Nuclear Export Determines the Cytokine Sensitivity of STAT Transcription Factors

Inga Lödige, Andreas Marg, Burkhard Wiesner, Barbara Malecová, Thomas Oelgeschläger, and Uwe Vinkemeier

From the Abteilung Zelluläre Signalverarbeitung, Leibniz-Forschungsinstitut für Molekulare Pharmakologie, Freie Universität Berlin, 13125 Berlin, Germany, the Abteilung Molekulare Medizin, Leibniz-Forschungsinstitut für Molekulare Pharmakologie, 13125 Berlin, Germany, and the Transcription Laboratory, Marie Curie Research Institute, Oxted, Surrey RH8 0TL, United Kingdom

Cytokine-dependent gene activation critically depends upon the tyrosine phosphorylation (activation) of STAT transcription factors at membrane-bound cytokine receptors. The extent of STAT activation and hence the specificity of signaling is primarily determined by structural complementarity between the SH2 domain of the STATs and the tyrosine-phosphorylated receptor chains. Here, we identified constitutive nucleocytoplasmic shuttling as another mechanism that controls the differential activation of STAT transcription factors. Our analysis of nucleocytoplasmic cycling of STAT1 revealed that the expression of the alternatively spliced transactivation domain and its signal-dependent serine phosphorylation maximized the rate of nuclear export. Export modulation occurred independently of retention factors or the export receptor CRM1, and was observed both before and during stimulation of cells with cytokines. Our data indicated a dual role for the transactivation domain. It enhanced the nuclear retention of activated STAT1, but had the opposite effect on inactivated molecules. Accordingly, and despite their identical receptor recognition, the STAT1 splice variants differed strongly in the amplitude of tyrosine phosphorylation and in the duration of the cytokine signal. Thus, regulated nuclear export determined the cytokine sensitivity of the shuttling STAT1 transcription factors by controlling their availability at the receptor kinase complex.

Cytokines such as interferon γ (IFNγ) engage the Janus kinase/signal transducer and activator of transcription (STAT) signaling pathway during inflammatory and immune responses (1). Activation of this pathway by IFNγ induces the phosphorylation of an intracellular tyrosine residue in the α-chain of the IFNγ receptor. This, in turn, creates the docking site specifically for the SH2 domain of STAT1. Subsequently, phosphorylation of residue tyrosine 701 of STAT1 triggers the dissociation from the receptor and SH2 domain-mediated STAT1 homodimerization that transforms the latent transcription factor into a high affinity DNA-binding protein (2). The STATs are nucleocytoplasmic shuttling proteins that make use of both carrier-independent and carrier-mediated translocation mechanisms (3) (Fig. 1A). Before tyrosine phosphorylation, direct binding to proteins of the nuclear pore (nucleoporins) allows carrier-independent transportation of STAT1 (4). Additionally, the exportin CRM1 supports nuclear export to a minor extent (4). Tyrosine-phosphorylated STAT1 dimers, on the other hand, are barred from further carrier-independent nucleocytoplasmic exchange (4). Instead, association with importin α5 and importin β is necessary for continued nuclear import (5, 6). However, tyrosine phosphorylation triggers the nuclear retention of the shuttling STAT1, which is kept in the nucleus until tyrosine dephosphorylation takes place. The inhibition of the dephosphorylation reaction afforded by DNA binding suffices to bring about the easily observable accumulation of STAT molecules in the nucleus (7). Despite that, given a half-life of no more than 15 min for activated STATs even at optimal DNA binding sites (8), the majority of STAT molecules returns to the cytoplasm in only a few minutes or less.

