STAT2 is a transcription factor critical to the signal transduction pathway of type I interferons (e.g. IFNα). STAT2 resides primarily in the cytoplasm and is tyrosine-phosphorylated after IFNα binds to cell surface receptors. In response to tyrosine phosphorylation, STAT2 rapidly localizes to the nucleus and acquires the ability to bind specific DNA targets in association with two other proteins, STAT1 and IFN regulatory factor-9 (IRF-9). To elucidate the mechanisms that regulate cellular localization of STAT2, we investigated STAT2 nuclear trafficking both prior to tyrosine phosphorylation and after phosphorylation. Prior to phosphorylation, STAT2 is primarily resident in the cytoplasm, however, we found that it dynamically shuttles between nuclear and cytoplasmic compartments. The nuclear translocation of latent unphosphorylated STAT2 was found to be dependent on its constitutive association with IRF-9, and the export of STAT2 from the nucleus was contingent on its constitutive association with IRF-9, and the export of STAT2 from the nucleus was contingent upon the function of an intrinsic nuclear export signal within the carboxyl terminus of STAT2. STAT2 export could be inhibited with leptomycin B, indicating a nuclear export signal (NES) within STAT2 is recognized by the CRM1 exportin carrier. In contrast, following tyrosine phosphorylation, STAT2 dimerizes with phosphorylated STAT1 and accumulates in the nucleus. In the absence of STAT1, STAT2 does not accumulate in the nucleus. In addition, subsequent to nuclear import of phosphorylated STAT2, it redistributes to the cytoplasm within an hour coordinate with its dephosphorylation in the cytoplasm. The nuclear translocation of latent unphosphorylated STAT2 was found to be dependent on its constitutive association with IRF-9, and the export of STAT2 from the nucleus was contingent upon the function of an intrinsic nuclear export signal within the carboxyl terminus of STAT2. STAT2 export could be inhibited with leptomycin B, indicating a nuclear export signal within STAT2 is recognized by the CRM1 exportin carrier. In contrast, following tyrosine phosphorylation, STAT2 dimerizes with phosphorylated STAT1 and accumulates in the nucleus. In the absence of STAT1, STAT2 does not accumulate in the nucleus. In addition, subsequent to nuclear import of phosphorylated STAT2, it redistributes to the cytoplasm within an hour coordinate with its dephosphorylation in the nucleus. The regulation of STAT2 nuclear trafficking is distinct from the previously characterized STAT1 factor.

The signal transducers and activators of transcription (STATs) remain the only characterized DNA binding factors that are regulated directly by tyrosine phosphorylation (1–4). Tyrosine phosphorylation confers new properties to the STAT factors by inducing dimerization via reciprocal phosphotyrosine and Src homology 2 (SH2) domain interactions. This conformational change can contribute to nuclear translocation and is essential for binding to specific DNA targets. The STAT1 and STAT2 factors are tyrosine-phosphorylated in the cytoplasm by Janus kinases (JAKs) activated in response to type I interferon (IFN) (5, 38). The phosphorylated STATs subsequently dimerize and localize to the nucleus. The STAT2 factor is unique among the STATs in that it is associated constitutively with a distinct transcription factor, interferon regulatory factor-9 (IRF-9) (7–9). After phosphorylation, a trimeric complex forms of STAT1-STAT2-IRF-9, known as IFN-stimulated gene factor 3 (ISGF3), that binds to a specific IFN-stimulated response element in the promoters of responsive genes (reviewed in Refs. 10–12). A successful innate immune response to viral infection requires the induced expression of genes by ISGF3. Targeted gene disruptions of STAT1, STAT2, or IRF-9 clearly demonstrate that animals lacking one of these factors succumb to infection (13–15).

STAT1 and STAT2 do not appear to possess consensus NESs that function autonomously, however, dimerization via SH2-phosphotyrosine phosphorylated STAT1 dimers and STAT1-STAT2 dimers are rapidly imported to the nucleus and have been shown to interact with at least one specific importin-α family member, importin-α5. The DNA binding domain of STAT1 is critical for NES function of the dimer and recognition of importin-α5. Nuclear export similarly is important in regulating the cellular localization of signaling proteins like the STATs. Binding to nuclear
components, including DNA, can mask the NES and prevent nuclear export. The DNA binding domain of STAT1 contains a NES that can function autonomously and appears to be masked when STAT1 dimers are bound to DNA. Dephosphorylation in the nucleus releases STAT1 from DNA and exposes the NES to CRM1 resulting in the redistribution of STAT1 back to the cytoplasm. The DNA binding domain of STAT1 appears to have co-evolved with nuclear import and export signals to ensure it is in the right place at the right time, an elegant integration of its function as a signal transducer and activator of transcription.

