Arginine/Lysine-rich Nuclear Localization Signals Mediate Interactions between Dimeric STATs and Importin α5*

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Interferon stimulation results in tyrosine phosphorylation, dimerization, and nuclear import of STATs (signal transducers and activators of transcription). Proteins to be targeted into the nucleus usually contain nuclear localization signals (NLSs), which interact with importin α. Importin α binds to importin β, which docks the protein complex to nuclear pores, and the complex translocates into the nucleus. Here we show that baculovirus-produced and -activated STAT1 homodimers and STAT1-STAT2 heterodimers directly interacted with importin α5 (NPI-1). This interaction was very stable and was dependent on lysines 410 and 413 of STAT1. Only STAT dimers that had two intact NLS elements, one in each monomer, were able to bind to importin α5. STAT-importin α5 complexes apparently consisted of two STAT and two importin α molecules. STAT NLS-dependent colocalization of importin α5 with STAT1 or STAT2 was seen in the nucleus of transfected cells. γ-Activated sequence DNA elements efficiently inhibited STAT binding to importin α5 suggesting that the DNA and importin α binding sites are close to each other in STAT dimers. Our results demonstrate that specific NLSs in STATs mediate direct interactions of STAT dimers with importin α, which activates the nuclear import process.

Signal transducers and activators of transcription (STATs) are latent transcription factors that are activated by cytokines and certain growth factors. Presently seven mammalian STAT proteins have been described. Binding of cytokines to their specific cell surface receptors leads to the activation of the Janus tyrosine kinase (JAK)-STAT pathway (1, 2). In response to type I IFN (IFN-α/β) stimulation, IFN-α/β receptor-associated JAK1 and Tyk2 are phosphorylated and activated (2–4). Activated JAKs in turn tyrosine-phosphorylate STAT-associated JAK1 and Tyk2 are phosphorylated and activated (2–4). Activated JAKs in turn tyrosine-phosphorylate STAT1 and STAT2 at Tyr-701 and Tyr-690, respectively, which results in dimerization and nuclear translocation of STAT1-STAT2 heterodimers. In the nucleus STATs interact with IRF-9/p48 protein to form ISGF3 complexes, which bind to well conserved interferon-stimulated response elements in the promoter regions of IFN-α/β-responsive genes and activate transcription (5–8). Binding of type II IFN (IFN-γ) to its receptor leads to the activation of JAK1 and JAK2 and tyrosine phosphorylation of STAT1 (also at Tyr-701). Activated STAT1 forms homodimers, which translocate into the nucleus and bind to GAS elements and activate transcription of IFN-γ-inducible genes (1, 2). Although the structure-function relationships of STATs have been carefully analyzed, the mechanisms of nuclear import of this important group of transcription factors have remained less well characterized. Recently we and others have shown that STAT1 and STAT2 have an arginine/lysine-rich nuclear localization signal (NLS) that mediates their nuclear translocation in dimeric complexes (9, 10).

Active nuclear transport of large macromolecules occurs via the nuclear pore complex (11). Proteins to be imported into the nucleus usually contain a mono- or bipartite basic-type NLS, which binds to a specific NLS receptor, importin α (12–14). The N-terminal importin β binding (IBB) domain of importin α interacts with importin β (15), which mediates the docking of NLS-containing cargo-importin α/β complex to the cytoplasmic side of the nuclear pore, and the complex translocates into the nucleus (16, 17). Inside the nucleus RanGTPase is involved in the disassembly of the cargo-importin complex (14, 18, 19). IFN-γ-induced nuclear import of STAT1 has been suggested to be dependent on one importin α subtype, importin α5 (20), and the RanGTPase (21). However, the elements that regulate STAT-importin α5 interactions have remained elusive.

In the present work we show, by using a baculovirus-reconstituted STAT activation system, that homodimeric STAT1 or heterodimeric STAT1-STAT2 complexes directly interact with importin α5. The interaction of STAT dimers with importin α is very stable and is dependent on NLS situated in the DNA binding domain of STATs. The STAT-importin α5 complex consists of two importin α and two STAT molecules. STAT-binding GAS oligonucleotides efficiently prevent the binding of dimeric STATs with importin α. We also demonstrate by confocal microscopy that wild type STATs colocalize with importin α, whereas NLS-mutated STATs do not.