In the nucleus, the activated STATs function as sequence-specific transcription factors. Gene activation by STAT1 is dependent on the C-terminal transactivation domain of 38 residues (9), expression of which is regulated by alternative splicing, generating full-length STAT1a comprising 750 residues or truncated STAT1b (10). Moreover, phosphorylation on serine 727 of a conserved PMSP motif in the transactivation domain maximizes gene transcription (11–13). In human cells serine phosphorylation occurs independent of STAT1 tyrosine phosphorylation (14), and several stimuli (UV irradiation, tumor necrosis factor α) can induce the serine phosphorylation of STAT1 in the absence of tyrosine phosphorylation (15). Interferon-γ, on the other hand, promotes both Ser727 as well as Tyr701 phosphorylation, but these events occur independently of one another (14). Given the diverse set of stimuli that induce serine phosphorylation of STAT1, it is not surprising that numerous kinases have been implicated in this process (15).

The duration and degree of gene activation in response to cytokine signals is primarily dictated by the number of activated cell surface receptors (16). Multiple mechanisms have been described that exert a negative influence on the activity of STAT proteins, e.g. tyrosine dephosphorylation, the inhibition of DNA binding, receptor degradation, or the inhibition of Jak kinases (17). Little is known, however, about signal enhancing mechanisms. Best characterized is the increased incorporation of STAT1s in transcription complexes at cytokine responsive promoters via their transactivation domain (TAD). The STAT1 TAD is responsible for the selective recruitment of histone acetyltransferases and other coactivators of transcription, several of which bind more strongly to serine 727-phosphorylated STAT1 (18–20).

We have analyzed the nucleocytoplasmic translocation of STAT1
STAT1 Activity Determined by Nuclear Export

both before and during the cytokine stimulation of cells. The results disclose a novel function of the transactivation domain and its serine phosphorylation in nucleocytoplasmic shuttling of STAT1, and demonstrate a stimulatory role for nuclear export in cytokine signaling.

MATERIALS AND METHODS

Cell Culture and Reagents—HeLa-S3 cells and U3A cells were grown as described (21). Human IFNγ (Caltbiochem) was used at a concentration of 50 units/ml (5 ng/ml) or as indicated; human Epo was purchased from Roche Applied Science. MG132 (50 μM final), cycloheximide (10 μg/ml final), and staurosporine were from Sigma. Ratjadone A was a kind gift of M. Kalles, Universität Hannover.

Purification of Recombinant STAT1, in Vitro Tyr701 Phosphorylation, and Microinjection—STAT proteins were expressed in baculovirus-infected S9 insect cells. Protein purification, in vitro tyrosine phosphorylation, purification of tyrosine-phosphorylated STAT1, and microinjection, were done essentially as described (4, 21).

In Vitro Ser727 Phosphorylation of STAT1—GST-p38 and malE-SKK3-DD were expressed in Escherichia coli BL21p(Lys)S. GST fusion protein was purified on a glutathione-Sepharose affinity matrix as described by the manufacturer (Amersham Biosciences). Expression and purification of the malE-fusion protein was essentially as described (22). STAT1 prepared from baculovirus-infected insect cells was virtually devoid of phosphorylation at position Ser727 (mass spectrometric limit of detection = 5%, data not shown). In vitro Ser727 phosphorylation of recombinant STAT1α was carried out for 3 h at 30 °C in a volume of up to 2 ml. Unphosphorylated or Tyr701-phosphorylated recombinant STAT1α (3 μM) was incubated in phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM Na₃VO₃, 5 mM glycerophosphate, 1 mM ATP, 2 mM dithiothreitol, and Complete (Roche Applied Science) protease inhibitor) with GST-p38 and malE-SKK3-DD (1.5 μM each).

The two kinases were removed by Tag-specific affinity purification. STAT1 Ser727 phosphorylation was confirmed by Western blotting with phosphospecific antibody (Upstate Biotech). Subsequent mass spectrometric analyses indicated Ser727 phosphorylation of at least 80% and 50% of the non-tyrosine-phosphorylated and Tyr701-phosphorylated STAT1, respectively (not shown).

EMSA—For EMSA analyses, cytoplasmic and nuclear extracts from reconstituted U3A cells were pooled and mixed with a radiolabeled M67 probe essentially as described (21). Where indicated, c-Eyk tyrosine kinase was coexpressed to maximize STAT1 activation (21).

