope.

5 RESULTS AND DISCUSSION

5.1 Nuclear import mechanisms of STAT1 homodimers and STAT1/STAT2 heterodimers (I, II)

Prior to this thesis study, observations in our laboratory have demonstrated that STAT1 and STAT2 have a structural arginine/lysine-rich element involved in their IFN-induced nuclear import (177). This unusual structural NLS was shown to be situated in the DNA binding domain of the molecule and become active only in dimers. The amino acids critical for nuclear import were mapped to lysines 410 and 413 of STAT1 and arginine 409 and lysine 415 of STAT2 (177). A few years before the unconventional dimer-specific NLSs were described, the mechanism regulating the nuclear localization of STAT1 was proposed to involve the function of importin α5 molecule (238). In that report, the authors showed that tyrosine-phosphorylation and homodimerization of STAT1 are essential for the interaction with importin α5. At that time, these two publications were the only reports describing NLSs or importin α interactions of STAT proteins.

5.1.1 STAT activation using baculovirus expression system

The critical event in cytokine-dependent STAT-mediated transcriptional regulation is phosphorylation of STATs on a single tyrosine residue at their C-terminus (54, 243). In response to an activating cytokine, such as IFNs, STAT1 homodimers or STAT1/STAT2 heterodimers are activated mostly by receptor accosiated Jak1/Jak2 or Jak1/Tyk2 kinases, respectively. However, the specificity of Jaks relies on their capacity to associate with particular cytokine receptor subunit rather than because of a high degree of intrinsic substrate specificity. In our experimental setting, coinfection of *Sf*9 cells with Tyk2 baculovirus construct and STAT1, STAT2 or STAT1-NLS$^-$ (K410A, K413A) protein expressing baculoviruses resulted in efficient tyrosine-phosphorylation of the STAT proteins (I, Figure 1). When expressed with Tyk2, Y701A mutant of STAT1 completely lacked tyrosine-phosphorylation required for formation of the active dimer. STAT2 does not form active tyrosine-phosphorylated homodimers. Tyrosine-phosphorylated STAT1 or STAT1-NLS$^-$ formed homodimers, and when expressed with STAT2, tyrosine-phosphorylated heterodimers of STAT1/STAT2 or STAT1-NLS$^-$/STAT2 were obtained (I, Figures 1 and 6). This enabled us to examine potential interactions between dimeric STAT complexes and importins with GST-importin protein binding assay.

5.1.2 Activated STAT1 homodimers and STAT1/STAT2 heterodimers directly bind to importin α5 via an unconventional structural NLS.

Since IFN-induced nuclear import of STAT1 had been suggested to be mediated by importin α5, and since it had been demonstrated that STAT1 and STAT2 proteins have a well conserved, unusual arginine/lysine-rich NLS, which regulates their nuclear import (177), we wanted to study whether this NLS element would be involved in direct binding of STATs to importin α molecules (I).

To determine the possible interactions of STAT1 homodimers and STAT1/STAT2 heterodimers with importin α5, pull-down assays were performed with GST-importin α5 fusion protein produced in *E. coli* and STAT dimers activated in the baculovirus expression system. We observed that wild type (wt) STAT homodimers and STAT1/STAT2 heterodimers bound strongly to importin α5 molecule (I, Figure 2). Instead, homodimers of STAT1-NLS$^-$ or STAT1-NLS$^-$/STAT2 heterodimers completely failed to bind to importin α5. Also monomeric STAT1 Y701A mutant and tyrosine phosphorylated wt STAT2 were devoid of importin α5 binding. The importin α5-STAT complexes were relatively stable, since washing with 2M NaCl or 2M urea did not disrupt the preformed importin α5-STAT interaction (I, Figure 3). After the observations from the GST-pull down experiments, the effects of STAT1 and STAT2 NLS mutants were studied in cultured HuH7 cells. When the cells were transfected with wt STAT1 and STAT2-NLS$^-$ (R409A, K415A) or with wt STAT2 and STAT1-NLS$^-$, we found that, both transfected proteins colocalized exclusively in the cell cytoplasm (I, Figure 4). Furthermore, when we transfected the cells with importin α5 and wt or NLS mutant forms of STAT1 or STAT2, we observed that, in contrast to wt molecules, no colocalization of importin α5 with STAT1 or STAT2 NLS mutants was found (I, Figure 5). These results indicate that STAT1 homodimers and STAT1/STAT2 heterodimers bind to importin α5 via unconventional NLSs that become active only in dimers.

