Arginine/Lysine-rich Structural Element Is Involved in Interferon-induced Nuclear Import of STATs*

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Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors, which mediate interferon (IFN), interleukin, and some growth factor and peptide hormone signaling in cells. IFN stimulation results in tyrosine phosphorylation, dimerization, and nuclear import of STATs. In response to IFN-γ stimulation, STAT1 forms homodimers, whereas IFN-α induction results in the formation of STAT1-STAT2 heterodimers, which assemble with p48 protein in the nucleus. Phosphorylation as such is not sufficient to target STATs into the nucleus; rather, the dimerization triggered by phosphorylation is essential. Although IFN-induced nuclear import of STATs is mediated by the importin/Ran transport system, no classic nuclear localization signal (NLS) has been found in STATs. In the three-dimensional structure of STAT1, we observed a structural arginine/lysine-rich element within the DNA-binding domain of the molecule. We created a series of point mutations in these elements of STAT1 and STAT2 and showed by transient transfection/IFN stimulation assay that this site is essential for the nuclear import of both STAT1 and STAT2. The results suggest that two arginine/lysine-rich elements, one in each STAT monomer, are required for IFN-induced nuclear import of STAT dimers. Import-defective STAT1 and STAT2 proteins were readily phosphorylated and dimerized, but they functioned as dominant negative molecules inhibiting the nuclear import of heterologous STAT protein.

MATERIALS AND METHODS

Cells—Human hepatocellular carcinoma HuH7 (26) cells were maintained in minimal essential medium, supplemented with penicillin (0.6 mg/ml), streptomycin (60 mg/ml), glutamine (2 mM), HEPES buffer, pH

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¶ The abbreviations used are: STAT, signal transducers and activators of transcription; JAK, Janus tyrosine kinase; IFN, interferon; NLS, nuclear localization signal; Ab, antibody; FITC, fluorescein isothiocyanate; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; wt, wild-type; IL-6, interleukin-6; IFNAR, IFN-α/β receptor; IFNγR, IFN-γ receptor; NES, nuclear export signal; NPI-1, nucleoprotein interact-actor 1; TRITC, tetramethylrhodamine isothiocyanate; EMSA, electrophoretic mobility shift assay.

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IFN-induced Nuclear Import of STATs

7.4 (20 mM), and 10% fetal calf serum (Integro, Zaandam, Netherlands). Before stimulation with IFNs, the cells were cultured in the growth medium supplemented with 2% fetal calf serum for 24 h. Monolayers and suspension cultures of Spodoptera frugiperda Sf9 cells were maintained in TNM-FH medium as described previously (27). Insect cells infected with baculovirus (10 8 IU/ml) was kindly provided by Dr. Kari Cantell at our Institute (28). Human IFN-γ (1 × 10 8 IU/ml) was obtained from the Finnish Red Cross Blood Transfusion Service and was prepared and purified as described (29).

Antibodies—ANTI-FLAG M5 Abs (Sigma Chemical Co., St. Louis, MO) were used in indirect immunofluorescence microscopy and in immunoprecipitation. 5% (1,000 dilution). Mouse anti-STAT1 alkaline phosphatase chain reaction products were digested with BamHI (Transduction Laboratories, Lexington, KY) and rabbit anti-human STAT1 (STAT1 p91 ϵ-24), STAT2 (STAT2 p113 ϵ-20), and p48 (IGSF-3 p48 p-c20) were obtained commercially (1:50–1:200 dilutions in immunofluorescence microscopy, Santa Cruz Biotechnology, Santa Cruz, CA). FITC- and TRITC-labeled goat anti-mouse and anti-rabbit immunoglobulins were used as secondary Abs (1:200 dilution, Cappel, Organon Teknika Co., West Chester, PA). In Western blotting secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (1:2000 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used. For the detection of tyrosine phosphorylation of STAT proteins, immunoprecipitated or gel-filtered samples were stained with rabbit or mouse anti-phosphotyrosine Abs (1,500 diluted, Transduction Laboratories) was performed by a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Plasmids and DNA Manipulations—Polymerase chain reaction was used to modify STAT1, STAT2, and p48 genes. The noncoding sequences were removed by inserting unique BamHI or BclI restriction sites immediately upstream of the first ATG codon and downstream of the stop codon of STAT1, STAT2, and p48 genes, respectively. The primers used for STAT1 were GCA CAA (GGC TCC) GCC ATG TCT CAG TGG TAC GAA CTT CAG (sense) and AAA AAT (TG ATG G) C TATA CTG TGT TCA TCA TAC TGT C (antisense); for STAT2 the primers were CTG TGA CCA GCC ATG TCT CAG TGG TAC GAA CTT CAG (sense); and for p48 the primers were GCA GAG CAT GCC ATG TCT CAG TGG TAC GAA CTT CAG (antisense) and GGA GAT CCC; and antisense, 5′-TCG AGG GAT CCC TTG TCA TCG (antisense); and for STAT2 the primers were GCA GAG CAT GCC ATG TCT CAG TGG TAC GAA CTT CAG (antisense) and AAA AAT T(GG ATC C) CT (antisense). The primers were underlined.)

