NF-κB Is Transported into the Nucleus by Importin α3 and Importin α4

NF-κB transcription factors are retained in the cytoplasm in an inactive form until they are activated and rapidly imported into the nucleus. We identified importin α3 and importin α4 as the main importin α isoforms mediating TNF-α-stimulated NF-κB p50/p65 heterodimer translocation into the nucleus. Importin α3 and α4 are close relatives in the human importin α family. We show that importin α3 isoform also mediates nuclear import of NF-κB p50 homodimer in nonstimulated cells. Importin α3 is shown to directly bind to previously characterized nuclear localization signals (NLSs) of NF-κB p50 and p65 proteins. Importin α molecules are known to have armadillo repeats that constitute the N-terminal and C-terminal NLS binding sites. We demonstrate by site-directed mutagenesis that NF-κB p50 binds to the N-terminal and p65 to the C-terminal NLS binding site of importin α3. In vitro competition experiments and analysis of cellular NF-κB suggest that NF-κB binds to importin α only when it is free of IκBa. The present study demonstrates that the nuclear import of NF-κB is a highly regulated process mediated by a subset of importin α molecules.

NF-κB1 p50/p65 transcription factor has a central role in controlling host cell immune and inflammatory responses, cell differentiation, and apoptosis (1, 2). Dysregulation of NF-κB has been associated with several common diseases such as cancer and diabetes (3). Cytoplasmic NF-κB can be rapidly activated by various physiological and nonphysiological stimuli such as cytokines, growth factors, bacterial or viral infection and UV irradiation. Activation of NF-κB is followed by its rapid translocation into the nucleus where it activates the transcription of numerous genes including those encoding for cytokines and cell adhesion molecules. Some genes can be transcriptionally up-regulated within minutes after NF-κB activation (2, 4).

NF-κB transcription factors are dimers belonging to the Rel family (5). All five mammalian NF-κB subunits, p65 (RelA), RelB, c-Rel, p50 (and its precursor p105), and p52 (and its precursor p100) contain an N-terminal Rel homology domain responsible for their dimerization, nuclear localization, and DNA binding (6, 7). NF-κB subunits can form various dimers, but the classical, best characterized form is composed of p50 and p65 (1, 7, 8). p65, RelB, and c-Rel contain a C-terminal transcription activation domain, and they can therefore form transcription-activating dimers with each other and with p50 or p52. p50 and p52 proteins lack the transcription activation domain, and the homodimers they form are mostly suppressors of gene expression (9).

NF-κB dimer is held in an inactive state in the cytoplasm by an inhibitor protein (IκB) that masks the NLSs of the subunits (10–13). IκBa preferentially inhibits the nuclear translocation of the p50/p65 heterodimer. Other IκB molecules found in higher vertebrates include IκBβ, IκBε, and Bcl3. All IκB molecules contain ankyrin repeats, which mediate specific interactions with the Rel-homology domains of NF-κB molecules. The C-terminal regions of p100 and p105 proteins also contain ankyrin repeats and they can function as an IκB (2, 4, 14). Crystal structures of most NF-κB Rel domains bound to DNA or IκB have been determined (15–22). p50/p65 heterodimers are activated by the IκB kinase (IKK) complex that phosphorylates IκBa on two N-terminal serine residues (23–29). Phosphorylation triggers polyubiquitination of IκBa, which is then rapidly degraded by the proteasome (29, 30). As a consequence, the NLSs of p50 and p65 proteins are unmasked, and the dimers are translocated into the nucleus where they activate NF-κB responsive genes.

p50/p65 heterodimers and p50 homodimers are considered the most abundant NF-κB types in most cells. p50 homodimer formation has been suggested to take place cotranslationally. During this process p50/p105 intermediates are formed, where the C-terminal ankyrin repeat containing domain of p105 functions as an IκB (IκBγ). Additional post-translational steps regulate p50 homodimer formation (14, 29, 31). Under nonstimulated conditions 10–20% of p105 proteins are processed to form p50 homodimers (32). The affinity between p50 and p65 proteins is stronger than between two p50 proteins, but the actual mechanism of p50/p65 heterodimer formation in the cytoplasm is poorly understood (18, 31). Eukaryotic cells are compartmentalized by the nuclear envelope into the cytoplasm and the nucleus. The nuclear envelope contains nuclear pore complexes (NPCs), which mediate the molecular traffic between the two compartments. The nucleocytoplasmic traffic of large molecules (>25 nm in diameter) is regulated by specific nuclear import and export systems. Proteins that contain classical NLSs are imported into the nucleus by importin α/β heterodimers. Importin α binds to NLSs...
containing proteins, and importin β 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 NPC (33, 34). A classical monopartite NLS consists of a stretch of basic amino acids, arginines and lysines (35, 36). Classical NLSs are found in p50 and p65 (37, 38). Recent studies have shown that some signaling molecules are transported into the nucleus by NLS- and importin-independent processes by associating directly with proteins of the NPC (39).