At the same time, an article demonstrating direct interaction of tyrosine-phosphorylated STAT1 homodimer with importin α5 was published (172). In that study the authors characterized a STAT1 L407A mutant, which forms tyrosine-phosphorylated homodimers and binds to DNA but does not bind to importin α5. The authors further proposed that NLS exists in an inactive state within a STAT1 monomer and becomes active upon dimerization. In addition, based on studies with the STAT1 L407A mutant in transfected cells, the authors suggested that an intact NLS in one STAT1 molecule is sufficient to translocate STAT1 homodimers or STAT1/STAT2 heterodimers into the nucleus. However, our results clearly showed that when STAT1 (K410A, K413A) NLS mutant was dimerized with wt STAT2 and

the dimer was tyrosine-phosphorylated, the complex did not show any binding to importin α5, nor was it transported into the nucleus in transfected cells (I, Figures 1, 2 and 4). Furthermore, when wt STAT1 was transfected with STAT2 (R409A, K415A) NLS mutant, both proteins colocalized exclusively in the cell cytoplasm (I, Figure 4). These results clearly indicate that two of these arginine/lysine-rich elements, one in each monomer, that form the conformational NLS of a STAT dimer, are required for recognition by importin α5 and subsequent nuclear import to take place. However, there is a possibility that when STAT1 L407A mutant is homodimerized, the two amino acid substitutions in the dimer are sufficient to alter the conformation of the dimer-specific NLS (presumably composed of L407 in addition to K410 and K413 in each STAT1 monomer) enough to retain the complex in the cytoplasm. But, when the L407A mutant of STAT1 is dimerized with wt STAT1 or wt STAT2, the single amino acid mutation does not have that effect, and the precence of the intact amino acids constructing the NLS are sufficient to retain the NLS recognizable by importin α5.

The importance of lysines 410 and 413 in the nuclear import of tyrosine-phosphorylated STAT1 has also been confirmed by another group (180). The authors found that the constitutive and IFN-γ-induced nuclear import of STAT1 proceed through independent pathways. In that report, Meyer and coworkers further suggested that the unconventional conformational NLS is not involved in the constitutive nuclear import of STAT1. Consistent with this observation, it has been suggested by another group that the constitutive import of latent STAT1 is independent of importin α/β and could be mediated by direct contacts with the proteins of the NPC (164). Meyer *et al.*, however, confirmed that lysines 410 and 413 are dispensable for STAT1 dimerization, yet they are critical for the nuclear import of the tyrosine-phosphorylated molecule, which is in line with our results. In addition, they found that two hydrophobic residues, leucines 407 and 409, are also important for nuclear accumulation of phosphorylated STAT1. However, no importin α-STAT interactions were analyzed. Arginines 414 and 417 of STAT3, coresponding to lysines 410 and 413 of STAT1, have been shown to mediate the nuclear import of STAT3 (157). In addition, leucine 411 of STAT3, corresponding to leucine 407 of STAT1, was not essential for STAT3 nuclear transport in that study. This further supports the concept that lysines 410 and 413 in STAT1 play an important role in the nuclear import of STAT1. However, it is notable that STAT3 nuclear import is likely to be mechanistically different from that of STAT1, since a region in the coiled coil domain of STAT3 with arginines 214 and 215 has been shown to mediate the importin α interaction and nuclear localization of STAT3 (156, 157). Furthermore, mutation of arginine 210 and lysine 211 of STAT1, corresponding to arginines 214 and 215 of STAT3, did not have any effect on the IFN-γ-induced nuclear import of STAT1 (157).