Quantitative Western Blotting—U3A or HeLaS3 cells growing on 10-cm dishes were transiently transfected with the indicated STAT1 constructs using the Lipofectamine (Invitrogen) method. After 24 h, cells were split according to the number of time/dosage points needed. For Epo stimulation, cells were cotransfected with a 3-fold molar excess of pRcCMV-EgαBglII. This construct misses the BglII fragment of the human gp130 receptor, therefore the major STAT3 phosphorylation site at residue Tyr877 is not expressed, whereas the STAT1 phosphorylation sites are retained (23). Cell lysis and Western blotting were performed essentially as described (21). The following primary antibodies were used: phosphospecific anti-Tyr701 STAT1 (Cell Signaling), phosphospecific anti-Ser727 STAT1 (Upstate), anti-STAT1 (p84/p91) (E23, Santa Cruz Biotechnology), anti-β-actin (Sigma). Blots were developed with an appropriate horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Biosciences). Signals were recorded on a Roche Lumi-Imager and quantitation (arbitrary units) was done using the LumiAnalyzer 3.0 software (Roche Applied Science). To allow for the comparison of signal intensities between different blots, blotting was done side by side on the same blotting appa-

tatus, and the blots were developed side by side in the same antibody solutions as indicated in the figures. To determine the specific tyrosine phosphorylation (ratio of [phosphospecific signal/STAT signal]), the bound phosphospecific anti-Tyr701 antibody was stripped off the blot, and the same blot was incubated with an anti-STAT1 antibody. Antigen concentrations and exposure times were adjusted not to exceed the linear signal range.

FRAP, Conventional, and Confocal Fluorescence Microscopy—Conventional fluorescence microscopy and image quantitation were as described (7). Confocal microscopy was done with a LSM410 laser scanning confocal microscope (Zeiss) using a 100×/1.3-numerical aperture oil immersion objective and a 30-milliwatt argon laser. Fluorescence images of single z-sections (optical slice of 0.7 μm) were scanned. High frequency imaging was done with transiently transfected HeLa cells that displayed similar GFP fluorescence in the nucleus. Stimulation with IFNγ was for 30 min. Approximately 100 images were acquired before bleaching, the last ten of which were used to calculate the average pre-bleach intensity. A region of interest (ROI) comprising 0.5% of the nucleus z-section was bleached (30 laser pulses of maximal intensity comprising a total bleach time of 4.35 ms over a time period of 0.55 s). Subsequently, imaging continued at the prebleach rate for 10 s. Fluorescence recovery of the ROI was subjected to curve fitting by using two exponentials (PRISM program) to determine bleach depth (first image after bleaching), equilibration (fluorescence recovery), and half time of equilibration (time until half recovery).

In Vitro Transcription and Reporter Gene Assays—Standard 60-min in vitro transcription and primer extension analysis was performed as reported previously (24). pDL(GAS2)HIV(−33/+80)(−) contains two STAT1 binding sites (Ly6E-GAS) in front of the HIV-1 core promoter from −33 to +80. See supplementary information for cloning details. To analyze STAT1 transcription activation, promoter templates were preincubated with recombinant STAT1 protein (50, 150, or 500 ng) for 15 min at 30 °C under transcription conditions. STAT1-activated transcription from pDL(GAS2)HIV(−33/+80)(−) and transcription from the control construct pTOG5HIV(−33/+68)(−) was measured in parallel by primer extension using the same radiolabeled oligonucleotide. The integrity of serine-phosphorylated recombinant STAT1 during in vitro transcription was confirmed by Western blotting with a phospho-Ser727-specific antibody (Upstate Biotech). Subsequent mass spectrometric analyses indicated Ser727 phosphorylation of at least 80% and 50% of the non-tyrosine-phosphorylated and Tyr701-phosphorylated STAT1, respectively (not shown).

Plasmids, Peptide Synthesis, and Coupling to GST-GFP—See supplementary information.