Six importin α family members have been identified in humans; importin α1 (Rch1, hSRP1α), importin α3 (Qip1), importin α4 (hSRP1γ), importin α5 (hSRP1, NP1), importin α6, and importin α7 (40–45). The crystal structure of two importin α molecules, yeast karyopherin α and mouse importin α2, have been determined (46, 47). Importin α molecules contain a large central domain that consists of 10 tandemly repeated armadillo (arm) motifs, which mediate the interactions with the NLS-containing cargo protein. Each importin α molecule has two potential NLS binding sites that directly interact with the NLS-containing cargo protein. The arm repeats 2–4 comprise the N-terminal NLS binding site and the arm repeats 7–9 the C-terminal NLS binding site (46–48).

We now report that TFN-α-induced nuclear import of NF-κB p50/p65 heterodimers is mediated by importin α3 and importin α4. Importin α3 is also involved in uninduced import of p50 homodimers. Importin α molecules bind to the previously identified NLSs of p50 and p65 proteins. Moreover, by site-directed mutagenesis we show that p50 is bound by the N-terminal and p65 by the C-terminal NLS binding site of importin α3.

MATERIALS AND METHODS

Antibodies—In Western blot analysis rabbit anti-p65 (sc-1095; 1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p50 (H-119; 1:2500; Santa Cruz Biotechnology), anti-iκBα (9242; 1:1000; Cell Signaling Technology, Inc., Beverly, MA) and anti-phospho(serine)-IκBα (9241; 1:1000; Cell Signaling Technology) antibodies were used as suggested by the manufacturer. Anti-importin α1 and α3 antibodies have been described previously (45). In Western blotting second horseradish peroxidase-conjugated goat anti-rabbit (1:2000; Dako, Glostrup, Denmark) immunoglobulins were used.

For immunoprecipitation anti-p50 (sc-1191; 5 μg/reaction; goat polyclonal; Santa Cruz Biotechnology) and anti-p65 (sc-372x; 5 μg/reaction; goat polyclonal; Santa Cruz Biotechnology) immunoglobulins were used.

For confocal laser microscopy rabbit anti-p50 (H-119; 1:50; Santa Cruz Biotechnology) and anti-c-Myc (sc-789; 1:1000; Santa Cruz Biotechnology) or mouse anti-FLAG M5 (1:400; Sigma) and anti-Penta-His (1:50; Qiagen Inc., Valencia, CA) antibodies were used. Secondary antibodies were FITC-labeled sheep anti-rabbit and anti-mouse IgG F(ab')2 fragment (1:100; Roche Applied Science, Mannheim, Germany) and Rhodamine Red-X-labeled goat anti-mouse and anti-rabbit immunoglobulins (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Cells, Media, and Other Reagents—Human A549 lung carcinoma cell line (ATCC, CCL 185) was maintained in continuous culture in minimum Eagle’s medium-α (Invitrogen) supplemented with 0.6 μg/ml penicillin, 80 μg/ml streptomycin, and 10% fetal calf serum (Integro, Zaandam, the Netherlands). Human hepatocellular carcinoma HuH7 (49 cells) were maintained in minimum Eagle’s medium-α with supplements as above. In transfection experiments the cells were cultured in the growth medium supplemented with 2% fetal calf serum. Human tumor necrosis factor-α (TNF-α) was purchased from R&D systems (Abingdon, UK). For cell stimulation 5 ng/ml of TNF-α was used. Leptomycin B (LMB) (10 μg/ml) was kindly provided by Dr. Minoru Yoshida from The University of Tokyo, Japan. Monolayers and suspension cultures of Spodoptera frugiperda Sf9 cells that were used for baculovirus expression were maintained in TNE-FH medium as described previously (50). For in vitro translation 35S-labeled PRO.MIX (~100 Ci/mmole) was used, and it was obtained from Amersham Biosciences (Buckinghamshire, UK).

Plasmids and DNA Manipulations—Escherichia coli-produced GST-importins α1, α3, α5, α7, and β as well as mutants in the arm repeats 3 and 8 of importin α3 have been described previously (48). To create arm repeat 7 mutations to GST-importin α3 we used Quick-Change™ site-directed mutagenesis kit (Stratagene). The primer used was 5’-GAG AAA ATT AAT AAA GGA GCA GTG TTC TCC TTC GCA ATC GCT AGA GAT CAG CAG CAG-3’.

Human p50 and p65 cDNAs in plasmids RecCMV and pCMV were kindly provided by Dr. Jorma Palvimä (University of Helsinki, Helsinki, Finland) and Dr. John Hiscott (McGill University, Montreal, Canada), respectively. To create NLS mutations to p50 and p65 the primers were 5’-CAA AGA TAA AAG AGA AGT GAC GGA GAC GGC TCA GGA GCT CAT GCC CAA TAT TTG C (p50 NLS: K362A, R383A) and 5’-GAT CGT CAC CGG ATT GAG GCA GCT AGG CAA TAT GAC TCT AGC (p65 NLS: K301A, R302A, K303A). To create FLAG-tagged p50 and c-Myc-Has-tagged p65 transient transfection constructs for indirect immunofluorescence and confocal laser microscopy, wild type and mutated cDNAs were modified by PCR to create N- and C-terminal BamHI and BglII sites, respectively, for further cloning into the BamHI site of FLAG-pcDNA3.1 (+) (51) and pcDNA3.1/Myc-His- (Invitrogen) expression vectors. The primers used were 5’-ATA TAT AGA TCT ACC ATG GCA GAT CAT GCA CAT CAA TTG (5’-oligodeoxynucleotide, BglII codons in bold face and initiation codon underlined) and 5’-ATA TAT AGA TCT ACC ATG TCC ATG CTG GAA CTT CAT CCC ACC ATC TAT TGT (5’-oligodeoxynucleotide, BamHI codons in bold face, and initiation codon underlined) and 5’-ATA TAT GGA TCT ACC ATG GAC GAA CTG TCC CCC ATC ATC (5’-oligodeoxynucleotide, BamHI sites in parentheses, ATG and STOP codons underlined, and the newly created BamHI cloning site in bold face). The PCR product was first digested with BglII and then subcloned into the BamHI cloning site of the pkcYMI baculovirus expression vector (50).