STAT2 is distinct among the STATs in its ability to bind to IRF-9 constitutively, and this property suggested that STAT2 localization may be regulated differently from STAT1 (7). IRF-9, also known as p48 or IFN-stimulated gene factor 3 γ (ISGF3γ), is a member of the IRF family of transcription factors that play diverse roles in immunity and cellular response to viral infections (8, 26–28). Characterized IRFs contain nuclear localization signals that allow their translocation to the nucleus, however, certain IRFs reside in a latent state in the cytoplasm of the cell and only redistribute to the nucleus following an activating trigger. The unifying characteristic of the members of the IRF family is the similarity of their amino-terminal DNA binding domains. Their diverse carboxyl domains are known to associate with distinct transcription factors, and this property contributes to their unique effects on gene expression. The carboxyl region of IRF-9 associates with the amino-terminal region of STAT2, and the ability of IRF-9 to bind DNA contributes to specific target recognition of the multimeric ISGF3.

In this report we provide evidence that the unphosphorylated STAT2 molecule constitutively shuttles in and out of the nucleus. Unphosphorylated STAT2 is imported into the nucleus via association with IRF-9, but a functional NES in the carboxyl terminus of STAT2 results in localization of the STAT2-IRF-9 complex to the cytoplasm. Following tyrosine phosphorylation in response to IFN signaling, STAT2 dimerizes with STAT1, and this conformational change results in a gain of NLS function, similar to what occurs with the STAT1 homodimer. STAT2 therefore appears to have evolved mechanisms that regulate its nuclear trafficking both prior to and subsequent to tyrosine phosphorylation.

RESULTS

STAT2 Shuttles between Cytoplasm and Nucleus in the Absence of IFN Signaling—To investigate the cellular localization of STAT2 prior to and subsequent to IFN stimulation, endogenous protein was visualized microscopically by immunofluorescence staining. The results clearly demonstrated a cytoplasmic localization of latent unphosphorylated STAT2 in the absence of IFN stimulation (Fig. 1A, panel a). To determine whether unphosphorylated STAT2 is restricted to the cytoplasm or whether it translocates to the nucleus but is efficiently transported back to the cytoplasm, we tested the effects of leptomycin B, an inhibitor of the CRM1 exportin carrier. Treatment of cells with leptomycin B in the absence of IFN stimulation was found to cause the nuclear accumulation of STAT2 (Fig. 1A, panel b). This finding indicates that STAT2 normally shuttles between the nucleus and the cytoplasm, but dominant nuclear export mediated by CRM1 maintains latent STAT2 levels in the cytoplasm.

STAT2 has been shown to interact with the non-STAT factor IRF-9 both prior to IFN stimulation and following IFN stimulation (7, 9). We previously identified the coiled-coil domain of STAT2 (138–230 amino acids) to be sufficient to interact with IRF-9 (7). The amino terminus of IRF-9 contains a DNA binding domain and a basic-rich NLS that mediates its nuclear localization. Cellular localization studies of IRF-9 have shown that overexpression produces a predominant nuclear presence; however, simultaneous overexpression of STAT2 leads to sequestration of IRF-9 to the cytoplasm (9). This result prompted us to ascertain whether association of STAT2 with IRF-9 is required for nuclear translocation of unphosphorylated STAT2, or whether a constitutive nuclear localization signal is intrinsic to STAT2. To this end we analyzed localization of STAT2 in cells that lack endogenous IRF-9 (U2A cells) (32). Immunostaining of STAT2 in U2A cells demonstrated that STAT2 resided in the cytoplasm of these cells regardless of leptomycin B treatment (Fig. 1A, panels c and d). The failure of STAT2 to accumulate in the nucleus of U2A cells after treatment with

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**Fibrosarcoma cell lines HT1080, U3A (STAT1-deficient, gift of G. R. Stark, Cleveland Clinic Foundation Research Institute), U2A (IRF-9-deficient), and U6A (STAT2-deficient) were cultured in Dulbecco’s modified Eagle’s medium with 8% fetal bovine serum. Cells were treated with 1000 units/ml IFN-α (gift from Hoffman-LaRoche, Nutley, NJ) and 10 nM leptomycin B (gift from Barbara Wolff-Winiski, Novartis Research Institute, Austria). DNA transfections were performed with FuGene-6 (Roche Applied Science).