Plasmids were digested with BamHI or BclI, isolated from the BamHI site of a modified transient FLAG-tagged pCDNA 3.1(+) expression vector (Invitrogen, Carlsbad, CA) or HA-tagged p12/CMV expression vector were constructed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were used 5′-GAA CTT CAG TGG TAC GAA CTT CAG (sense) and 5′-CTT GTG TGC TGG TAC TGG TGC (antisense). The transport kinetics of endogenous STAT1 and STAT2 was dependent on IFN dose, because low doses of IFN-α (10 IU/ml) resulted in reduced transport kinetics (not shown). As detected by confocal laser microscopy (Fig. 1), nuclear import of STAT1 and STAT2 was observed within 30 min after IFN-α treatment. The nuclear accumulation was at its maximum within 30–60 min, and the cytoplasmic recycling was completed within 3 h. The kinetics of nuclear transport of STAT1 and STAT2 was dependent on IFN dose, because low doses of IFN-α (1–10 IU/ml) resulted in reduced transport kinetics (not shown). As visualized by confocal laser microscopy (Fig. 2, a and b), in untreated HuH7 cells STAT1 was located evenly in the cytoplasm and nucleus, whereas STAT2 was predominantly cytoplasmic and no colocalization with STAT1 could be seen in the cell nucleus. 30 min after IFN-α treatment both STAT1 and STAT2 was detected in both the cell nucleus and showed marked nuclear colocalization (Fig. 2, c and d). To study the intracellular localization of p48 protein, we carried out double-staining experiments in HuH7 cells (Fig. 3), p48 protein was predominantly localized in the nucleus already in untreated HuH7 cells and no detectable change in intracellular distribution was seen after IFN-α treatment. STAT1, instead, was effectively imported into the nucleus and showed marked colocalization with p48 protein 30 min after IFN-α treatment (Fig. 3).

RESULTS

Kinetik of IFN-induced Nuclear Import of STAT Proteins—The transport kinetics of endogenous STAT1 and STAT2 was analyzed by treating HuH7 cells with 1000 IU/ml of IFN-α. As detected by confocal laser microscopy (Fig. 1), nuclear import of STAT1 and STAT2 could be seen starting at 10 min after IFN-α treatment. The nuclear accumulation was at its maximum within 30–60 min, and the cytoplasmic recycling was completed within 3 h. The kinetics of nuclear transport of STAT1 and STAT2 was dependent on IFN dose, because low doses of IFN-α (1–10 IU/ml) resulted in reduced transport kinetics (not shown). As visualized by confocal laser microscopy (Fig. 2, a and b), in untreated HuH7 cells STAT1 was located evenly in the cytoplasm and nucleus, whereas STAT2 was predominantly cytoplasmic and no colocalization with STAT1 could be seen in the cell nucleus. 30 min after IFN-α treatment both STAT1 and STAT2 was detected in both the cell nucleus and showed marked nuclear colocalization (Fig. 2, c and d). To study the intracellular localization of p48 protein, we carried out double-staining experiments in HuH7 cells (Fig. 3), p48 protein was predominantly localized in the nucleus already in untreated HuH7 cells and no detectable change in intracellular distribution was seen after IFN-α treatment. STAT1, instead, was effectively imported into the nucleus and showed marked colocalization with p48 protein 30 min after IFN-α treatment (Fig. 3).