MATERIALS AND METHODS

Cells—Monolayers and suspension cultures of Spodoptera frugiperda Sf9 cells that were used for baculovirus expression were maintained in TNM-FH medium as described previously (22). Human hepatocellular carcinoma HuH7 (23) cells were maintained in minimal essential medium supplemented with 0.6 mg/ml penicillin, 60 mg/ml streptomycin, 2 mM glutamine, 20 mM HEPES buffer, pH 7.4, and 10% fetal calf serum (Integro, Zaandam, the Netherlands). In transfection experiments the cells were cultured in the growth medium supplemented with 2% fetal calf serum.

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§ The abbreviations used are: STAT, signal transducers and activators of transcription; IFN, interferon; NLS, nuclear localization signal; GAS, γ-activated sequence; JAK, Janus tyrosine kinase; IBB, importin β binding; NP, nucleoprotein; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; wt, wild type; FPLC, fast protein liquid chromatography; arm, armadillo; IP, immunoprecipitation.
Antibodies—In Western blot analysis rabbit anti-Tyk2 (H-135, 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-STAT1 (c-24, 1:20,000; Santa Cruz Biotechnology), rabbit anti-STAT2 (c-20, 1:2000; Santa Cruz Biotechnology), rabbit anti-phosphotyrosine (PY99, 1:200; Santa Cruz Biotechnology), and mouse monoclonal anti-phospho-tyrosine (Y701) (Y701, 1:2000; Sigma) were allowed to bind to Sepharose-immobilized GST-importin a in IP buffer using a 24-ml Superose 12 fast protein liquid chromatography (FPLC) (Amersham Biosciences) gel filtration column. To study STAT a complex formation STAT protein-containing baculovirus cell extracts were mixed with E. coli cell extracts containing GST-importin a. Proteins in gel filtration fractions were separated by SDS-PAGE and transferred to nitrocellulose filters followed by staining with anti-GST, anti-STAT1, anti-phospho-STAT1, and anti-phosphotyrosine antibodies as described above. To estimate the relative amounts of STAT and importin a proteins in STAT1/STAT2-importin a complexes, the proteins in gel filtration fractions were separated by SDS-PAGE followed by Coomassie Blue staining. Quantitation of Coomassie Blue-stained protein bands was carried out with the Kodak densitometry and analysis system 120. The MW-GF-200 kit containing activated STAT1 proteins followed by STAT1 binding to Sepharose-immobilized GST-importin a. Sepharose-importin a-bound proteins were boiled in Laemmli sample buffer, separated by SDS-PAGE, and analyzed by Western blotting as described above. Gel Filtration and Protein Oligomerization Analysis—Baculovirus-infected Sf9 cells or E. coli lysates were gel filtrated in the above lysis buffer for 30 min at room temperature. Cell extracts containing STAT proteins were separated by a Sephacryl S-200 column following gel filtration. Protein samples were analyzed by Western blotting using antibodies as described above.

Expression and Tyrosine Phosphorylation of STAT Proteins in Baculovirus System—To study possible interactions of wild type or NLS-mutated STATs with importins we used a baculovirus expression system to reconstitute the STAT activation system. We created a Tyk2 baculovirus construct that was found to express Tyk2 protein in relatively high levels. Coinfection of Sf9 cells with recombinant Tyk2 baculovirus and STAT1, STAT2, or NLS mutant STAT1 protein-expressing baculoviruses resulted in efficient expression and tyrosine phosphorylation of wt STAT1, wt STAT2, and STAT1 Y701A mutant protein completely lacked tyrosine phosphorylation (Fig. 1). Tyrosine-phosphorylated wt STAT1 or STAT1 Y701A formed dimers (result not shown and Ref. 9), which enabled us to analyze potential interactions of dimeric STAT complexes with importins.