**Plasmid Constructs—**Expression plasmids pCDNA3-STAT1, pCDNA3-STAT2, and pCDNA3-IRF-9 were described previously (7). STAT2 deletion mutations and gene fragments were amplified by PCR with Pfu polymerase (Stratagene) and cloned into PCR-Blunt II TOPO (Stratagene) prior to subcloning upstream of green fluorescent protein (GFP) gene at the EcoRI site of EGF-N1 (Clontech). Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene), and all mutations were confirmed by DNA sequencing.

**Antibodies—**Rabbit anti-STAT2 antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunofluorescence (1:500 dilution), Western blot analyses (1:1000 dilution), and immunoprecipitation (1.0 μg/ml). Rabbit anti-STAT2 phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) was used in Western blot analyses (1:1000 dilution). Rabbit anti-ISGF3γ p48 antibody (C-20, Santa Cruz Biotechnology) was used for Western blot analysis (1:500 dilution) and immunofluorescence. Fluorescein isothiocyanate- or TRITC-labeled goat anti-rabbit immunoglobulins were used as secondary antibodies (1:200; Jackson ImmunoResearch Laboratories) for immunofluorescent staining. Horse radish peroxidase-conjugated anti-rabbit immunoglobulin was used as secondary antibody for Western blot analyses (Amersham Biosciences). Immunocomplexes were collected by binding to protein G-agarose beads (Invitrogen).

**Fluorescent Microscopy—**Cells were plated on glass coverslips and fixed with 4% paraformaldehyde. Endogenous proteins or exogenous proteins expressed in cells following 16-h transfection were evaluated using a Zeiss Axioskop (Carl Zeiss) equipped for epifluorescence with a GFP and rhodamine filter set (Chroma Technology). Images were captured with a Diagnostic Instruments Spot 2 camera (Sterling Heights, MI) and presented in Adobe Photoshop 4.0. Images are representative of reproducible results obtained from two to five independent experiments. 

**Western Blot Analysis—**Cells were lysed in 50 mM Tris, pH 8.0, 400 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 with protease inhibitors. Proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Proteins were detected by Western blotting with anti-STAT2 phosphotyrosine or anti-STAT2 antibodies, followed by secondary antibody and the enhanced chemiluminescence system (PerkinElmer Life Sciences).

**Importin Binding—**The plasmids encoding human importin-α family members were described previously (29) or were gifts of Drs. E. Hartmann and M. Kohler (University of Gottingen) (30). The importins were synthesized in vitro in the presence of [35S]methionine (IPT kit Promega). Radiolabeled proteins were incubated with bacterially expressed GST or GST-IRF-9 bound to glutathione beads (Sigma). The binding buffer contained 20 mM HEPES (pH 7.3), 110 mM potassium acetate, 5 mM sodium acetate, 1.5 mM magnesium acetate, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.2% Tween 20, and 1% nonfat dry milk. Following incubation, the complexes bound to glutathione beads were washed, and proteins were eluted and analyzed by SDS-PAGE and autoradiography.
leptomycin B indicated that IRF-9 was required for STAT2 nuclear import in the absence of IFN.

Although STAT2 localized in the nucleus following leptomycin B in the presence of endogenous IRF-9, the nuclear accumulation was not complete. This result suggested that the endogenous levels of IRF-9 were limiting. To investigate this possibility we overexpressed IRF-9 in U2A cells by transient transfection and evaluated localization of endogenous STAT2. Immunofluorescent staining of endogenous STAT2 demonstrated that STAT2 primarily resided in the cytoplasm of untreated cells overexpressing IRF-9 (Fig. 1B, panel a). Immunostaining of overexpressed IRF-9 in these same cells revealed a nuclear presence of IRF-9, suggesting endogenous STAT2 was limiting, and free IRF-9 accumulated in the nucleus (panel b).