We also wanted to examine the possible interactions of endogenous, as well as baculovirus-produced STAT1/STAT2 dimers with other importin α isoforms in addition to importin α5 (II). Pull-down assays were performed with GST-importin α1, α3, α5 and α7 fusion proteins produced in *E. coli* and IFN-α-stimulated NK-92 cell lysates or STAT1 homodimers or STAT1/STAT2 heterodimers activated with baculovirus expression system. Endogenous and baculovirus-activated STAT1 and STAT2 bound effectively to GST-immobilized importin α5, whereas no binding to importin α1, α3 or α7 was observed (II, Figures 2 and 3). This further confirmed the specific interaction of STAT1 and STAT2 with importin α5.

NLSs have also been described to exist in some secreted cytokines, like IFN-γ (253). It has also been proposed that, after endocytosis with the receptor subunit, IFN-γ forms a trimeric complex with STAT1 and importin α5, and that the nuclear import of this complex is mediated by the IFN-γ NLS (252). However, this most likely is not the nuclear import mechanism for STAT1, since STAT1 NLS mutants, which form tyrosine phosphorylated dimers, do not localize into the nucleus (I) (172, 177, 180).

5.1.3 STAT-importin α5 complexes consist of two STAT and two importin α5 molecules.

To determine the protein composition of importin α-STAT complex, we examined the preformed importin α5-STAT complex by FPLC. We analyzed the fraction corresponding to the major peak (at 300-350 kDa) of importin α5-STAT1/STAT2 complexes by SDS-PAGE. Based on the intensity of Coomassie-blue stained bands, it was possible to directly quantitate the STAT1, STAT2 and importin α5 protein-specific bands. The protein levels indicated that in the complex, the relative amounts of STAT1 and STAT2 were equal, whereas the complex contained twice as much importin α5 (I, Figure 6). This suggests that the nuclear import complex of STATs is composed of two STAT molecules attached to two importin α5 molecules.

5.1.4 STAT1 and STAT2 bind to the C-terminal Arm repeats 8 and 9 of importin α5.

Previously, it has been suggested that the importin α5 binding site for STAT1 is distinct from the classical NLS binding site and is located at the very C-terminal end of importin α5 (238). By deletion analysis the binding site was located to the region between amino acids 475-538. However, the exact binding site was not determined (238).

The classical NLS binding sites of importin α molecules are situated in regions, which consist of Arm repeats 2-4 (amino acids 118-245 of importin α5) and 7-8

(amino acids 331-412). These sites have been termed as the major and the minor NLS binding sites, respectively. The helical grooves of these sites contain the well conserved trypthophan and asparagine residues, which directly interact with the arginines and lysines of NLSs. It has been shown that the major binding pocket binds to the monopartite NLS and to the larger strech of basic residues in bipartite NLS, whereas the minor pocket interacts with the smaller strech of basic residues in bipartite NLS (47, 75). However, by using deletion analyses several molecules with classical and nonclassical NLS have been suggested to be able to bind to the C-terminal regions of importin α1 and importin α5 molecules (72, 80, 90, 101).

To determine the exact binding site of importin α5 for STAT1 and STAT2 we created mutations to the conserved trypthophan and asparagine residues in Arm repeats 2, 3, 4, 7, 8 and 9 of importin α5 molecule. We included Arm repeat 9 in our studies, because the two asparagine residues appeared as a part of a possible NLS binding pocket. In addition, different combinations of these Arm repeat mutations were constructed. By using GST-importin protein binding assay we found that mutations in Arm repeats 2, 3, 4, or 7 or different combinations of these had no effect on STAT1 or STAT2 binding to importin α5. Instead, the two amino acid mutations in Arm repeats 8 or 9 completely abolished the binding of STAT1 homodimers and STAT1/STAT2 heterodimers to importin α5 molecule (II, Figure 5). These results indicate that, instead of the very C-terminal end proposed previously (238), the exact binding site for STAT1 and STAT2 NLSs is the Arm repeat domains 8 and 9 of importin α5 molecule. This NLS binding site differs from the previously described minor NLS binding site composed of Arm repeats 7 and 8. Together with previous reports, this indicates that importin α molecules are flexible in using both, the N-terminal and the C-terminal, NLS binding pockets for binding to specific substrates.