Transfections—HuH7 cells were transfected with HA-STAT1- and HA-STAT2-p12/CMV or FLAG-STAT1- and FLAG-STAT2-pCDNA 3.1(+) gene constructs, using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany).

Immunoprecipitation, SDS-PAGE, and Western Blotting—Transfected HuH7 cells were left untreated or were treated with IFN-α (1000 IU/ml, 30 min). The cells were collected, washed with phosphate-buffered saline, and lysed in immunoprecipitation binding buffer (IP buffer) (PBS, 0.5% NP-40, 0.5% sodium deoxycholate, pH 7.4, 24°C). Immunoprecipitates were washed three times, and Laemmli sample buffer (35) was added. SDS-PAGE was performed on 6–12% polyacrylamide gels. Proteins were separated on gels and transferred onto Immobilon-P membranes (polyvinylidene difluoride, Millipore, Bedford, MA) and visualized with the enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK) as recommended.

Electrophoretic Mobility Shift Assay (EMSA)—Monolayers of Sf9 cells were infected with STAT1, STAT1 Y701A, STAT1 K410A,K413A, STAT2, p48, wtJAK2, or knJAK2 recombinant baculoviruses. 28 or 38 h after infection, cells were disrupted in lysis buffer (10 mM HEPES-KOH, pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 100 μM NaN3). Lysates were cleared of insoluble material by centrifugation and used for electrophoretic mobility shift assay using IRSF15 and GAS consensus sequence oligonucleotides as described (34).

Gel Filtration—Lysates (above) of baculovirus-infected Sf9 cells were gel-filtered in the above lysis buffer using a 24-ml Superose 12 fast protein liquid chromatography (Amersham Pharmacia Biotech) gel filtration column. Molecular weight standards were obtained from Sigma.

Indirect Immunofluorescence and Confocal Laser Microscopy—For indirect immunofluorescence and confocal laser microscopy, transiently transfected HuH7 cells were grown on glass coverslips, either treated with IFN-α (1000 IU/ml) for 45 min or left untreated and performed as described previously (35). The cells positive for FLAG tag were visualized and photographed on a Zeiss Axiopt photomicroscope or a Leica TCS NT confocal microscope. In some experiments, to better visualize STAT and p48 proteins in immunofluorescence, the cells were primed with IFN-γ (10 IU/ml for 24 h), which is known to up-regulate their expression (34, 36).
main (37). Although STATs do not contain a classic NLS, computer analysis of the STAT1 structure (38) revealed a cluster of basic amino acids in the DNA-binding domain of the molecule within the DNA-binding domain (Fig. 4, a and c). This element includes amino acids Arg-378, Lys-379, Lys-410, Lys-413, and Arg-418. All of these amino acids are conserved in STAT1, STAT3, and STAT4 proteins and are mostly conserved in STAT2 (human and pig), which is lacking the lysine corresponding to K410 of STAT1. STAT5a, STAT5b, and STAT6 are more distantly related to the other STAT molecules (Fig. 4 b), and they lack the basic residues corresponding to Arg-378 and Lys-379 of STAT1. Instead, they have two additional arginine or lysine residues further downstream (Fig. 4 a).

To analyze whether this structural site rich in basic amino acids is involved in nuclear import of human STAT1 and STAT2, we made a series of double point mutations and studied the nuclear import of the FLAG-tagged, mutated STAT proteins in transiently transfected HuH7 cells by indirect immunofluorescence microscopy (Fig. 5). HuH7 cells were selected, because they were effectively transfected and possessed excellent cellular morphology in microscopic studies. Although HuH7 cells had their own functional STAT1 and STAT2 proteins, using FLAG-tagged gene constructs, we were able to follow the transport of transfected mutant proteins. To rule out the possibility that the FLAG-tag affected the transport, we also carried out the experiments with influenza virus hemagglutinin (HA)-tagged STAT gene constructs with identical results (not shown). Weak basal nuclear accumulation of intrinsic (Fig. 1) as well as of transiently expressed (Fig. 5) STAT1 and to a lesser extent of STAT2 was observed in HuH7 as well as in several other cell lines studied.