Leptomycin B treatment of these cells led to a significant localization of STAT2 to the nucleus, but only in cells overexpressing IRF-9 (panels c and d). These results suggest that endogenous STAT2:IRF-9 complexes normally shuttle between the nucleus and the cytoplasm in untransfected cells, and STAT2 provides a functional NES that localizes the complexes to the cytoplasm.

**IRF-9 Interacts with Specific Importins**—A classic NLS has been identified within the amino terminus of IRF-9 that appears to be required for IRF-9 nuclear localization (9). There are six characterized mammalian importin-α family members that share homologous domains. Although importin-α carriers are homologous, there is evidence for tissue-specific expression, inducible expression, and functional specificity of different mammalian family members (30, 33–35). To investigate whether IRF-9 is recognized by specific members of the importin-α family, we performed in vitro importin-α binding assays. Five importin-α family members were synthesized in vitro in the presence of [35S]methionine and incubated with bacterially expressed glutathione S-transferase (GST) or GST-IRF-9 bound to glutathione beads. The bound importin proteins were eluted from the beads and detected by SDS-PAGE and autoradiography (Fig. 2). IRF-9 was found to bind to a specific subset of importin-α family proteins. Interaction was readily detected with importin-α3 and importin-α4, and less well with importin-α7. This is in distinct contrast to the binding of STAT1 tyrosine-phosphorylated dimers to importin-α5 (21).

**STAT2 Contains a Functional NES within Its Carboxy-terminal Domain**—To identify the region of STAT2 responsible for its nuclear export, we generated STAT2 amino- and carboxy-terminal deletion mutations linked to GFP and evaluated the cellular localization of truncated proteins in the presence of IRF-9. Linear depictions of the mutations tested are shown in Fig. 3A. STAT2 mutations that retain the coiled-coil domain would be predicted to interact with IRF-9 and be imported into the nucleus by the constitutive NLS in IRF-9 (7). A functional NES in STAT2 will redistribute the STAT2:IRF-9 complexes to the cytoplasm, however, the use of leptomycin B will maintain STAT2 nuclear presence by blocking CRM1-mediated export. Deletion of a functional NES in STAT2 but maintenance of binding to IRF-9 should result in accumulation of STAT2 in the nucleus in either the absence or presence of leptomycin B.

The STAT2-GFP constructs were co-transfected with IRF-9 into STAT2-deficient U6A cells to eliminate the contribution of endogenous STAT2 (Fig. 3B). Full-length STAT2 and STAT2 ΔN111 localized to the cytoplasm, but in the presence of leptomycin B they accumulated in the nucleus, consistent with the presence of a functional NES. The STAT2 ΔN235 lacks the coiled-coil domain that is responsible for binding to IRF-9, and, as expected, it did not translocate to the nucleus, demonstrating the IRF-9 requirement for nuclear import of STAT2. The carboxy-terminal deletions STAT2 397ΔC, STAT2 682ΔC, and STAT2 732ΔC accumulated in the nucleus in the absence of leptomycin B, indicating that these proteins lost the functional NES activity. The smallest carboxy-terminal deletion, STAT2 753ΔC, behaved as full-length STAT2 and accumulated in the cytoplasm unless cells were treated with leptomycin B, indicating the presence of the NES. Proper size and expression of deletion constructs were confirmed by Western blot (data not shown). These results together define a region between 732 and 753 amino acids that contains a sequence critical for STAT2 nuclear export.