5.2 Nuclear import mechanisms of NF-κB proteins (III, IV)

In mammals, NF-κB family of transcription factors comprises of five members: p50, p65, p52, RelB and c-Rel, which associate to form transcriptionally competent homo- and heterodimers. The most abundant and best characterized of these dimers is the prototypical NF-κB p50/p65 heterodimer. p50, p65, p52 and c-Rel have been shown to contain arginine/lysine-rich classical monopartite NLSs, which mediate the nuclear localization of the proteins (22, 88, 143, 149). However, prior to our studies there were no published data on the possible interactions of NF-κB proteins with importin molecules. In order to understand the molecular mechanisms of nuclear import of NF-κB, we studied the interactions of NF-κB with importins by pull-down assays and immunoprecipitation experiments.

5.2.1 NF-κB components of the classical and the alternative pathway are transported into the nucleus via a subset of importin α molecules

In the beginning of this study (III), we had *E. coli* expression constructs for GST-importin α1, α3, α5, α7 and β and baculovirus expression constructs for GST-importin α1, α3, α4 and α7. Different expression systems were used, since at that time all isoforms could not be expressed in *E. coli* or by baculovirus expression system. First, with this limited set of GST-importins, we analyzed the interactions of the classical NF-κB pathway components p50 and p65 with importin molecules by GST-pull-down experiments. We found that endogenous p50 and p65, extracted from A549 cells, interacted specifically with importin α3 and α4 in a TNF-α stimulation-specific fashion (III, Figure 1AB). When we used GST-p50 and GST-p65 fusion proteins in similar experiments, we found that GST-immobilized p50 and p65 bound importin α3 from A549 cell lysates (III, Figure 1D). The specific interaction was further confirmed by coprecipitating importin α3 with p50 and p65 proteins from TNF-α-stimulated cell extracts using p50 or p65-specific antibodies (III, Figure 2). Consistent with endogenous proteins, also *in vitro*-translated p50 and p65 bound to GST-immobilized importin α3 (III, Figure 5). These data indicated that p50 and p65 are transported into the nucleus by importin α3 and importin α4.

In the next set of experiments covering the nuclear import of NF-κB we had all different importin α isoforms as GST-fusion proteins (IV). Importin α6 was expressed in *E. coli* and other importin α isoforms in *Sf*9-cells (we were not able to produce functional importin α6 by baculovirus expression system). Using this full set of importin α molecules, we found that, consistent with our previous observations, endogenous p50 and p65 proteins bound to importin α3 and α4 in a stimulus specific fashion, while no binding to importin α1 or α7 was detected (IV, Figure 2). However, in this case we also observed that *Sf*9-expressed importin α5 was able to bind p50 and p65 proteins, a result not obtained with *E. coli*-produced importin α5. Moreover, also importin α6 was found to interact with *in vitro*-translated p50 and p65 (IV, Figure 1B). To further confirm the binding specificities of different importin α isoforms for p50 and p65 proteins, we performed immunoprecipitation experiments with Myc-tagged importin α constructs (all but α6), which were transfected in Huh7 cells. In coimmunoprecipitation analyses, importin α3 and α4 and to a lesser extent importin α5 readily bound p50 and p65 from TNF-α-stimulated cell extracts (IV, Figure 3). These data together with the data above confirmed that importin α3 and α4 are mediating the nuclear import of p50 and p65 proteins. In addition, our results, obtained with the *Sf*9-produced GST-importin α5 and with the transfected importin α constructs, revealed that also importin α5 is specifically involved in the nuclear import of p50 and p65. Since

importin α6 is expressed only in testis, it can be concluded that the primarily nuclear import of the classical NF-κB pathway components p50 and p65 is mediated via importin α3, importin α4 and importin α5.