RESULTS

The Transactivation Domain Facilitates the Nuclear Export of STAT1—We have previously examined the nucleocytoplasmic translocation of non-phosphorylated STAT1 in resting cells (4). It was found that truncated STAT1 (STAT1tc), a mutant generated by the concurrent deletion of N- and C-terminal domains, was still capable of constitutive nucleocytoplasmic shuttling. Yet, whereas nuclear import remained unaffected, the nuclear export of truncated STAT1 was diminished. To dissect which domain deletion reduced export, nuclear microinjections of purified recombinant full-length STAT1α, STAT1β (the splice variant lacking the C-domain), STAT1ΔN (lacking the N-terminal 126 residues), and STAT1tc were performed (Fig. 1B). As is shown in Fig. 1C, removal of the N-domain was without effect on nuclear export (1ΔN), whereas the translocation of the C-terminally truncated STAT1β and STAT1tc was mark-
**STAT1 Activity Determined by Nuclear Export**

**FIGURE 1**: Influence of the C-domain on STAT1 nuclear export in resting cells. A, model of STAT1 nucleocytoplasmic cycling (adopted from Ref. 3). Non-Tyr701-phosphorylated STAT1 (gray arrows) constantly shuttles between the nuclear and cytoplasmic compartments in a carrier- and energy-independent fashion; in addition, the export receptor CRM1 promotes nuclear export of non-Tyr701-phosphorylated STAT1 to a minor extent. Tyr701-phosphorylated STAT1 dimers (blue arrows) are barred from carrier-free nucleocytoplasmic cycling and depend on importin α5 for nuclear import. Nuclear export requires prior Tyr701 dephosphorylation. (+P) indicate Tyr701 phosphorylation and dephosphorylation, respectively. Arrow widths are proportional to their corresponding flux rates at the onset of IFN stimulation. B, domain structure of the STAT1 derivatives used in this work with the span of N (N); coiled coll (CC), DNA binding (DNA), linker or nuclear pore complex binding (NPC), SH2 (SH2), and transactivation domain (TAD). Phosphorylation sites (Y701, S727) and the regions required for importin binding (dsNLS) are indicated. C, comicroinjection of non-phosphorylated STAT1 variant proteins and fluorescein isothiocyanate-labeled bovine serum albumin (injection site marker) into the nuclei of resting HeLa cells. After incubation for 2 h and fixation of the cells, immunocytochemistry with a STAT1 antibody and staining of nuclei with Hoechst dye followed. Asterisks mark two cells that received microinjection in the cytoplasm. Bar, 20 μm.

The transactivation domain regulates STAT1 nuclear export during cytokine stimulation of cells—stimulation of cells with cytokines triggers the nuclear accumulation of the tyrosine-phosphorylated dimers, which precludes the direct observation of the concurrent nuclear export of non-phosphorylated molecules. To circumvent this problem we devised an assay that takes advantage of the different translocation requirements of tyrosine-phosphorylated and non-tyrosine-phosphorylated STAT1. We reasoned that precluding the binding of tyrosine-phosphorylated STAT1 dimers to importin α should abrogate the nuclear accumulation of STAT1 during cytokine stimulation, whereas the nuclear export should not be affected. Under these conditions the ensuing accumulation of activated STAT1 in the cytoplasm might therefore lay bare the ongoing nuclear export of STAT1 from the nucleus. A schematic outline of this assay is shown in Fig. 2A. STAT1 harbors two non-conventional nuclear localization signals that both are necessary specifically for the import of activated dimers. One is a short stretch of amino acids in the DNA binding domain, whereas the amino-terminal domain of 120 residues constitutes the other one (5, 21, 25). Inactivation of either signal precludes the binding of tyrosine-phosphorylated STAT1 to importin α (5, 25), and thus prevents the nuclear import (supplementary Fig. S1A). In addition to its effect on nuclear import, the deletion of the N-domain is associated with a dephosphorylation failure that renders the activation of STAT1 virtually irreversible (25–27). We found that the N-domain deletion mutants used in this study did not differ in their very low dephosphorylation rates (supplementary Fig. S2A). Thus, during the cytokine stimulation of cells, the pool of non-phosphorylated STAT1 variants available for carrier-independent nuclear import will be depleted. Nevertheless, in all other respects that are of importance here the ΔN mutants are indistinguishable from wild-type STAT1. The N-domain is not required for receptor recognition and tyrosine phosphorylation (26, 27). Moreover, the deletion of the N-domain does not affect SH2 domain-mediated dimerization and DNA binding (supplementary Fig. S1B; see also Refs. 26 and 27), and the N-domain is not required for the constitutive nucleocytoplasmic transport of unphosphorylated STAT1 (Ref. 4 and Fig. 1C). Therefore, N-domain deletion mutants were used in this assay.