**The Carboxyl Terminus of STAT2 Functions as an NES When Linked to GFP**—To confirm that the STAT2 carboxyl
The carboxyl terminus of STAT2 contains a functional NES. A, diagram of STAT2 and location of the IRF-9 binding region within the coiled-coil (C-C) domain, the DNA binding domain (DBD), the SH2 domain, and the site of tyrosine phosphorylation (pY). Deletions of the amino terminus (ΔN) or carboxyl terminus (ΔC) are shown with numbers corresponding to amino acids. Localization results are noted for each construct in the right panel as cytoplasmic (C) or nuclear (N) in cells expressing IRF-9 in the absence (−) or presence of leptomycin B (LMB) treatment. B, full-length STAT2-GFP (STAT2), as well as amino- and carboxyl-terminal deletion mutants of STAT2-GFP (ΔN or ΔC), were co-transfected with an IRF-9-expressing plasmid into STAT2-deficient U6A cells. Cells were untreated (left panel) or treated with leptomycin B (LMB) for 2 h (right panel). Localization of STAT2-GFP proteins was visualized by fluorescent microscopy. Fluorescent images for each transfection corresponds to 82–95% of the cell population and are representative of three independent experiments.

Fig. 3. The carboxyl terminus of STAT2 contains a functional NES. A, diagram of STAT2 and location of the IRF-9 binding region within the coiled-coil (C-C) domain, the DNA binding domain (DBD), the SH2 domain, and the site of tyrosine phosphorylation (pY). Deletions of the amino terminus (ΔN) or carboxyl terminus (ΔC) are shown with numbers corresponding to amino acids. Localization results are noted for each construct in the right panel as cytoplasmic (C) or nuclear (N) in cells expressing IRF-9 in the absence (−) or presence of leptomycin B (LMB) treatment. B, full-length STAT2-GFP (STAT2), as well as amino- and carboxyl-terminal deletion mutants of STAT2-GFP (ΔN or ΔC), were co-transfected with an IRF-9-expressing plasmid into STAT2-deficient U6A cells. Cells were untreated (left panel) or treated with leptomycin B (LMB) for 2 h (right panel). Localization of STAT2-GFP proteins was visualized by fluorescent microscopy. Fluorescent images for each transfection corresponds to 82–95% of the cell population and are representative of three independent experiments.

The carboxyl terminus contains a functional NES, we evaluated whether this region could promote nuclear exclusion of a heterologous protein, GFP. The small size of GFP (~27 kDa) and its lack of an intrinsic NLS or NES allow the protein to distribute between the nuclear and cytoplasmic compartments (Fig. 4A, panel a). Fusion of an NES-containing sequence to GFP should lead to its exclusion from the nucleus by active nuclear export. The carboxyl-terminal 200 amino acids (652–851) of STAT2 were fused to GFP and expressed in STAT2-deficient U6A cells to visualize its localization by fluorescence microscopy. The STAT2 652–851-GFP fusion was excluded from the nucleus, indicating the presence of a functional NES (panel b). A more carboxyl-terminal region of STAT2 (766–801 amino acids) linked to GFP was not excluded from the nucleus (panel d). A region of the DNA binding domain of STAT2 (389–407 amino acids) that shares homology to the NES region of STAT1 provided some cytoplasmic exclusion of GFP but notably only in cells expressing low levels of the construct (panel c).

The predominant NES of unphosphorylated STAT2 therefore appears to span the carboxyl-terminal domain region of STAT2 between amino acids 732–753. This region contains several stretches of leucine residues that could contribute to nuclear export activity (Fig. 4B). To determine the contribution of individual leucine residues in this region, site-directed mutagenesis was performed on the STAT2 652–851-GFP fusion. Leucine residues corresponding to amino acids 733, 740/741, 745, and 751 were replaced with alanine, and the mutations were tested for their effect on nuclear export (Fig. 4C). The L733A substitution had no apparent effect on export activity (panel a). However, L740/741/AA, L745A, and L751A ablated the nuclear export function. These results indicate that a functional NES in the carboxyl terminus of STAT2 exists in amino acid region 740–751.