In addition to the data obtained with importin α5 here, we have recently observed that the nature of the expression system (prokaryotic vs. eukaryotic) can greatly affect the binding specificity of different importin α isoforms to the same cargo molecules (176). The differences in the binding specificities of *E. coli*- and *Sf*9-expressed importin α isoforms suggest that post-translational modifications may be needed to alter the binding of some molecules to certain importin α molecules. It has, in fact, been demonstrated that the function of importin α1 can be regulated by phosphorylation and acetylation (272). The authors presented evidence that importin α1 contributes to the nuclear import of human mRNA binding protein HuR through two AMP-activating protein kinase (AMPK)-modulated mechanisms. First, AMPK was shown to trigger the acetylation of importin α1 at lysine 22, a process dependent on the activity of acetylase CBP/p300. Second, AMPK was shown to directly phosphorylate importin α1 at serine 105. The authors further demonstrated that importin α1 proteins bearing K22R or S105A mutations failed to mediate the nuclear import of HuR. Moreover, the authors observed that also importin α3 and α7 appear to be adequate *in vitro*-targets for AMPK-mediated phosphorylation. However, the significance of these modifications in the import functions was not dicussed. In addition, another report identified importin α1 and α7, but not importin α3, as substrates for CBP/p300 mediated acetylation (10). While it is known that the NLS-dependent nuclear import can be regulated by phosphorylation in the vicinity of the NLS (120-122), these findings indicate that the binding specificities of at least some importins can be regulated by phosphorylation and/or by acetylation. The lack of these regulation strategies could explain why we observed different binding specificities with bacteria-produced and *Sf*9-produced importin α5.

To further analyze the nuclear import of NF-κB transcription factors, we studied the possible interactions of the alternative NF-κB pathway components p52, RelB and c-Rel with importin α molecules with similar pull-down experiments (IV). We found that *in vitro*-translated p52 (like p50 and p65 proteins) bound mainly to importin α3, α4, α5 and α6. *In vitro*-translated RelB and c-Rel, instead, bound to importin α5, α6 and α7 (IV, Figure 1B). When we analyzed the interaction of endogenous p52 to ubiquitously expressed importin α isoforms (that is all but α6), the result was consistent with the binding observed for *in vitro*-translated protein; importin α3, α4 and to a lesser extent α5 bound cellular p52 from Sendai virus-infected or TNF-α-stimulated cell extracts (IV, Figure 2).

Interestingly, the binding pattern of TNF-α-stimulated and Sendai virus infection-induced endogenous RelB was clearly different from the *in vitro*-translated RelB.

Cellular RelB (like *in vitro*-translated protein) bound strongly to importin α5. In addition, cellular RelB also effectively bound to importin α4, whereas *in vitro*-translated protein did not show any interaction with importin α4. Furthermore, in contrast to *in vitro*-translated protein, endogenous RelB did not show any binding to importin α7 (IV, Figures 1B and 2). This difference in the binding pattern of *in vitro*-translated and cellular RelB could be explained by the observation that RelB is the only NF-κB protein that does not form functional homodimers *in vivo* (113). Our data strongly suggest that when RelB is expressed *in vitro*, that is without other NF-κB family members, the binding specificity of the protein to importin α molecules is different from the endogenous cellular RelB, which in its functional form exists only as heterodimers with other NF-κB proteins. These data also indicate that importin α3 and α4 are the likely candidates for the nuclear import of functional endogenous RelB containing dimers. Moreover, in contrast to p50, p52 and p65-containing NF-κB dimers, it seems that importin α3 does not contribute significantly to the nuclear import of RelB-containing dimers. The data further suggest that importin α3 and α4, which are very closely related isoforms and comprise one importin α subfamily, can still have different substrate specificities for certain NF-κB family members.

5.2.2 p50 binds to the N-terminal and p65 to the C-terminal NLS binding site of importin α3

Since we had previously observed that importin α molecules are able to use either the N- or C-terminal NLS binding pockets for binding to different nuclear-targeted proteins (II), we wanted to analyze whether p50 and p65 would bind to the N-terminal or C-terminal NLS binding sites of importin α3 (III).

To locate the binding sites for p50 and p65 we performed pull-down assays with GST-importin α3 constructs bearing mutations in the N-terminal Arm repeat 3 or C-terminal Arm repeats 7 and 8. Interestingly, we found that importin α3 uses different binding sites for binding p50 and p65 proteins. We found that mutations in Arm repeat 3 abolished the binding of p50 to importin α3 but had no effect on p65-importin α3 interaction. In contrast, Arm repeats 7+8 mutation eliminated the binding of p65 to the importin but had no effect on p50 binding. Equal results were obtained with *in vitro*-translated and endogenous p50 and p65 proteins (III, Figure 7). These results indicate that p50 and p65 are bound by different NLS binding sites of importin α3.