Although FLAG-tagged transiently expressed wild-type STAT1 protein was readily transported into the nucleus after IFN-α or IFN-γ stimulation, all arginine/lysine mutant STAT1 proteins showed impaired IFN-induced nuclear import (Fig. 5). Fig. 6 shows the nuclear import of the corresponding STAT forms in a quantitative manner. In STAT1 R378A,K379A and K413A,R418A mutant proteins nuclear import was partially inhibited, whereas STAT1 K410A,K413A protein appeared to be fully nuclear transport incompetent after stimulation with either IFN type (Fig. 5, j, k, l). A revertant, where residues 413 and 418 were mutated back to those of the wild-type STAT1, showed normal IFN-induced nuclear translocation (Fig. 5, p, q, r and Fig. 3). Mutation in tyrosine 701 of STAT1 (STAT1 Y701A in Fig. 5), which has previously been shown to result in lack of tyrosine phosphorylation and subsequent dimerization (39), rendered the protein cytoplasmic. The corresponding mu-
tations in STAT2 molecule showed similar effects to those seen in STAT1 (Figs. 5 and 6). IFN-α-induced nuclear import of STAT2 R374A,K375A and STAT2 R409A,K415A proteins were partially and completely blocked, respectively (Fig. 5, \( u, v, w \), x). It is unlikely that p48 protein, which together with STAT1 and STAT2 proteins forms the ISGF3 complex (4), would be involved in STAT1\( \times \)STAT2 heterodimer transport, because both transfected as well as intrinsic p48 protein was always found in the nucleus and its cellular distribution was not altered by IFN treatment (Fig. 3).

**Tyrosine Phosphorylation of STAT Mutant Proteins**—Although the tyrosine phosphorylation site is far from the arginine/lysine-rich element of STAT proteins (Fig. 4c), we considered the possibility that mutations in the arginine/lysine-rich element of STAT1 or STAT2 proteins would affect their tyrosine phosphorylation properties. Therefore, we transfected HuH7 cells with wt or mutant STAT genes for 48 h, followed by stimulation with IFN-α for 1 h. Cells were collected, and transgene STAT protein expression was analyzed by anti-FLAG Abs. All STAT1 constructs were equally well expressed, and in response to IFN-α stimulation there was a clear increase in the molecular weight of all tested STAT1 proteins except Y701A mutant construct (Fig. 7a). Immunoprecipitation, followed by Western blotting with antibody to P-Tyr Abs, revealed that all STAT forms except STAT1 Y701A were readily tyrosine-phosphorylated (Fig. 7b). This result suggests that point mutations in the arginine/lysine-rich element do not affect IFN-induced phosphorylation status of STAT proteins.

**Nuclear Import Defective STAT Mutant Proteins Inhibit the Nuclear Import of Heterologous STAT Proteins**—Because STAT1 and STAT2 heterodimerize after stimulation with IFN-α, we studied whether the transport-defective STATs in-
hibit the nuclear import of heterologous STAT molecules. wt and transport-defective STAT proteins were transiently expressed in HuH7 cells, and colocalization of the DNA-binding domain of both STAT1 and STAT2 were shown. Amino acids mutated to alanine are shown in black squares. HuH7 cells were transiently transfected with FLAG-tagged wt or point mutated STAT1 and STAT2 gene constructs as shown in the figure. At 48 h post-transfection the cells were treated with IFN-α or IFN-γ (1000 IU/ml) for 30 min and fixed. Staining was performed with monoclonal Abs against the FLAG-epitope, followed by staining with FITC-labeled anti-mouse immunoglobulins. All experiments were repeated three to four times, and typical subcellular localization of STATs is presented in each picture. The relative effectiveness of IFN-induced nuclear import is presented after the sequence of each gene construct (+ + +, +, −). Bar, 10 μm.