Binding of STAT1 Homodimers or STAT1-STAT2 Heterodimers to Importin a Is Regulated by a Lysine-rich NLS of STAT1—IFN-induced nuclear import of STAT1 has been suggested to be mediated by importin a (20). We recently proposed that STAT proteins have a well conserved arginine/lysine-rich NLS in their DNA binding domain that regulates their nuclear import (9). To study whether this element is involved in direct binding of STATs to importins in vitro we carried out binding experiments with E. coli-produced GST-importin a fusion proteins and baculovirus-expressed STAT proteins. WT or mutant STAT baculovirus-expressed STAT proteins were expressed as N- or C-terminally with Tyk2 baculovirus construct, cell extracts were contained in STAT1/STAT2-importin a complexes. The proteins in gel filtration fractions were separated by SDS-PAGE and analyzed by Western blotting as described above.
modimeric STAT1 K410A,K413A, or heterodimeric STAT1 K410A,K413A-STAT2 complexes completely failed to bind to importin α5 (Fig. 2). Tyrosine-phosphorylated STAT2 was also devoid of importin α5 binding activity suggesting that STAT2 can only dimerize with STAT1. No binding of STATs to importin α5 was seen (see Fig. 7 and results not shown).

To study the stability of STAT-importin α5 complex we washed Sepharose-bound importin α5-STAT complexes with buffers containing high concentrations of NaCl or urea. Some reduction in the amounts of importin α5-bound STAT1 or STAT1/STAT2 dimers was seen after washing with 1 or 2 M NaCl, whereas 2 M urea was not able to disrupt the STAT-importin α complex (Fig. 3). In higher urea concentrations Sepharose-bound STATs were released, but apparently this was due to the release of GST-importin α fusion protein from the resin (Fig. 3). Influenza A virus NP that is known to bind to importin α5 (26) was used as a positive control in binding stability experiments.

Co-localization of STATs with Importin α—Biochemical evidence suggested that STAT1 binding to importin α5 takes place via lysine residues at positions 410 and 413 of the STAT1 protein. To study whether the arginine/lysine-rich NLS of STAT1 or STAT2 regulates STAT interactions with importin α5 also in cultured cells we carried out co-localization experiments with transfected STAT and importin α5 gene constructs. We used confocal laser microscopy to analyze the colocalization of wt and NLS-mutated STATs in transfected human HuH7 hepatoma cells. In transiently transfected and IFN-α-treated (1000 IU/ml, 45 min) cells tyrosine-phosphorylated STAT1 clearly colocalized with STAT2 in the cell nucleus (Fig. 4). When nuclear import-defective STAT1 K410A,K413A or STAT2 R409A,K415A were cotransfected with heterologous wt STAT gene constructs no IFN-α-induced nuclear accumulation of STATs was seen. However, mutant and wt STAT forms colocalized in the cell cytoplasm (Fig. 4) suggesting that STAT1 and STAT2 directly interact with each other. The data indicates that NLS-defective STAT1 functions as a dominant negative for nuclear import of wt STAT2 and vice versa.

Next we transfected HuH7 cells with importin α5 and wt or NLS-mutated STAT gene constructs. Transfected cells were
treated with IFN-α for 45 min, and the cells were fixed and stained with STAT- and importin α5-specific antibodies. wt STAT1 or STAT2 was found to colocalize with importin α5 especially in the cell nucleus. Such a colocalization was not observed between importin α5 and NLS-mutated STAT1 K410A,K413A proteins (Fig. 5) suggesting that STAT1-importin α5 interaction is mediated by lysines 410 and 413 of STAT1 also in living cells. Similar results were observed with NLS-mutated STAT2 R409A,K415A protein. While in IFN-α-stimulated cells wt STAT2 was transported into the nucleus with intrinsic STAT1, the NLS-mutated STAT2 was not (Fig. 5).