This gave us a working model that one importin α3 molecule interacts with heterodimeric p50/p65 complex in a manner that the N-terminal NLS binding site interacts with p50 and the C-terminal binding site with p65. This interaction creates a stable transport complex with no space for IκBα. Biochemical experiments

supported this model, since increasing amounts of *in vitro*-translated IκBα competed with the interaction of p50/p65 complex for importin α3 (III, Figure 5B). Furthermore, when we studied the kinetics of TNF-α-induced p50/p65 activation and binding to importin α, we found that NF-κB binding to importin α3 was seen only after IκBα was degraded from the complex (III, Figure 3). Moreover, importin α3 coprecipitated with p50 and p65 proteins only after TNF-α stimulation, when the complex was free of IκBα (III, Figure 2). We propose that p50/p65 complex can, thus, bind to importin α3 only in the absence of IκBα. In addition, we did not observe any significant binding of cellular p105 or p100 to importin α molecules further indicating that NF-κB complexes containing the inhibitor IκBα do not bind to importin α molecules (III, Figure 3B; IV, Figures 2 and 4).

The original model for NF-κB function postulated that in unstimulated cells the transcription factor is anchored in the cytoplasm by association with IκB molecules, which mask the NLSs of the subunits in the dimer (85). Later, the crystallocraphic structures of IκBα bound to p50/p65 dimers revealed that IκBα protein masks only the NLS of p65, whereas the p50 NLS remains accessible (116, 119). The precence of this accessible NLS on p50 coupled with NESs that are present on IκBα and p65 was proposed to result in constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm observed with *in vivo* immunofluorescence studies in unstimulated cells (114, 123, 260). Similarly, crystal structure of IκBβ/NF-κB p65 homodimer complex showed that IκBβ contacts only one p65 NLS of the homodimer (162). In addition, it has been observed that in the IκBγ/NF-κB p50/p65 heterodimeric complexes the p65 NLS is free from IκBγ contacts (184).

However, based on several published reports, IκBα/NF-κB complexes are thought to shuttle continuously in and out of the nucleus, whereas IκBβ/NF-κB, IκBε/NF-κB and IκBγ/NF-κB complexes remain constitutively cytoplasmic in resting cells (84, 114, 115, 123, 161, 260, 261). It has been proposed that the cytoplasmic retention of these complexes is regulated by additional proteins that mask their exposed NLSs (38, 184) or that the solvent exposed NLS is not just that accessible when the dimer is bound to IκBβ compared to the IκBα bound NF-κB dimer (261, 262). However, whether the NLS is more or less accessible, our results clearly indicate that when NF-κB is bound to importin α molecules, the complex does not contain IκBα. Our results rather support the more recent reports, which propose a dissociation model, in where the constant shuttling of IκBα/NF-κB complexes observed in resting cells, is the result of inhibitor dissociation and separate nuclear import of IκBα and NF-κB rather than the complex as a whole (20, 30). However, our results do not rule out the possibility that IκBα/NF-κB complexes could enter the nucleus by the means of other than the classical importin α/β pathway. In fact, it

has been suggested that nuclear import of IκBα could be mediated by direct contacts with the components of the NPC (229).

5.2.3 Identification of NLSs of NF-κB proteins regulating importin α interaction and nuclear import

p50, p65, p52 and c-Rel proteins have been shown to contain arginine/lysine-rich NLSs that regulate their nuclear import. However, the possible NLS of RelB has remained uncharacterized. The amino acid sequence of RelB has two potential arginine/lysine-rich regions that could function as NLSs. These regions comprise amino acids 409-413 (KKRKR; NLS1) and 433-438 (KRRKKK; NLS2). To determine whether the previously identified NLSs of p50, p65, p52 and c-Rel and the potential NLSs of RelB mediate the interactions of these proteins with importin α molecules, we carried out binding experiments with GST-importins and *in vitro*-translated wt or NLS mutant forms of NF-κB proteins. Details of the mutated amino acids are described in III and IV.