To analyze the possible effect of STAT2 expression on IFN-γ induction in HuH7 cells both wt and nuclear transport-defective STAT2 were transiently expressed, and colocalization of STAT1 and STAT2 was detected. Overexpression of both wt and nuclear transport-defective FLAG-STAT2 blocked IFN-γ induction in transiently transfected HuH7 cells (Fig. 8b).

Transiently transfected FLAG-STAT1 Y701A did not inhibit IFN-α-induced nuclear import of endogenous STAT2, suggesting that mutant STAT1 Y701A is incapable of forming dimers with endogenous STAT2 and thus does not block its nuclear import. It is likely that the weak nuclear accumulation of STAT2 was due to the endogenous STAT1 in HuH7 cells (Fig. 8a). Transiently transfected transport-defective FLAG-STAT2 R409A,K415A, instead, completely blocked IFN-α-induced nuclear import of endogenous STAT1. This suggests that FLAG-STAT2 R409A,K415A mutant protein and endogenous STAT1 formed complexes in the cytoplasm but they were not translocated into the cell nucleus after IFN-α induction (Fig. 8a).

The results suggest that in a heterodimer both arginine/lysine-rich elements, one in each STAT, have to be intact before IFN-induced nuclear import of STAT dimers can take place.

**Nuclear Import Defective STAT1 K410A,K413A Is Tyrosine-phosphorylated and Dimerized but It Does Not Bind DNA**—To study STAT dimerization and DNA binding in a system lacking intrinsic human STAT proteins we reconstituted STAT activation and DNA binding analysis using a baculovirus expression system. We infected Sf9 cells with STAT1, STAT2, p48, and JAK2 protein-expressing recombinant baculoviruses. Sf9 cells and a baculovirus expression system is selected, because JAK2 kinase has earlier been shown to tyrosine-phosphorylate and activate STAT1 protein (32). As shown in Fig. 9(a and b), wtSTAT1 was tyrosine-phosphorylated, dimerized, and exhibited DNA binding to the GAS oligonucleotide probe in the presence of wtJAK2 kinase, but not with that of knJAK2, as analyzed by gel filtration, anti-phosphotyrosine blotting, and EMSA, respectively. The nuclear import-defective FLAG-
STAT1 K410A,K413A mutant was also tyrosine-phosphorylated, and dimerized, but it was unable to bind to the GAS oligonucleotide probe. The active ISGF3 complex bound ISRE15 oligonucleotide probe when Sf9 cells were coinfected with STAT1, STAT2, p48, and wtJAK2 protein-expressing gene constructs, as detected by EMSA (Fig. 9c). Instead, when STAT1 was replaced with the nuclear import-defective STAT1 K410A,K413A mutant, or when wt JAK2 was replaced with knJAK2, DNA-binding activity of the ISGF3 complex was not seen or it was dramatically reduced, respectively (Fig. 9c).

DISCUSSION

In the present work, we have characterized the kinetics of nuclear import of IFN-stimulated STAT proteins and identified an arginine/lysine-rich element in the DNA-binding domains of STAT1 and STAT2 that regulates their IFN-induced nuclear import. By indirect immunofluorescence analysis, we observed that the kinetics of nuclear import of STAT proteins was fast and transient, because already within 10 min after IFN-α stimulation, STAT proteins were found in the nucleus and within 2–3 h they were recycled back to the cytoplasm (Fig. 1). The kinetics of nuclear import of STATs correlates well with the IFN-stimulated DNA-binding activity of ISGF3 and GAS complexes (34, 40). p48 protein was apparently not associated with STAT import, because it was constitutively expressed in the cell nucleus and its intracellular distribution was not changed during IFN-α stimulation. In addition, colocalization of STAT1, STAT2, and p48 (Figs. 1, 2, and 3) was clearly visualized by confocal laser microscopy preferentially in the nucleus, suggesting that heterodimers of STAT1 and STAT2 are first transported into the nucleus where they then associate with the p48 protein to form the ISGF3 complex.