**Comigration of Importin α5-STAT Complexes in Gel Filtration**—As shown above we have demonstrated that dimeric STATs bind to importin α5 in an NLS-dependent manner (Fig. 2). To estimate the molecular size and protein composition of importin α5-STAT complexes we carried out comigration experiments using gel filtration. We mixed Tyk2-activated STAT cell extracts with E. coli-produced importin α5 and analyzed the migration pattern of these complexes by FPLC using a Superose 12 gel filtration column. First GST-importin α5 (by itself) was subjected to gel filtration analysis, and it was found in fractions corresponding to 70–90-kDa proteins (Fig. 6A) indicating that importin α existed as a monomer. Unphosphorylated STAT1 and phosphorylated STAT1 dimers eluted in the range of 90 and 160–200 kDa, respectively (Fig. 6A). To estimate the size of importin α5-STAT1/STAT2 complex we allowed phosphorylated STAT1/STAT2 dimers to bind to Sepharose-immobilized GST-importin α5 followed by washing and elution of the proteins from the resin by glutathione. Purified importin α5-STAT1/STAT2 complex was analyzed by gel filtration, and it was found to elute in fractions correspond-
produced GST-importin α5 protein-expressing recombinant baculoviruses. After 42 h the cells were collected, cell extracts were prepared, and protein samples were subjected to a Superose 12 gel filtration column by themselves or after binding the proteins to Sepharose-immobilized E. coli-produced GST-importin α5. The gel filtration profile of GST-importin α5 was also analyzed. Samples from gel filtration fractions were subjected to 8% SDS-PAGE followed by Western blot analysis with anti-GST, anti-STAT1, anti-phospho(tyrosine)-STAT1, or anti-phosphotyrosine antibodies as shown in the figure. A, gel filtration patterns of Sf9 cell-produced monomeric STAT1 (unphosphorylated, expressed alone) and phosphorylated STAT1 dimers (coexpressed with Tyk2) and E. coli-produced GST-importin α5 protein. B, gel filtration pattern of GST-importin α5-STAT1/STAT2 complexes. STAT1/STAT2 complexes were allowed to bind to immobilized importin α5. Glutathione-Sepharose-bond GST-importin α5-STAT1/STAT2 complexes were released by glutathione treatment followed by analysis on a Superose 12 gel filtration column. Protein samples in gel filtration fractions were separated by 8% SDS-PAGE followed by Western blot analysis with anti-GST, anti-phosphotyrosine or anti-GST antibodies. C, peak fractions of importin α5-STAT1/STAT2 complexes (B, arrow) were separated by 8% SDS-PAGE, stained with Coomassie Brilliant Blue, and quantitated. Blue dextran (void), β-amylase (200 kDa), and bovine serum albumin (66 kDa) functioned as molecular mass markers. p, phospho; imp, importin.

**Fig. 6.** Gel filtration analysis of STAT-importin α5 complexes. Sf9 cells were infected with STAT1, Tyk2 + STAT1, or Tyk2 + STAT1 + STAT2 protein-expressing recombinant baculoviruses. After 42 h the cells were collected, cell extracts were prepared, and protein samples were subjected to a Superose 12 gel filtration column by themselves or after binding the proteins to Sepharose-immobilized E. coli-produced GST-importin α5. The gel filtration profile of GST-importin α5 was also analyzed. Samples from gel filtration fractions were subjected to 8% SDS-PAGE followed by Western blot analysis with anti-GST, anti-STAT1, anti-phospho(tyrosine)-STAT1, or anti-phosphotyrosine antibodies as shown in the figure. A, gel filtration patterns of Sf9 cell-produced monomeric STAT1 (unphosphorylated, expressed alone) and phosphorylated STAT1 dimers (coexpressed with Tyk2) and E. coli-produced GST-importin α5 protein. B, gel filtration pattern of GST-importin α5-STAT1/STAT2 complexes. STAT1/STAT2 complexes were allowed to bind to immobilized importin α5. Glutathione-Sepharose-bond GST-importin α5-STAT1/STAT2 complexes were released by glutathione treatment followed by analysis on a Superose 12 gel filtration column. Protein samples in gel filtration fractions were separated by 8% SDS-PAGE followed by Western blot analysis with anti-GST, anti-phosphotyrosine or anti-GST antibodies. C, peak fractions of importin α5-STAT1/STAT2 complexes (B, arrow) were separated by 8% SDS-PAGE, stained with Coomassie Brilliant Blue, and quantitated. Blue dextran (void), β-amylase (200 kDa), and bovine serum albumin (66 kDa) functioned as molecular mass markers. p, phospho; imp, importin.