Human importin α1, α3, α4, α7, p50, and p65 cDNAs were modified by PCR to create N- and C-terminal BglII (for importin α4 and p50) or BamHI (for importin α3, α7, and α6) sites for further cloning into the BamHI site of pGAP-CMV1 vector. Primers used were: 5’-GCA CAA GGA TCC ACC ATG TCC ACC AAC GAG AAT CAT AC (5’-oligodeoxynucleotide, BamHI codons in boldface and initiation codon underlined) and 5’-GTC TGG TGA TCC TCA AAA GTT AAA GGT CCC AGG ACC CCC (5’-oligodeoxynucleotide, for importin α1, 5’-GCA CAA GGA TCC ACC ATG GCG GAC AAC TGG CAT GAC (5’-oligodeoxynucleotide) and 5’-GTC TGG TGA TCC TTA AAA CTA AAA CTG GAA CCC TTC TGG TAC (5’-oligodeoxynucleotide) for importin α3, 5’-GGA AGC AGA TCT ACC ATG GCC GAG AAC CCC ATG TTG (5’-oligodeoxynucleotide) and 5’-ATA TAT AGA TCT ACC ATG TCC TTA AAA ATT AAA TTG TTC TGT TTG (5’-oligodeoxynucleotide) for importin α5, 5’-GAG AGC AGA TCC ACC ATG GCG GAC AAC TGG CAT GAC (5’-oligodeoxynucleotide) and 5’-ATA TAT GGA TCC TTC TTA GGA GCT CAT GTC AGC GGC GCC (5’-oligodeoxynucleotide) for p50 and 5’-ATA TAT GGA TCC ACC ATG GAC GAA CTG TCC CCC ATC ATC (5’-oligodeoxynucleotide) and 5’-ATA TAT GGA TCC TTC TTA GGA GCT CAT GTC AGC GGC GCC (5’-oligodeoxynucleotide) for p65. Recombinant viruses were obtained as described previously (50).

Production of GST Fusion Proteins in E. coli and Sf9 Cells, and Preparation of Cell Lysate from A549 Cells—Human importins α1, α3, α4, α7, p50, and p65 cells were first infected with importin, p50 or p65-expressing baculoviruses for 42 h and then collected, and whole cell extracts were prepared by disrupting the cells in 1-μl buffer on ice for 10 min. The cells were disrupted by passing them through a syringe. Cell extracts were clarified by Eppendorf centrifugation (13,000 rpm, 10 min).

NF-κB Nuclear Transport by Importin α3 and Importin α4
For preparation of cell lysate, A549 cells were stimulated with TNF-α (5 ng/ml) or left nonstimulated. The cells were washed, harvested, and lysed in l-buffer containing 1 mM NaVO₄ and protease inhibitors on ice for 10 min. The cells were disrupted by passing them through a syringe. Cell debris was removed by centrifugation at 13,000 rpm at 4 °C for 10 min.

**Importin Binding Assay, Immunoprecipitation, SDS-PAGE, and Western Blotting**—For GST pull-down experiments, GST fusion proteins were first allowed to bind to 25 μl of glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences) at +4 °C for 60 min in l-buffer followed by washing twice with the buffer. 25 μl of glutathione-Sepharose-immobilized GST fusion proteins was mixed with 200 μl of cell lysate and rotated at +4 °C for 2 h followed by washing three times with l-buffer. Sepharose beads were dissolved in 30 μl of 2× Laemmli sample buffer, and the proteins were separated on 10% SDS-PAGE. The gels were fixed and stained with Coomassie Brilliant Blue or transferred onto Immobilon-P membranes (polyvinylidene difluoride; Millipore, Bedford, MA) followed by staining with primary and secondary antibodies and visualization of the proteins with the enhanced chemiluminescence system (ECL) (Amersham Biosciences) as recommended by the manufacturer.

To detect the binding of in vitro translated proteins in GST pull-down experiments, 25 μl of in vitro translated p50, p65, or IκBα proteins (TNF-coupled reticulocyte lysate systems, Promega) were allowed to bind to 25 μl of Sepharose-immobilized GST-importin on ice for 60 min followed by washing three times with l-buffer. GST importin-bound 35S-labeled proteins were dissolved in 30 μl of 2× Laemmli sample buffer and separated on 10% SDS-PAGE. The gels were fixed and treated with Amplify reagent (Amersham Biosciences) as specified by the manufacturer and autoradiographed.