STAT2 Requires STAT1 but Not IRF-9 for Nuclear Localization following IFN-α Stimulation—In response to IFN-α, STAT1 and STAT2 become tyrosine-phosphorylated and associate via SH2-phosphotyrosine interactions. The STAT1–STAT2–IRF-9 complex, known as interferon stimulated factor-3 (ISGF3), forms in the cytoplasm prior to nuclear translocation (36, 37). Nuclear transport of the tyrosine-phosphorylated ISGF3 complex appears to result from a conformational change of the proteins and recognition by the importin-α5 carrier (21, 23, 25). Although IRF-9 is known to be essential for the ability of ISGF3 to bind the interferon-stimulated response element DNA sequence, its role in the nuclear import of ISGF3 remains to be evaluated. To determine whether IRF-9 binding to STAT2 was required for proper nuclear localization in response to IFN-α, we analyzed localization of endogenous STAT2 by immunofluorescence in the presence or absence of IRF-9. In the absence of IFN stimulation, unphosphorylated STAT2 resided primarily in the cytoplasm of human fibrosarcoma cells (HT1080) and a cell line derivative that lacks endogenous IRF-9 (U2A) (32) (Fig. 5). In response to IFN-α signaling, tyrosine-phosphorylated STAT2 accumulated in the nucleus of both HT1080 and U2A cells, demonstrating that IRF-9 is not required for IFN-α-induced nuclear import of STAT2. We also evaluated the localization of tyrosine-phosphorylated STAT2 in a cell line derivative lacking STAT1 (U3A) (38). In contrast to HT1080 or U2A cells, STAT2 did not accumulate in the nucleus of U3A cells after IFN-α treatment, although STAT2 was accurately tyrosine-phosphorylated (Ref. 39 and data not shown). Therefore the formation of a heterodimer of STAT1–STAT2 is required for nuclear accumulation of tyrosine-phosphorylated STAT2. The specific importin that was previously shown to bind the STAT1–STAT2 heterodimer, importin-α5, is notably distinct from the importin-α members that recognize IRF-9 (Fig. 2) (23).

STAT2 Nuclear Trafficking in Response to IFN-α Signaling—To investigate the dynamics of STAT2 nuclear trafficking,
we used immunofluorescence of endogenous STAT2 to follow its localization with time after IFN-α. HT1080 cells were treated with IFN-α for a 30-min pulse, then the IFN-α was removed from the culture, and STAT2 localization was evaluated at various times subsequent to IFN-α removal from 0 to 120 min (Fig. 6A, left panels). STAT2 efficiently accumulated in the nucleus of cells after a 30-min IFN-α pulse (0 min), but by 30 min after removal of IFN-α, STAT2 began to reappear in the cytoplasm (30 min). Between 60 and 120 min after IFN-α removal, the majority of STAT2 returned to the cytoplasm. The reappearance of STAT2 in the cytoplasm indicated an active nuclear export of the molecule. To determine whether export of STAT2 was mediated by CRM1, the same experiment was performed in the presence of the specific CRM1 inhibitor, leptomycin B (Fig. 6A, right panels). Leptomycin B dramatically inhibited the redistribution of STAT2 to the cytoplasm following IFN-α treatment. The effect was readily apparent at 60–120 min after IFN-α stimulation. These results provide evidence that CRM1 plays a major role in the nuclear export of STAT2 following activation by the IFN-α/H9251 signal pathway.

To investigate whether STAT2 nuclear export following IFN stimulation correlated with the state of STAT2 tyrosine phosphorylation, we examined the phosphorylation status of STAT2 after IFN-α treatment in the absence or presence of leptomycin B. A similar IFN pulse-chase experiment as described in Fig. 6A was performed, but cell lysates were prepared and tyrosine-phosphorylated STAT2 was detected by Western blot with specific STAT2 phosphotyrosine antibodies (Fig. 6B). 30 min of IFN treatment led to a dramatic increase in STAT2 tyrosine phosphorylation, however, this phosphorylation decreased following the removal of IFN. Densitometric analyses of the STAT2 phosphotyrosine signal (anti-STAT2pY) normalized to the total STAT2 signal (anti-STAT2) revealed a decrease of about 25% after 30 min, 70% by 60 min, and greater than 90% by 120 min (left panel). The kinetics of dephosphorylation correlated well with the redistribution of STAT2 from the nucleus back to the cytoplasm. Blocking STAT2 nuclear export with leptomycin B treatment did not alter the kinetics of dephosphorylation (right panel), indicating that dephosphorylation of STAT2 can occur in the nucleus. Dephosphorylation of STAT2 results in its dissociation from STAT1 and consequent dissociation from DNA target sites. The change in conformation and displacement of STAT2 from DNA may correlate with CRM1 recognition of the STAT2 export signal.