To examine nuclear export during cytokine stimulation, mammalian cells were transfected with plasmids encoding the N-domain deletion mutants STAT1ΔN, STAT1tc, or STAT1ΔN Ser727→Ala. First, the respective proteins were characterized in terms of expression levels and DNA binding ability. Differences among the mutants or with wild-type STAT1 were not found (see supplementary Fig. S1B, S1C, and S1D). Moreover, similar to wild-type STAT1, these STAT1 variant proteins were present in both the cytoplasm and the nucleus (Fig. 2B). Next, the response to IFNγ was tested. As expected, control cells expressing full-length STAT1α showed the well described nuclear accumulation during the stimulation with IFN (Fig. 2B). In striking contrast, but in accordance with the model of STAT1 nucleocytoplasmic shuttling depicted in Fig. 2A, the N-terminally truncated mutants reacted inversely to IFNγ and gradually disappeared from the nucleus (Fig. 2B). Notably, the mutation to phenylalanine of the Tyr701 phosphorylation site abolished the IFN responsiveness (Fig. 2B), indicating that phosphorylation of STAT1 were required for the observed effects. Treatment of cells with MG132 to inhibit proteosomal degradation, or with cycloheximide to suppress protein translation did not influence the clearance of STAT1 from the nucleus (not shown). However, time course experiments with IFN-stimulated cells revealed differences in the rate of clearance from the nucleus. STAT1ΔN achieved discernible nuclear clearance in more than 60% of the cells already after 1 h of IFNγ stimulation, and after 2 or 3 h in the presence of IFNγ. STAT1ΔN was depleted from the nucleus in more than 80% of the cells. The nuclear clearance of STAT1tc, which additionally lacks the transactivation domain, occurred much more slowly. After 1 h of IFNγ stimulation, STAT1tc was depleted from the nucleus in about 10% of the cells. Nevertheless, after IFNγ treatment for 3 h or longer STAT1tc was cleared from the nuclei of more than 80% of cells. Remarkably, the mutation to

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FIGURE 2. Influence of the STAT1 C-domain on nuclear export in cytokine-stimulated cells. 

A, model of nucleocytoplasmic cycling of STAT1 N-domain deletion mutants at the onset of cytokine stimulation. Carrier-free nucleocytoplasmic cycling and CRM1-dependent nuclear export of non-Tyr701-phosphorylated STAT1 occur irrespective of the presence of the N-domain (gray arrows). In contrast, the nuclear import of the Tyr701-phosphorylated N-domain deletion mutant is blocked (blue arrow). 