We found that wt proteins bound effectively to the tested importin α molecules (eg. p50 and p65 to importin α3 and p52, c-Rel and RelB to importin α5), whereas NLS mutant forms of p50, p65, p52 and c-Rel as well as both NLS1 and NLS2 mutant forms of RelB failed to do so (III, Figure 6AB; IV, Figure 5). To confirm the results obtained in biochemical experiments also in cultured human cells, we analyzed the nuclear accumulation of wt and NLS defective proteins in transiently transfected HuH7 cells. We found that wt p50, p65, p52 and c-Rel proteins accumulated effectively in the nucleus, whereas the NLS mutants were localized in the cytoplasm (III, Figure 6C; IV, Figure 6AB). However, when the cells were transfected with RelB NLS1 or NLS2 mutant, the nuclear localization of RelB was somewhat impaired but still clearly detectable. Only after both of the potential NLS sequences were simultaneously mutated the nuclear import of RelB was eliminated (IV, Figure 6C). These results show that the previously identified monopartite NLSs of p50, p65, p52 and c-Rel mediate the binding of these proteins to importin α molecules and regulate their nuclear import. Furthermore, the results presented here demonstrate that RelB, instead, has a bipartite NLS sequence, which mediates the binding of RelB to importin α molecules and regulates the nuclear import of the protein in transfected cells.

5.2.4 Certain NF-κB family members are regulating the nuclear accumulation of a heterologous family member

To further analyze the function of the NLS signals of NF-κB proteins in the nuclear import of specific dimers, we carried out cotransfection experiments with wt or NLS mutant forms of p50, p65, p52 and RelB proteins. The nuclear accumulation of the transfected proteins was studied with immunofluorescence microscopy (III, IV).

When we compared the effects of transfected wt or NLS mutant form of p65 protein on the cellular localization of p50, we found that NLS defective p65 did not have any effect on the distribution of cellular p50 (III, Figure 8). This suggested that significant part of p50 homodimers is transported into the nucleus independently of p65 and NLS-deficient p65 cannot function as dominant negative for nuclear import of cellular p50. Furthermore, when we cotransfected the cells with NLS defective p52 and wt p50 or vice versa, we found that the NLS defective forms of these proteins did not have any effect on the cellular distribution of the heterologous wt proteins (IV, Figure 9). This indicates the tendency of p50 and p52 to form homodimers, at least when transfected and overexpressed together.

To study whether there is a cross-regulation in the nuclear import of RelB and p52, we carried out cotransfection experiments with wt and NLS defective forms of p52 and RelB. As expected, when transfected together, wt p52 and RelB colocalized in the cell nucleus. Interestingly, when NLS-mutant p52 was transfected with wt RelB, both proteins were transported into the nucleus, where they clearly colocalized. Furthermore, when the cells were transfected with wt p52 and NLS mutant RelB, wt p52 colocalized effectively with NLS defective RelB in the cell cytoplasm (IV, Figure 7). These data indicate that the nuclear import of p52/RelB heterodimers is regulated exclusively by the newly characterized NLS of RelB. An additional interesting observation was that the NLS of p52 protein was the dominant signal for the nuclear localization of the heterodimeric p52/p65 complexes (IV, Figure 8). The data show that, in contrast to the p52/RelB heterodimer, the nuclear import of heterodimeric p52/p65 complexes is regulated by the NLS of the p52 protein.

The data presented here indicate that importin α family members clearly have distinct substrate specificities in nuclear import of NF-κB proteins. Based on the results from the binding experiments of *in vitro*-translated and endogenous NF-κB proteins presented above, it can be concluded that the specificity to a given NF-κB protein is also different and changes upon the composition of the imported dimer. Furthermore, the results presented here show that the NF-κB components of the classical and alternative pathways have somewhat different specificities for importin α molecules.