STAT2 Nuclear Export following IFN Stimulation—The carboxyl-terminal NES of STAT2 clearly plays an active role in
nuclear export of unphosphorylated STAT2/IRF-9 complexes prior to IFN-α signaling. To determine whether this newly identified NES contributes to the export of STAT2 following tyrosine phosphorylation and dimerization with STAT1, we evaluated the cellular localization of full-length STAT2 with specific NES point mutations following IFN-α stimulation. STAT2-deficient U6A cells were transfected with wild type STAT2-GFP or a carboxyl-terminal NES point mutant, STAT2-GFP L740A,L741A, and stimulated with IFN-α for 30 min (Fig. 7). The cellular localization of STAT2 was evaluated by fluorescence microscopy following IFN-α removal. In comparison to wild type STAT2, which was exported by 60 min following IFN withdrawal, the carboxyl-terminal NES mutation remained nuclear at 60 min of withdrawal, indicating defective export (compare panels e and f). However, by 120 min much of the NES mutant protein reappeared in the cytoplasm. There were no significant differences in phosphorylation between the wild type and the NES mutation as judged by a Western blot to evaluate tyrosine-phosphorylated STAT2 following IFN-α (data not shown). Together our results indicate that there is a strong NES in the carboxyl terminus, and apparently one or more different weaker NESs in STAT2 or an associated molecule that serve to export STAT2 from the nucleus subsequent to its dephosphorylation.

**DISCUSSION**

Proper cellular localization is critical to the function of signaling molecules, particularly transcription factors. For this reason we investigated the nuclear-cytoplasmic localization of STAT2, a transcription factor essential for the innate immune response to type I IFN signaling. Fluorescence microscopy was used to evaluate the localization of unphosphorylated STAT2 in comparison to tyrosine-phosphorylated STAT2. Although STAT2 predominantly resides in the cytoplasm of unstimulated cells, the use of an inhibitor of nuclear export, leptomycin B, clearly revealed its presence in the nucleus. This result indicated that unphosphorylated STAT2 is not static but dynamically shuttles between the nucleus and cytoplasm. STAT2 is distinct among the STAT members in that it binds to a non-STAT factor, IRF-9 (7). Because IRF-9 possesses a constitutive NLS, we tested the possibility that the STAT2/IRF-9 complex is transported into the nucleus by IRF-9 (9). Evaluation of cells that lack IRF-9 and mutations of STAT2 that do not bind IRF-9 demonstrated the nuclear import of unphosphorylated STAT2 (Fig. 8, top). Nuclear entry of STAT2 in the absence of IFN signaling depended on the interaction of its amino-terminal coiled-coil domain with IRF-9, and IRF-9 was demonstrated to interact directly with importin-α3, -α4, and -α7. Mutational analyses of STAT2 were performed to identify a functional NES that must mediate export of STAT2/IRF-9 from the nucleus in the absence of IFN signaling. By analyzing deletion mutations of STAT2, we identified a distinct functional NES in its carboxyl terminus. The carboxyl NES plays a dominant role in nuclear export of the unphosphorylated STAT2. The nuclear-cytoplasmic shuttling of the STAT2/IRF-9 complex is regulated by nuclear export of unphosphorylated STAT2/IRF-9 following IFN-α signaling.
complexes may contribute to a rapid reaction in response to IFN signaling. Alternatively, the NES of STAT2 may contribute to signal silencing by sequestering IRF-9 to the cytoplasm to eliminate an active DNA binding factor from the nucleus. Because overexpression of IRF-9 alone was found by our group to eliminate an active DNA binding factor from the nucleus. It is not certain how nuclear-cytoplasmic shuttling is a benefit to a signaling response. There is evidence that factors such as Smad4 may have dual roles in the nucleus and bind to repressors when inactive (31). STAT2-IRF9 complexes shuttle dynamically between the nucleus and cytoplasm, and the function of a constitutive NES in STAT2 may serve to remove a transcription factor from the nucleus and decrease untoward effects on gene expression.

Although the members of the STAT transcription factor family share many properties, they are distinguished by their activation in response to different ligands and kinases, their interaction with distinct transcription factors, and their regulation of distinct gene subsets. As we describe in this report, the regulation of STAT2 nuclear trafficking is distinct from that of STAT1. These unique characteristics of STAT molecules contribute to their individual roles in specific biological responses.

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