**Fig. 7.** Inhibition of STAT1 dimer binding to importin α5 by IRF-1 GAS oligonucleotide. Sf9 cell extracts containing activated STAT1 dimers were preincubated with different amounts of IRF-1 GAS oligonucleotide (from 40 ng to 5 μg) for 30 min followed by binding to Sepharose-immobilized GST-importin α5 (GST-imp-α5) in the presence of the same amount of IRF-1 GAS DNA. Consensus NF-κB GAS-oligonucleotide was used as control DNA. Plain Sepharose (S) and Sepharose-importin β (GST-imp-β) beads were used as control beads. Sepharose-bound proteins were boiled in Laemmli sample buffer, subjected to 8% SDS-PAGE, and stained in Western blotting with anti-phosphotyrosine-STAT1 antibodies.

**Fig. 7.** Inhibition of STAT1 dimer binding to importin α5 by IRF-1 GAS oligonucleotide. STAT1 dimers were preincubated with different concentrations of IRF-1 GAS oligonucleotide (from 40 ng to 5 μg) for 30 min followed by binding to Sepharose-immobilized GST-importin α5 (GST-imp-α5) in the presence of the same amount of IRF-1 GAS DNA. Consensus NF-κB GAS-oligonucleotide was used as control DNA. Plain Sepharose (S) and Sepharose-importin β (GST-imp-β) beads were used as control beads. Sepharose-bound proteins were boiled in Laemmli sample buffer, subjected to 8% SDS-PAGE, and stained in Western blotting with anti-phosphotyrosine-STAT1 antibodies.

**DISCUSSION**

In the present work we have demonstrated that activated STAT dimers are able to directly bind to two importin α5 (NPI-1) molecules with relatively high affinity. STAT1-importin α5 interaction was regulated by dimerization of STATs and by a lysine-rich element in the DNA binding domain of STAT1 since the mutation of lysines 410 and 413 to alanines completely abolished STAT1 binding to importin α5. By confocal laser microscopy we also found that in IFN-α-stimulated cells STAT1 and STAT2 colocalized with importin α5 in the cell nucleus. Consistent with biochemical analysis NLS-mutated STAT1 or STAT2 failed to show colocalization with importin α5. In addition, we observed that STAT1-specific GAS oligonucleotide was able to inhibit STAT1-importin α interaction suggesting that STAT1 binding sites to importin α5 or target DNA elements are very close to each other.

It is well established that STATs have to undergo cytokine receptor-mediated tyrosine phosphorylation by JAKs and dimerization via phosphotyrosine residues and Src homology 2 domains of each of the monomers before nuclear translocation can take place (2, 31). The key regulatory event controlling the nuclear import of STATs appears to be dimerization (32, 33). Sekimoto and coworkers (20) demonstrated that importin α5 interacted with activated STATs, but in their work specific STAT NLSs were not identified. Now we know more of the details of the regulation of nuclear import and export of STATs. Mutational analyses revealed that lysines 410 and 413 of STAT1 and corresponding basic residues of STAT2 regulate IFN-induced nuclear import of STAT1 homodimers and STAT1/STAT2 heterodimers (9, 10). Several groups have also shown IFN-independent nuclear localization of STAT1, which...
appears to occur constitutively and by a different mechanism than that of IFN-induced import of STAT1 (9, 10, 34). Nuclear export of STAT1 is regulated by CRM1/exportin 1 protein, which binds to a DNA-free form of STAT1 via a leucine-rich consensus-like nuclear export signal situated at positions 400–409 of STAT1 (35). It is of great interest that the STAT1 nuclear export signal is in the immediate vicinity of its NLS residing at positions 410–413 (9, 35). Mutations in the STAT1 nuclear export signal may also interfere with STAT1-importin α interaction (36).