For immunoprecipitation experiments, 25 μl of protein A-Sepharose (Amersham Biosciences) was incubated with 5 μg of goat immunoglobulins against NF-κB p50 or NF-κB p65 proteins in l-buffer for 1 h, followed by washing three times with the buffer. Protein A-Sepharose beads were then mixed with 1 ml of A549 cell lysate (stimulated with TNF-α (5 ng/ml) for 30 min or left nonstimulated), rotated at +4 °C for 6 h, followed by washing twice with l-buffer and once with washing buffer (10 mM Tris-Cl, pH 6.8, 1 mM EDTA). Proteins were separated on 12% SDS-PAGE and Western blots were stained with rabbit immunoglobulin against p50, p65, importin α3, and IκBα.

**Oligonucleotide Precipitation**—A549 cells were stimulated with 5 ng/ml of TNF-α for 0, 15, 30, or 60 min. The cells were collected, and samples were treated as described by Rosen et al. (53). Upper strands of CCL5 (RANTES) (5′-ggattctc ttc ccc tta ggg gag gat gcc cct caa ct) and CXCL10 (IP-10) (5′-ggattctc gag gga aat tcc gta act tgg) promoter NF-κB elements were synthesized with BanHI overhangs as spacers, and they were 5′-biotinylated (DNA Technologies Inc., Gaithersburg, MD). Lower strands were nonbiotinylated. Oligonucleotides were annealed in 0.5 mM NaCl and incubated with streptavidinagarose beads (Neutradin; Pierce) at +4 °C for 2 h in a ratio to yield maximum saturation of the beads with the biotinylated oligonucleotide. The beads were incubated with the agaro agarose beads saturated with the oligonucleotide at +4 °C for 2 h in binding buffer containing 10 mM HEPES, 133 mM KCl, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.01% Triton X-100, 0.5 mM dithiothreitol, 1 mM NaVO₄, and protease inhibitors. After washing oligonucleotide-bound proteins were dissolved in 75 μl of 2× Laemmli sample buffer and separated on 10% SDS-PAGE. Proteins were transferred onto Immobilon-P membranes and visualized with antibodies against p50 and p65 proteins.

**Indirect Immunofluorescence and Confocal Laser Microscopy.**—For indirect immunofluorescence and confocal laser microscopy transiently transfected HuH7 cells were grown on glass coverslips. Cells were left untreated or pretreated with LMB (2 ng/ml) (54) for 20 min followed by stimulation with TNF-α (100 ng/ml) in the presence of LMB (2 ng/ml) for 30 min. The cells were fixed with methanol at −20 °C for 10 min and processed for immunofluorescence as previously described (55). The cells positive for FLAG, c-Myc, and His tags or expressing native p50 protein, as indicated in the figures, were visualized and photographed on a Leica TCS NT confocal microscope.

**RESULTS**

**NF-κB Binds to Importin α3 and Importin α4.**—Upon activation NF-κB is translocated from the cytoplasm into the nucleus. The nucleocytoplasmic shuttling of NF-κB has been thought to be mediated via importin β/ and exportin 1-dependent pathways. However, the molecular mechanisms of the nuclear import of NF-κB have remained unknown. To investigate the possible interactions of NF-κB with different importin isoforms, we stimulated human A549 lung carcinoma cells with TNF-α. Different expression systems were used for importin α3, since all isoforms could not be expressed in E. coli or by baculovirus expression. Cell extracts were prepared, and the cellular proteins were allowed to bind to Sepharose-immobilized bacterially expressed GST-importins α1, α3, α5, α7, or β (A) or baculovirus-expressed GST-importins α1, α3, or α7 (B) at +4 °C for 2 h. Sepharose-bound proteins were dissolved in Laemmli sample buffer followed by SDS-PAGE and Western blotting with anti-p50 or anti-p65 antibodies. A similar gel was also stained with Coomassie Blue to visualize the amount of Sepharose-immobilized GST-importin isoforms.

**To determine the specificity of anti-importin antibodies bacterially expressed GST-importins α1, α3, α5, α7, or β (A) or baculovirus-expressed GST-importins α1, α3, or α7 (B) at +4 °C for 2 h, Sepharose-bound proteins were dissolved in Laemmli sample buffer followed by SDS-PAGE and Western blotting with anti-p50 or anti-p65 antibodies.**

**D** cell extracts were prepared from cultured A549 cells, and the proteins in the cell extracts were allowed to bind to Sepharose-immobilized GST-p50 and GST-p65 proteins (first lane shows uninfected 5/9 cell extract bound to Sepharose as a control). Sepharose-bound proteins were dissolved in Laemmli sample buffer followed by SDS-PAGE and Western blotting with anti-importin α1 and α3 antibodies.

**FIG. 1.** TNF-α-activated NF-κB binds to importin α3 and α4. A and B, cultured A549 cells were stimulated with 5 ng/ml of TNF-α for 30 min or left untreated as indicated. Cell extracts were prepared, and the proteins in the cell extracts were allowed to bind to Sepharose-immobilized bacterially expressed GST-importins α1, α3, α5, α7, or β (A) or baculovirus-expressed GST-importins α1, α3, or α7 (B) at +4 °C for 2 h. Sepharose-bound proteins were dissolved in Laemmli sample buffer followed by SDS-PAGE and Western blotting with anti-p50 or anti-p65 antibodies. A similar gel was also stained with Coomassie Blue to visualize the amount of Sepharose-immobilized GST-importin isoforms. C, to determine the specificity of anti-importin antibodies bacterially expressed GST-importins α1, α3, α5, and α7 were separated on SDS-PAGE followed by Western blotting with anti-importin α1 and α3 antibodies. D, cell extracts were prepared from cultured A549 cells, and the proteins in the cell extracts were allowed to bind to Sepharose-immobilized GST-p50 and GST-p65 proteins (first lane shows uninfected 5/9 cell extract bound to Sepharose as a control). Sepharose-bound proteins were dissolved in Laemmli sample buffer followed by SDS-PAGE and Western blotting with anti-importin α1 and α3 antibodies.
extracts were allowed to bind to Sepharose-immobilized GST-p50 and GST-p65 proteins followed by Western blotting for the presence of importin α3 and α4. We did not observe any importin α1 binding to GST-p50 or GST-p65.