In unstimulated cells STAT1 was evenly distributed in the cytoplasm and the nucleus, whereas STAT2 was predominantly cytoplasmic (Figs. 1 and 2). IFN stimulation resulted in rapid nuclear accumulation and colocalization of STATs (Figs. 1 and 5). This suggests that the mechanism of constitutive nuclear accumulation of intrinsic or transiently expressed STAT1 (and to lesser extent STAT2) may differ from that of IFN-induced nuclear import. It is possible that in the absence of IFN stimulation STATs recycle between the cytoplasm and the nucleus with other transport proteins. Recently, it was shown that STAT3 is present as high molecular mass complexes in the size range of 200–400 kDa and 1–2 MDA both in

FIG. 6. A graphical representation of subcellular localization of transiently expressed wt and mutant STAT1 and STAT2 proteins in IFN-α- and IFN-γ-stimulated HuH7 cells as presented in the Fig. 5. Subcellular localization of STAT1 and STAT2 proteins was divided in three categories; I, mainly cytoplasmic (open bars); II, cytoplasmic and nuclear (shaded bars); and III, mainly nuclear (solid bars). The results represent the means of three individual experiments, in which 100 cells were counted.

STAT1 R376A,K379A mutant was also tyrosine-phosphorylated, and dimerized, but it was unable to bind to the GAS oligonucleotide probe. The active ISGF3 complex bound ISRE15 oligonucleotide probe when Sf9 cells were coinfected with STAT1, STAT2, p48, and wtJAK2 protein-expressing gene constructs, as detected by EMSA (Fig. 9c). Instead, when STAT1 was replaced with the nuclear import-defective STAT1 K410A,K413A mutant, or when wt JAK2 was replaced with knJAK2, DNA-binding activity of the ISGF3 complex was not seen or it was dramatically reduced, respectively (Fig. 9c).

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FIG. 7. IFN-α-induced tyrosine phosphorylation of wild-type and mutant STAT1 and STAT2 proteins. HuH7 cells were transiently transfected with wt or mutated STAT gene constructs for 48 h. a, transfected cells were left untreated or stimulated with IFN-α (1000 IU/ml for 30 min). The cells were collected and prepared for Western blot analysis. Transfected FLAG-STAT1 was detected with monoclonal anti-FLAG Abs, followed by ECL detection. Tyrosine-phosphorylated and unphosphorylated forms of STAT1 s are shown by arrows. b, transiently transfected HuH7 cells were either stimulated with IFN-α (1000 IU/ml for 30 min) or left unstimulated. Immunoprecipitation was carried out with anti-FLAG Abs, and tyrosine phosphorylation was detected on Western blots with anti-phosphotyrosine Abs, followed by ECL detection. Tyrosine-phosphorylated STAT1 and STAT2 are shown by arrows (STAT1 P-Tyr and STAT2 P-Tyr).
One of the proteins that was found to interact with STAT3 in an IL-6-dependent manner was chaperone GRP58 ER-60 ERp57. The results suggest that STATs interact with a number of other cellular proteins some of which could be involved in intracellular protein transport. In uninduced cells, STAT1 and STAT2 have been shown to exist as unphosphorylated heterodimers. This weak association is accomplished by regions apart from the SH2 domain, which is crucial in IFN-induced nuclear import competent dimer formation (42).

IFN-α/β receptor (IFNAR) and IFN-γ receptor (IFNGR) are each composed of two integral membrane glycoproteins (IFNAR1 and IFNAR2; IFNGR1 and IFNG2). Cross-communication between these two receptors seems to be important at least in IFN-γ signaling, where a constitutive subthreshold of IFN-α/β signaling and an association between IFNAR1 and IFNGR2 is needed for efficient signal transduction (43). The only constitutive STAT binding site in uninduced cells is located in IFNAR2, the ligand binding component of IFNAR. IFNAR2 binds constitutively the weakly associated STAT1-STAT2 heterodimer via the STAT2 component. During IFN-γ induction, a specific binding site for STAT1 is formed on IFNGR1. It seems possible that IFNAR serves as an efficient source for STAT1 during IFN-γ induction. In cells lacking IFNAR1 IFN-γ-induced antiviral response is seriously impaired (43). Interestingly, also STAT4 dimer formation by the IFN-α/β receptor in human T-cells occurs via intermediates involving STAT2 (44).