6 CONCLUSIONS

Cells respond to various extracellular and intracellular stimuli with the activation of specific signal transduction pathways, leading to changes in host cell gene expression profiles in the nucleus. Many signalling molecules, which function as transcription factors must traverse the barrier of the nuclear envelope to gain access to the target genes. The main focus of this study was to characterize the mechanisms involved in the nuclear import of STAT1, STAT2 and NF-κB transcription factors.

The present study demonstrates that STAT1 homodimers and STAT1/STAT2 heterodimers are transported into the nucleus specifically by importin α5 molecule. These observations, togethter with previous findings, indicate that STATs use two different pathways for nuclear import. Before cytokine induction unphosphorylated STATs translocate into the nucleus by a carrier- or, at least, importin α-independent mechanism, that could involve interactions with nucleoporins. Nuclear import of tyrosine-phosphorylated STAT dimers, on the other hand, is dependent on the dimer-specific NLSs of STATs, importin α5 molecules, RanGTPase and metabolic energy.

We found that all NF-κB proteins are transported into the nucleus by a subset of importin α molecules. These results are the first to describe interactions of NF-κB proteins with importin α molecules. In our experimental settings we did not observe any association of IκBα with the NF-κB/importin α complexes. This indicates that NF-κB/IκBα complexes are not transported into the nucleus as a whole or, at least, not by the classical importin α mediated nuclear import pathway. Furthermore, the results presented here show that the NF-κB components of the classical and alternative pathways have somewhat different specificities to importin α molecules. Moreover, most likely due to the different NF-κB dimers existing under different cellular conditions, the specificity of importin α molecules to a given NF-κB family member is dependent on and changed by the composition of the proteins in the transported dimer. However, at present it is still unclear what is the contribution of specific NF-κB complexes to a given physiological response. Therefore, it is difficult to determine what would be the specificity of a given NF-κB dimer to distinct importin α family members under particular stimulus, and whether this specificity changes among different cellular conditions.

The observations in the present work have taken a clear step forward in understanding the molecular mechanisms mediating the transport of STAT and NF-κB transcription factors from the cytoplasm into the nucleus. Furthermore, these results provide additional evidence for the existence of distinct substrate specificities among importin α isoforms. In addition, this work shows that importin α molecules

are apparently more flexible than previously anticipated, since they can use both the N-terminal and the C-terminal NLS binding sites for binding to various proteins destined for nuclear import.

7 ACKNOWLEDGEMENTS

This study was carried out at the Laboratory of Infectious Disease Immunology at the Department of Viral Diseases and Immunology, National Public Health Institute, Helsinki. Professor Pekka Puska, Head of the institute and Professor Tapani Hovi, Former Head of the Department, are gratefully acknowledged for providing excellent working facilities.

The Finnish Cultural Foundation, the Maud Kuistila Memorial Foundation, the Alfred Kordelin Foundation, the Paulo Foundation, the Finnish Cancer Foundation, the Magnus Ehrnrooth Foundation, the Farmos Research Foundation and the Finnish Foundation for the Research of Viral Diseases are acknowledged for their financial support during this research work.

I warmly thank my supervisors Dr. Krister Melén and Professor Ilkka Julkunen. Their excellent support, optimism and professional expertise have made it possible to complete this thesis. Ilkka is thanked for providing the great working environment placed at my disposal and for being always an optimistic and supportive boss. I wish to express my deepest gratitude to Krister who has truly thaugt me just about everything in the lab along my way from an undergraduate student to a research scientist. Thanks man!

I thank Docent Päivi Ojala and Professor Jorma Palvimo for the rapid review process and their valuable comments.

I wish to express my sincere thanks to all my co-authors and collaborators who have contributed to this thesis. I also express my heartfull thanks to all the former and present members in Ilkka's lab. It has been a real pleasure to work with you! I especially thank the technical assistants and secretaries, Mari, Hanna, Teija, Johanna, Sari, Kirsi-Mari, Tia and Minna for indispensable assistance and patient help with everything. I also warmly thank the cell-ladies, Sinikka and Raija, for providing me excellent cell cultures and being always so kind and extremely friendly whatever I needed. Finally, Ville and Pame are deeply respected for the experimental activities outside the lab!

Helsinki, January 2008

Riku Fagerlund

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