We wanted to verify these in vitro results with in vivo experiments made by immunoprecipitation. Cultured A549 cells were stimulated with TNF-α or left nonstimulated. Cell extracts were prepared and the proteins in the cell extracts were allowed to bind to Protein A Sepharose-immobilized anti-p50 (A) or anti-p65 (B) immunoglobulins. Bound proteins were analyzed by Western blotting for the presence of p50, p65, importin α3, and IκBα. As is shown in Fig. 2, importin α3 coprecipitated with p50 and p65 only after TNF-α stimulation when the complex was free of IκBα.

Kinetics of TNF-α-induced p50/p65 Activation—Inactive p50/p65 heterodimers preexist in the cytoplasm associated with their specific inhibitory molecule IκBα. A diverse range of stimuli, including TNF-α induction, results in serine phosphorylation of IκBα and its subsequent degradation followed by translocation of the free p50/p65 heterodimer into the nucleus and activation of genes containing NF-κB binding sites. It has been suggested that in nonstimulated cells NF-κB/IκBα complexes are shuttling between the nucleus and cytoplasm (56–59). It has been proposed that this shuttling is mediated by the NLS of p50 that would be exposed in the NF-κB/IκBα complex (60). To investigate the kinetics of NF-κB binding to importin α3, we stimulated A549 cells with TNF-α for different time periods (Fig. 3). Whole cell extracts were prepared, and the proteins in the cell extracts were analyzed by Western blotting with anti-p50, anti-p65, anti-IκBα and anti-phospho(Thr32)-IκBα (P-IκBα) antibodies. The protein levels of p50/p105 and p65 remained constant throughout the experiment (Fig. 3A). IκBα was degraded rapidly after TNF-α induction. IκBα levels started to decrease at 1 min after TNF-α stimulation, and the protein was completely degraded at 8 min after TNF-α stimulation. The appearance of the phosphorylated form of IκBα was also a very rapid phenomenon. After 1 min of TNF-α stimulation the phosphorylation of IκBα was detectable and it was strongly phosphorylated after 4 min of induction. The phosphorylated form of IκBα disappeared after 8 min of TNF-α stimulation due to the degradation of IκBα (Fig. 3A). To further investigate the ability of NF-κB to bind to importin α3, we prepared whole cell extracts of A549 cells and analyzed by Western blotting for the presence of p50 and p65. As shown in Fig. 3B, p65 binding to importin α3 was seen only after IκBα was degraded (≥8 min) from the NF-κB/IκBα complexes.

Importin α3 bound small amounts of p50 also under nonstimulated conditions (Figs. 1A and 3B). The complex containing p50 under nonstimulated conditions did not include p105 (Fig. 3B). A similar gel as in Fig. 3B was also stained with anti-IκBα antibodies but no IκBα was detectable in the protein complexes bound to importin α3 (results not shown, Fig. 5). This suggests that the p50 protein bound to importin α3 under nonstimulated conditions is in the form of free p50 homodimer. No p65 binding to any importin α isoform under nonstimulated conditions was seen (Figs. 1A and 3B).

To see whether p50 homodimers were also able to bind DNA under nonstimulated conditions we carried out oligonucleotide precipitation experiments using NF-κB promoter elements. A549 cells were stimulated with TNF-α for 0, 15, 30, and 60 min. Cellular proteins were precipitated with oligonucleotides containing NF-κB binding sites from CCL5 and CXCL10 genes followed by Western blotting with anti-p50 and anti-p65 antibodies. p65 containing dimers were bound to NF-κB promoter elements only after TNF-α stimulation, but small amounts of p50 homodimers were also bound under nonstimulated conditions (Fig. 4). These data are well in line with the experiments described in Figs. 1A, 3B, 5A, and 7C.

IκBα Inhibits the Binding of p65 Homodimers and p50/p65 Heterodimers but Not p50 Homodimers to Importin α3—To further characterize NF-κB binding to importins α3 and α1, we used in vitro translated p50 and p65 proteins in GST-importin binding experiments. In vitro translated p50 homodimers are...
rather stable while p65 homodimers dissociate more easily. Stable p50/p65 dimers are formed when mRNAs encoding p50 and p65 are cotranslated (31).

First, we wanted to ensure that in vitro translated proteins behave in a similar fashion to native TNF-α-activated NF-κB proteins in importin α binding experiments (Fig. 1). We observed that also in vitro translated p50 homodimers bound strongly to importin α3 and to a lesser extent to importin α1 (Fig. 5A). p65 homodimers were bound strongly to importin α3 but no marked binding to importin α1 was seen (Fig. 5A).