The above results (43, 44) may give an explanation for our results showing that STAT2 overexpression (wt or nuclear import-defective STAT2) inhibits IFN-γ-induced STAT1 nuclear import. Apparently, there is a very delicate balance between the amount of STAT1 and STAT2 in the cell, because overexpression of STAT2 completely blocked STAT1 nuclear import after IFN-γ stimulation (Fig. 8b). It may be that in STAT2 excess IFNAR2 is occupied by monomeric STAT2 molecules and hence IFNAR cannot serve as the source for STAT1 molecules required by IFNGR. In a situation of STAT2 overexpression also the low basal level transport of STAT1 is blocked in uninduced cells (Fig. 8b). It is thus apparent that STAT1 molecules in the cytoplasm interact, at least weakly, with the unphosphorylated forms of STAT2 (42).

FIG. 8. Subcellular localization and nuclear import of transiently transfected, FLAG-tagged STAT1 and STAT2 gene constructs in IFN-α-treated HuH7 cells detected by confocal microscopy. The cells were first transfected with FLAG-tagged gene constructs as indicated in the figure. After 12 h, the cells were primed with 10 IU/ml of IFN-α or IFN-γ for 24 h (IFN-α-treated cells were primed with IFN-γ and vice versa), then treated with 1000 IU/ml of IFN-α or IFN-γ for 45 min, or left untreated, fixed, and double-stained with anti-FLAG, anti-STAT1 (b) or anti-STAT2 (a) Abs as indicated in the figure. Bar, 10 μm.
suggested that STAT1 is imported into the nucleus by the aid of one importin-α family member, NPI-1 (24). The 3-D structure of NPI-1 has not been resolved, but crystallographic analysis of yeast karyopherin α, another member of this highly conserved importin-α family, has been resolved (45). Karyopherin-α consists of a tandem array of ten armadillo repeats forming a long helical surface groove that harbors the binding sites for the classic mono- and bipartite NLSs (45). The binding site of STAT1 was suggested to be located in the C-terminal region of NPI-1, distinct from the binding site for classic NLS (24). The STAT NLS might thus differ from the classic mono- or bipartite NLSs. We reasoned that STAT1 could, however, contain an NLS consisting of basic amino acids, but its structure would be different from classic NLSs. In other transcription factors and DNA-binding proteins ~80% of the NLSs overlap or are immediately adjacent to the nucleic acid-binding domains (37). Analysis of the crystal structure of STAT1 (38) and STAT3 (46) revealed a structural arginine/lysine-rich element in the DNA-binding domain of the proteins. This element seemed like a good candidate for a STAT NLS, because it was very well conserved in STAT1 to STAT4. Site-directed mutagenesis of the conserved basic amino acids in the arginine/lysine-rich structural element (Fig. 4) revealed that this site was involved in the nuclear import of both STAT1 and STAT2. A novel feature was that two intact elements, one in each monomer, were required for the IFN-induced nuclear import of STAT dimers. Nuclear import-defective STAT1 K410A,K413A or STAT2 R409A,K415A mutant proteins were able to block the IFN-α-induced import of heterologous endogenous STAT molecules (Fig. 8b), thus functioning as dominant negative STAT forms. Therefore, we think that in STAT dimers both arginine/lysine-rich structural elements are involved in binding to transport proteins, which act as the first step in the cascade leading to nuclear import. The question rises, whether this site binds to an unknown “adapter” protein or is itself an NLS binding directly to importin-α. A defect in STAT1 nuclear import is not due to lack of dimerization, because in a reconstituted baculovirus STAT activation system STAT1 K410A,K413A mutant was tyrosine-phosphorylated and dimerized. Mutant STAT1 complexes were, however, not able to bind GAS or ISGF3 oligonucleotide probes (Fig. 9). This could be expected, because the site regulating nuclear import of STAT1 is in the immediate vicinity of the STAT1-DNA interaction site (Fig. 4). A recent study by McBride and coworkers (47) showed that STAT1 nuclear export is leptomycin B-sensitive and regulated by CMR1 export protein. They also demonstrated that the nuclear export signal (NES) of STAT1 is situated immediately adjacent (amino acids 400–409) to the putative NLS of STAT1 described in the present paper. The close proximity of NLS and NES in STAT1 would enable either import or export function to take place. Leptomycin B treatment of IFN-stimulated STAT1-transfected cells failed to show any nuclear accumulation of STAT1 K410A,K413A (results not shown) further supporting the view that the protein is fully incapable of entering the nucleus.