B, top, HeLa-S3 cells transiently expressing GFP fusion proteins of the indicated STAT1 variants were left untreated or stimulated with IFNγ for the indicated times. 10 ng/ml ratjadone A (+Ratj) were added 1 h before IFNγ stimulation. Shown is the GFP fluorescence of fixed cells. Bar, 20 μm. Bottom, quantitative analysis of B. At the indicated time points, the ratio of nuclear to cytoplasmic fluorescence density was determined for 30–60 cells that expressed STAT1ΔN, STAT1tc, or the Ser727 to Ala mutant of STAT1ΔN. Cells were scored positive for cytoplasmic accumulation if the nuclear fluorescence density/cytoplasmic fluorescence density ≤ 0.5. C, graph showing the results of live cell fluorescence imaging of STAT1-negative U3A cells coexpressing STAT1ΔN-GFP or STAT1tc-GFP and chimeric Epo receptor (3-fold molar excess of DNA). After taking the first image (t = 0), cells were stimulated with Epo (7 units/ml), and further confocal microscopical images of the median slice were taken every 10 min for another 3 h. Shown is the ratio of nuclear and cytoplasmic fluorescence density (mean ± S.D.) of five cells for each STAT1 mutant.
alanine of the Ser277 phosphorylation site in the transactivation domain generated an intermediate phenotype. Although cytoplasmic accumulation of the serine mutant was apparent in 80% of the cells after 2 h of IFN/β/IFN/H253, the rise in nuclear clearance was slower. This became apparent at the 1-h time point, when only slightly more than 20% of cells displayed nuclear clearance (Fig. 2B), indicating that serine phosphorylation of the transactivation domain is required for maximal nuclear export.

To rule out IFN/γ-specific effects and to exclude interference from endogenous wild-type STAT1, the nuclear clearance experiments were repeated with STAT1ΔN and STAT1tc in erythropoietin (Epo)-stimulated STAT1-negative U3A cells (9). For these experiments, cells were cotransfected with the STAT1 mutants, and a chimera comprising the extracellular domain of the Epo receptor fused to the transmembrane and intracellular regions of a modified gp130 receptor chain that strongly activates STAT13 (23). Twenty-four hours after the transfection, the cells were treated with Epo, and the response of the N-terminally deleted STAT1 molecules was recorded by confocal microscopy in living cells. As was seen before in response to IFN/γ, the activation of truncated STATs by Epo resulted in their clearance from the nucleus. In accordance with the results obtained by comparison of cell pools (Fig. 2B), the time lapse microscopy of individual cells also revealed reduced nuclear export of C-terminally deleted molecules (Fig. 2C). In summary, these results demonstrated that the transactivation domain facilitated the nuclear export of STAT1. In addition, these observations with cytokine-stimulated transfected cells confirmed the results that were obtained by the microinjection of recombinant proteins into resting cells (Fig. 1C).

**The Transactivation Domain Modulates Nuclear Export Independent of CRM1 and Retention Factors**—In addition to carrier-independent export modes, the export receptor CRM1 was demonstrated to contribute to nuclear export of STAT1. Therefore, we used ratjadone A, a highly specific inhibitor of CRM1, to explore the role of this transport receptor in the export modulation of STAT1 (28). Whereas there is no discernible leucine-rich canonical NES in the transactivation domain that may serve as CRM1 recognition site (not shown), the absence of the C-domain might nevertheless diminish the interaction with CRM1. As shown in Fig. 2B, the pharmacological inactivation of CRM1 by ratjadone unexpectedly decelerated nuclear clearance of the STAT1 variants, but their nuclear export continued. Importantly, the inactivation of CRM1 did not abolish the translocation differences that were caused by