Because it has previously been shown that IκBα is also found in the nucleus, we wanted to study whether IκBα uses the importin α/β import machinery and binds to importins. As is seen in Fig. 5A, no IκBα binding to any GST-importins was detected, which support the previous findings that IκBα is not transported into the nucleus by the classical importin α/β pathway. Nuclear import of IκBα may be mediated through direct interactions with components of the NPC (61), or it is transported into the nucleus by a piggy-back mechanism with NLS-containing proteins (62).

Because it is well-known that IκBα inhibits nuclear translocation of NF-κB, we wanted to analyze whether IκBα could inhibit the binding of NF-κB in our importin α3 binding assay. IκBα preferentially binds to p50/p65 heterodimers and with slightly lower affinity to p65 homodimers and with significantly lower affinity to p50 homodimers (63). As shown in Fig. 5B, IκBα inhibited the binding of p65 homodimers but not p50 homodimers to importin α3. IκBα also inhibited the binding of p50/p65 heterodimers to importin α3 (p50+p65 in Fig. 5B).

IκBα was not found in a complex with the NF-κB dimers that were bound to Sepharose-immobilized GST-importin α3 (Fig. 5B). This, together with the data in Figs. 2 and 3, imply that the NF-κB complexes that are bound to importin α3 and are further transported into the nucleus are free of IκBα.

Classical NLSs of p50 and p65 Mediate Interactions with Importins α3 and α1—p50 and p65 proteins have been shown to contain arginine/lysine-rich NLSs (37, 38). To study whether these elements are involved in direct binding of p50 and p65 proteins to importins α3 and α1, we carried out binding experiments with E. coli-produced GST-importins and in vitro translated p50 and p65 proteins. In vitro translated [35S]methionine-labeled wild-type or NLS mutant p50 (K362A, R363A) and p65 (K301A, R302A, K303A) were allowed to bind to Sepharose-immobilized GST-importins α3 or α1 followed by identification of importin bound p50 and p65 by SDS-PAGE and autoradiography. Wild-type p50 and p65 proteins bound to importins α3 and α1 whereas NLS-mutated p50 and p65 completely failed to bind to either importin α isoform (Fig. 6, A and B). To confirm the biochemical observations also in cultured cells the nuclear import of the wild-type and NLS-mutated p50 and p65 was studied in transiently transfected HuH7 cells by indirect immunofluorescence microscopy. As seen in Fig. 6C, wild-type p50 and p65 were transported into the nucleus whereas NLS-mutated proteins were not. These data show that the NLSs of p50 and p65 mediate the binding of NF-κB to importins α3 and α1 and its subsequent nuclear localization. p65 is known to exhibit a strong CRM1-dependent nuclear export signal (NES), which mediates its transport back to the cytoplasm. An inhibitor specific for CRM1-dependent export, LMB was used in Fig. 6C to inhibit the nuclear export of p65. p50 Is Bound to the N terminus and p65 to the C-terminal NLS Binding Site of Importin α3—Importin α3 are relatively well conserved molecules (Fig. 7A) that contain 10 arm repeats, which mediate the interactions with NLS-containing proteins. N-terminal arm repeats 2–4 have been considered as the major NLS binding site and C-terminal arm repeats 7–9 have been referred to as the minor NLS binding site (46, 47) (Fig. 7B). An alignment of the arm repeats comprising the N- and C-terminal NLS binding sites shows that the tryptophan and asparagine residues are conserved in all arm repeats except arm 9. The primary sequence of the surrounding residues is not conserved between the arm repeats although the arm repeats are conserved in sequence and order when human importins are compared with each other (Fig. 7, A and B). To further characterize the mechanism of NF-κB binding to importin α3 molecule, we created mutations in the N-terminal and C-terminal NLS binding sites of importin α3. Proteins in TNF-α-stimulated or non-stimulated A549 cell extracts were allowed to bind to wild type or arm 3- or arm 7-8-mutated importin α3 molecules followed by analysis of bound p50 and p65 by Western blotting. Mutations in the arm repeat 3 had no effect on p65 binding, whereas mutations in the arm repeats 7+8 lead to an almost complete inhibition of p65 binding (Fig. 7C). Unlike in the case of p65, arm repeat 3 mutations in importin α3 completely prevented
p50 binding whereas mutations in the arm repeats 7+8 had no effect. To confirm the results obtained by cellular TNF-α-stimulated NF-κB proteins, we used in vitro translated p50 and p65 proteins and the arm repeat mutants in Importin α3 binding experiments. In this experiment also an arm 3+8 mutated importin α3 was used. As is seen in Fig. 7D in vitro translated p50 and p65 gave identical results with the TNF-α-stimulated cellular p50 and p65 proteins. Arm repeat 3 mutation in Importin α3 prevented the binding of both p50, and mutations in arm repeats 7+8 prevented the binding of p65. Mutations in arm repeats 3+8 in importin α3 prevented the binding of both proteins. These results show that the N-terminal arm repeats of importin α3 form the binding site for p50 whereas p65 is bound to the C-terminal NLS binding site.