Karyopherin-α, the yeast homolog of NPI-1, is known to exist as dimers, because it has been crystallized in this form (45). Amino acids mediating the dimerization of karyopherin-α are highly conserved and they are also found in other importin-α molecules, including NPI-1. It has been hypothesized that importin-α dimers are inactive, because the groove that harbors the binding sites for the mono- and bipartite NLSs is not accessible in dimeric forms of importin-α. However, it is possible that the C-terminal binding sites for STATs are accessible also or only in NPI-1 dimers. The NPI-1 dimer would have the binding sites for both of the two arginine/lysine-rich elements

**FIG. 9.** Gel filtration and EMSA analysis of GAS and ISGF3 complex formation and DNA binding in baculovirus-infected Sf9 cells. a, Sf9 cells were infected with STAT1, wild type (wt) JAK2, and kinase negative (kn) JAK2 protein-expressing baculovirus gene constructs as indicated in the figure. After 38 h the cells were collected, and cell lysates were subjected to gel filtration in a Superose 12 column. Samples from gel filtration fractions were run on 12% SDS-PAGE followed by Western blots with anti-STAT1 or anti-phosphotyrosine Abs. The positions of STAT1 monomers (85 kDa) and dimers (170 kDa) are marked with arrows. b, Sf9 cells were infected with wt baculovirus (E2) or coinfected with STAT1 and JAK2 protein-expressing baculovirus gene constructs as indicated in the figure. After 28 h the cells were collected, and cell lysates were analyzed by EMSA using GAS oligonucleotide probe. In b and c, supershift analyses were conducted with anti-STAT1 and anti-STAT2 antisera. Normal rabbit serum (NRS) was used as a control antiserum. GAS and ISGF3 complexes are marked with arrows.
in STAT dimers. This would lead to stabilization of the STAT-importin-α heterotetramer complex, importin-β binding, and nuclear translocation.

Different members of the STAT family are known to form homo- and heterodimers (9, 40), and presumably the mechanism of nuclear import is identical for most, if not all STAT proteins. Both the evolutionary conservation in the arginine/lysine-rich elements in STAT1, STAT2, STAT3, and STAT4 (Fig. 4, a and b) and the similar three-dimensional structure of STAT1 and STAT3 favors the idea that the nuclear import of these STAT molecules is regulated by the structural element described by us in the present work. STAT5α, STAT5b, and STAT6 seem to be different from STAT1–4, because they lack the N-terminal basic cluster and instead have six basic amino acids within nine residues at a site corresponding to residues Lys410 to Arg418 of STAT1 protein (Fig. 4a). Presently, the three-dimensional structure of STAT5 or STAT6 is not available, and therefore, it is not known whether this site is situated on the surface of the molecule. Experimental analysis of the possible involvement of this arginine/lysine-rich element in STAT5 or STAT6 nuclear import is yet lacking. It has also been suggested that the most N-terminal amino acids (first 129 residues) may regulate STAT nuclear translocation, because chimeric STAT1-STAT2 and STAT1-STAT5 proteins showed marked defects in their activation and nuclear translocation (48). These chimeric STAT proteins were, however, structurally altered, because they showed e.g. changed DNA-binding properties (48). Recently, it has also been suggested that the C-terminal nuclear localization sequence of IFN-γ regulates STAT1 alpha nuclear import at an intracellular site (49).

In the present report we have shown that the IFN-α-induced STAT nuclear import involves tyrosine phosphorylation and dimerization of STAT1 and STAT2, followed by rapid nuclear translocation and association with the p48 protein. We present evidence that a structural arginine/lysine-rich element in the DNA-binding domain of STATs regulates their nuclear import, a mechanism that could be common to all STAT proteins. We also demonstrate that the site regulating nuclear import of STATs has to be intact in both molecules of STAT dimers for the nuclear translocation to take place.

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