In the present work previous cell biological observations (9, 10) were extended to a biochemical level. For these studies we chose to use the baculovirus expression system since it has been shown to efficiently produce biologically active components of the JAK/STAT pathway (9, 37). Here we show that importin α can directly bind to STAT1 homodimers or STAT1/STAT2 heterodimers evidently with a relatively high affinity since even high molar concentrations of NaCl or urea are not able to disrupt the interaction of STAT dimers with importin α. We also show that no binding of monomeric STAT1 to importin α is taking place. In addition, neither monomeric nor dimeric STAT bind to importin β. This suggests that IFN-induced nuclear import of STAT1 homodimers or STAT1/STAT2 heterodimers is initiated by tyrosine phosphorylation-triggered dimerization of STATs followed by efficient and direct binding of STAT dimers to two importin α molecules. In our experiments the key residues regulating importin α/STAT dimer interaction were lysines 410 and 413 of STAT1, which apparently mediate direct interaction between dimeric STAT complexes and importin α. Some signaling proteins, such as Smads, can directly interact with importin β, which mediates their translocation into the nucleus (38, 39). Importin β is a snail-like molecule that has a narrow canyon that functions as the binding pocket for the helical arginine/lysine-rich N-terminal domain (IBB) of importin α (15). The NLS of STATs is situated on the surface of the DNA binding domain (9), and therefore it is hard to vision that this element could mediate the interaction between nuclearly targeted molecules and importin α. This is consistent with the finding that STAT1(40, 41), and it is thus likely that the other members of the STAT family use the same importin α-mediated nuclear import pathway.

Gel filtration experiments show that the nuclear import complex of STATs is composed of two STAT molecules attached to two importin α molecules. Importin α alone was found to exist as a monomer (Fig. 6), but in the presence of STAT dimers it formed complexes that migrated in the 300–350-kDa range in gel filtration analysis. Coomassie Blue staining and quantitation of the protein composition of these complexes indicated that STAT1/STAT2 dimers bound to two importin α molecules. Dimeric STAT binding to two importin α molecules may represent a previously uncharacterized type of interaction between nuclearly targeted molecules and importin α. Based on the three-dimensional structure of mouse importin α and karyopherin α, a yeast homolog of importin α, the molecule has two binding sites that bind classical NLS peptides (e.g. SV40 T-antigen NLS). The overall structure of importin α/karyopherin α includes well conserved armadillo (arm) repeats that mediate NLS binding (42, 43). Arm repeats 2–4 form the major NLS binding site, whereas arm repeats 7 and 8 form the minor binding site. The major binding site (arm 2–4) also functions as the binding site for the autoinhibitory N-terminal IBB domain of importin α (44, 45). Sekimoto and coworkers (20) suggested, based on extensive deletion analysis, that neither of the NLS binding sites functions as the binding site for STAT1, but rather it is the C-terminal end of importin α that regulates STAT1-importin α interaction. To reveal the question of STAT binding site(s) in importin α a more fine-tuned mutational analysis should be carried out. Alternatively, a three-dimensional structural analysis of the STAT-importin α complex should be obtained.

In our previous study we observed that the nuclear import-defective STAT1 K410A,K413A mutant was also defective in its DNA binding activity to GAS or interferon-stimulated response elements (9). This prompted us to study whether GAS oligonucleotides would be able to interfere with STAT1-importin α interaction. We observed that high concentrations of GAS DNA was able to almost completely inhibit STAT1 dimer binding to importin α. This suggests that STAT1 binding to importin α or target DNA occurs at sites that are very close to each other.

In the present work we have taken a clear step forward in understanding the mechanisms of nuclear import of STATs. We have evidence that dimeric STAT complexes interact with two importin α molecules via a lysine-rich conformation NLS within the STAT1 DNA binding domain. However, it still remains an open question why only STAT dimers are able to bind to importin α and which of the NLS binding sites of importin α are involved in this interaction.

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