**NLS Defective p65 Does Not Have Any Effect on the Distribution of Cellular p50**—It has been suggested that p50 and p65 proteins have their own independently functioning NLSs (37, 38). Here we have shown that these NLSs mediate p50 and p65 interactions with importin α3 (and importin α1) and regulate their nuclear import (Figs. 1, 2, 5, 6, and 7). Using confocal laser microscopy we compared the effects of transfected wild-type and NLS-mutated p65 protein on cellular location of p50. LMB was used to prevent the nuclear export of p65. When cells were transfected with wild-type or NLS-mutated p65 the distribution of the cellular p50 remained the same. p50 was mainly nuclear and showed a granular expression pattern both in TNF-α-stimulated and in untreated cells (Fig. 8). Transfected wild-type p65 was mainly cytoplasmic and after TNF-α stimulation it colocalized with p50 in the cell nucleus (Fig. 8). NLS-mutated p65 remained cytoplasmic also after TNF-α stimulation (Fig. 8). This suggests that a significant portion of p50 homodimers is transported into the nucleus independently of p65 and transport-deficient p65 (NLS-) cannot function as a dominant negative for nuclear import of p50 under these circumstances.

**DISCUSSION**

*Importin α3 and Importin α4 Are Novel Members of the NF-κB Signal Transduction Pathway*—Translocation of NF-κB transcription factors from the cytoplasm into the nucleus is a critical step in their signal transduction pathway. NF-κB contains classical NLS motifs, and they are imported into the nucleus via the importin α/β pathway. In the present study we show strong evidence that TNF-α-activated NF-κB p50/p65 heterodimers are transported into the nucleus preferentially by importin α3 and importin α4. Importin α3 also seems to be involved in the nuclear import of p50 homodimers in uninduced cells. It is shown that the previously identified NLSs of p50 and p65 molecules are the direct targets for importin α binding. In our study also bacterially expressed importin α1 bound to NF-κB after TNF-α stimulation although to a lesser extent than importin α3. This binding was mediated by the NLSs of p50 and p65 proteins. However, no binding of baculovirus-expressed importin α1 to NF-κB was detected under the same circumstances.
**Fig. 7.** p50 binds to the N-terminal and p65 to the C-terminal NLS binding site of importin α3. A, relationships between the six human importin α isoforms. Human importin α isoforms can be grouped into three subfamilies (shaded). The dendrogram was generated using the PILEUP program in the GCG software package (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI). Protein sequences of the importin α arm domains were used in the comparison. The GenBank™ accession numbers are as follows: importin α1 (P52292), α3 (O00629), α4 (O00505), α5 (P52294), α6 (O15131), and α7 (AF060543). B, an alignment of human importins α1, α3, α4, α5, and α7 arm domains that are involved in NLS binding (upper panel). Identical amino acids are shown in blue. Locations of the conserved tryptophan (W) and asparagine (N) residues in each arm repeat are shown by yellow vertical lines. A representation of importin α arm repeat domain (middle panel). The amino acids supposedly interacting with NLSs are shown in purple. In the lower panel the p65 homodimer dimerization domain is shown in a complex with (left) and without (right). The amino acids forming p65 NLSs are shown in different colors. DNA is shown as a black circle. The representations were done using the program Protein Explorer. Protein Data Bank accession numbers are 1BK5 (importin α1) and 1K3Z (p65). C, A549 cells were left untreated or stimulated with 5 ng/ml of TNF-α for 30 min. Cell extracts were prepared, and the proteins in the cell extracts
NF-κB Nuclear Transport by Importin α3 and Importin α4

Moreover, we did not observe any binding of cellular importin α1 to GST-p50 or GST-p65 proteins. On the basis of these findings, it cannot be predicted if importin α1 is specifically and significantly involved in NF-κB nuclear transport.

Importin α isoforms are expressed in the same cells and tissues, but they may have distinct substrate specificities. It is likely that the whole NLS binding groove of importin α contributes to the specificity of target protein binding, not only the few critical amino acids directly interacting with NLS. Similarly, the flanking sequences of the NLS contribute to the importin α binding affinity and specificity. For example, SV40 T antigen NLS peptide binds to all importin isoforms (40–45), whereas the full-length T antigen binds primarily to importin α3 and to a lesser extent to importin α1 (48).

Importin α molecules have two NLS binding sites that directly interact with the NLS of the cargo. Arm repeats 2–4 comprise the N-terminal NLS binding site and arm repeats 7–9 the C-terminal NLS binding site. We have previously shown that importin α3 is able to use either its N- or C-terminal binding sites for binding different nucleus-targeted proteins. Influenza A virus nucleoprotein is bound to the C-terminal NLS binding site whereas simian virus 40 large T antigen is bound to the N-terminal NLS binding site (48). Our present study demonstrates another variation in theme, since NF-κB p50 and p65 molecules are bound by different NLS binding sites of importin α3, p50 is bound by the N-term and p65 by the C-terminal NLS binding site.

It is an intriguing possibility that p50 and p65 NLSs may be bound simultaneously to the different NLS binding sites of the same importin α molecule (Fig. 9). This kind of binding strategy may stabilize the complex during nuclear import. However, the question arises how p50 homodimers are bound to importin α because p50 NLS seems to be bound only by the N-terminal binding site.

Importin α3 and α4 belong to the same importin subfamily and display a high degree of sequence homology (45) (Fig. 7, A and B). We believe that importin α4 can function much in the same way as NF-κB interactions as importin α3. It is likely that these importins also have distinct substrate specificities (45, 64).

Like NF-κB, signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors activated by cytokines. Upon activation STATs are phosphorylated, dimerized, and transported into the nucleus by the classical importin α/β pathway. In our previous study we showed that STAT1 and STAT2 contain nonclassical NLSs that become operative only in dimers. The NLSs of both partners of a STAT dimer have to be intact for nuclear import to take place (65). STAT1 homodimers and STAT1/STAT2 heterodimers bind to the C-terminal NLS binding site of importin α5 and two importin α molecules are needed to transport the dimer into the nucleus (48, 66). In contrast to STATs it seems that in NF-κB dimers each NLS may function independently (37). However, it has also been suggested that both partners of a Rel homodimer require an intact NLS for proper nuclear import (67).

Are IκB-bound NF-κB Dimers Shuttling between the Cytoplasm and Nucleus?—Since p65 possesses both an NLS and a strong NES, IκB free p50/p65 heterodimers and p65 homodimers are continuously transported into and out of the nucleus. In the presence of leptomycin B, an inhibitor of CRM1-dependent nuclear export, p65 containing dimers accumulate in the nucleus (57–59). IκBα contains its own unconventional NLS that is masked when the protein is bound to NF-κB dimers (16, 17, 68). When IκBα is not bound to NF-κB dimers it can shuttle between the cytoplasm and the nucleus by means of its NLS and NES.

It has been suggested by several groups that in uninduced cells NF-κB/IκBα complexes continuously shuttle between the cytoplasm and the nucleus (56–60). According to our results importin α3 does not bind to NF-κB dimers when they are associated with IκBα. Our data suggest that NF-κB and IκBα are not transported into the nucleus as a complex. Our data rather support the previous findings that in resting cells cytoplasmic dissociation of the NF-κB/IκBα complex is followed by nuclear import of the single subunits rather than the complex as a whole (69, 70). After re-association of NF-κB and IκBα in the nucleus, the complex is then transported back to the cytoplasm. This transport is mediated by IκBα (56, 57), or by the inherent NES of p65 (71).

We found that small amounts of p50 can associate with importin α3 under uninduced conditions. This form of p50 was free of IκBγ (the C-terminal-half of p105). In uninduced cells p105 is partially degraded, generating p50 (14, 32, 72, 73). We assume that the basal binding of p50 to importin α3 detected is because of the continuous degradation of the p105, which generally would not bind to IκBα.

were allowed to bind to Sepharose-immobilized wild type or arm repeat 3 or 7 + 8 mutants of GST-importin α3 at +4°C for 2 h. The binding experiments were carried out as described in the legend for Fig. 1. Western blots were stained with anti-p50 and anti-p65 antibodies as indicated. A similar gel was also stained with Coomassie Blue to visualize the amount of GST-importin α3. D, in vitro translated p65 (upper panel) and p50 (middle panel) or p50 + p65 (lower panel) were allowed to bind to Sepharose-immobilized wild type or arm repeat 3, 7 + 8, or 3 + 8 mutants of GST-importin α3. Sepharose-bound proteins were dissolved in Laemmli sample buffer and separated on 10% SDS-PAGE. An autoradiogram of [35S]methionine-labeled p50 and p65 proteins is shown. In vitro translation products (c) were used as controls. Coo massie Blue-stained gel is shown to visualize the amount of GST-importin α3.
of p50/p65 heterodimers to importin α3? It is possible that when IkBα is degraded from the p65/p105 dimer the subsequent p50/p65 heterodimer rapidly binds IkBα (or IkBβ). After that, the continuous shuttling of p50/p65 may occur only after IkBα dissociation is taking place (69, 70). Nearly all of the studies on the accumulation of NF-κB and IkBα in the nucleus under uninduced conditions depend on the use of LMB for relatively long time periods. This indicates that the shuttling is a slow phenomenon and was not detectable in our GST-importin interaction experiments. Unlike p50/p65 heterodimers, p50 homodimers are not effectively regulated by IkBs other than IkBα. Thus, the continuous degradation of p105 associated with p50 could provide the resting cell the small amount of p50 homodimers required. A conceptual model of NF-κB transport into the nucleus is presented in Fig. 9.

The shuttling of the p50/p65-IkBα complex has been suggested to be mediated by the free NLS of p50 (60). However, p50/p50-IkBα, p50/p65-IkBβ, and p50/p65-IkBβ complexes do not shuttle between the cytoplasm and nucleus. It has been suggested that the cytoplasmic retention of these complexes could involve additional factors that mask their exposed NLSs (73). Alternatively, it is possible that when IkB is bound to NF-κB, importin molecules simply do not fit to interact with the free NLS.

Taken together, our data allow us to conclude that NF-κB binds to importin α3 and importin α4 and is transported into the nucleus only when it is free of IkB molecules. In the future, structural analysis of NF-κB bound to importin molecules is needed to reveal the exact mechanism of interaction between these molecules.

Acknowledgments—We thank Dr. Tapani Hovi for critically reading the manuscript and Drs. Jorma Palvimo and John Hiscott for the human p50 and p65 cDNAs. We also thank Mari Aaltonen for excellent technical assistance and Sinikka Sopanen and Raija Tyni for providing us with the cells.

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