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**FIGURE 3. Influence of the transactivation domain on the intranuclear mobility of STAT1 variant proteins.** High speed FRAP analyses of single z-sections through the nucleus of unstimulated (A) or IFN-γ-stimulated cells (B). To achieve comparable tyrosine phosphorylation of STAT1, cells expressing STAT1α, STAT1-DNAmin, or GFP were treated with 1.0 ng/ml IFN/γ, whereas 5.0 ng/ml IFN/γ were used with STAT1β, STAT1ΔN, or STAT1tc-expressing cells. Cells were imaged every 16 ms. Top, shown is the prebleach fluorescence intensity, the bleach period, and the fluorescence recovery kinetics during the FRAP experiment. Bottom, enlargement of A, showing the first 1.5 s of the fluorescence recovery curves immediately after photobleaching. The intersection with the y-axis indicates the bleach depth. Each curve represents the mean fluorescence ± S.D. of at least five cells. See supplementary information for a statistical analysis.
FIGURE 4. Nuclear export determines the amplitude of STAT1 tyrosine phosphorylation. A, Western blot of purified in vitro tyrosine-phosphorylated STAT1α and -β (5 ng each, degree of Tyr<sup>701</sup> phosphorylation >95% as determined by mass spectroscopy, data not shown). Shown are results with a phospho-Tyr<sup>701</sup>-specific antibody (top) and the reprobing with a STAT1 antibody (bottom). B, U3A cells transiently expressing STAT1αN or STAT1tc were stimulated with IFNγ for the indicated times. Quantitative Western blot analyses of whole cell extracts probed with Tyr<sup>701</sup>-phosphospecific antibody and reprobed with a STAT1 antibody are shown. C, identical to B, but the STAT1 derivatives expressed a canonical
the expression of the transactivation domain. Clearly, even in the absence of functional CRM1, the STAT1ΔN protein was exported much faster than the C-terminally deleted STAT1tc (Fig. 2B, +RatJ). We therefore concluded that CRM1 was not involved in the export modulation by the transactivation domain. Moreover, we found no evidence for a transferable export function in the transactivation domain. We expressed the STAT1 C terminus as GFP fusion proteins, and additionally, carrier-coupled synthetic peptides comprising the C-terminal 38 residues (without or with Ser27 phosphorylation; not shown) were injected into the nucleus of HeLa cells. These experiments resulted in the pancellular distribution of the C-domain fusion constructs similar to GFP alone, but not in their accumulation in the cytoplasm (not shown).

In addition, the mobility of STAT1 variant proteins was determined to explore the relationship between nuclear export and intranuclear mobility. We used fluorescence recovery after photobleaching (FRAP) analyses and time-lapse microscopy of HeLa cells that expressed GFP fusion proteins of STAT1α, STAT1β, STAT1ΔN, or STAT1tc. As controls, GFP or the mutant STAT1αDNA−min, which is devoid of DNA binding, was included (7). First, the mobility in the nucleus of unstimulated cells was examined. Images were acquired every 16 ms already before bleaching (fluorescence intensity = 100%), and the measurement was continued at this speed for another 10-s postbleach. As shown in Fig. 3A, all STAT1 variants as well as the green fluorescent protein displayed a fast nuclear fluorescence recovery. Notably, when the half-maximal recovery times were calculated, there was no statistically significant difference between the STAT1-GFP fusion proteins and GFP (data not shown), thus stressing the rapid and transient interaction of STAT1 at nuclear binding sites. Therefore, we compared the bleach depths, defined as (prebleach intensity minus postbleach intensity/prebleach intensity) × 100, of the variant proteins. For very dynamic processes and limited laser power, exchange processes during the laser bleach can limit the bleach depth that can be achieved locally. Thus, high mobility is associated with a decreased bleach depth. However, the bleach depths that were achieved for the STAT1 variant proteins did not differ significantly, whereas the bleach depth of GFP remained significantly lower (Fig. 3A, bottom, and see supplemental Table S1 for statistical analyses). These results indicated the higher mobility of GFP in comparison to the STAT1 fusion proteins. Importantly, mobility differences between the STAT1 variants were not found. Thus, we concluded that the reduced export that was seen with mutants that lacked the transactivation domain was unlikely to be caused by their immobilization because of the binding to nuclear retention factors.

As another control, we examined the effects of tyrosine phosphorylation and concomitant DNA binding on the intranuclear mobility. To this end, cells that expressed the STAT1 variant proteins were stimulated with IFNγ for 30 min before the FRAP analysis was performed (Fig. 3B). The half-maximal recovery times did not differ from the values obtained in unstimulated cells (data not shown). Moreover, the bleach depths of the N-terminally deleted mutants STAT1ΔN and STAT1tc remained unchanged during the stimulation with IFNγ. This outcome was expected, since these proteins are barred from nuclear import after tyrosine phosphorylation, and only non-tyrosine-phosphorylated molecules are present in the nucleus even after cytokine stimulation. Next, we examined STAT1α and STAT1β as well as STAT1α DNA−min. Because of the low IFN sensitivity of STAT1β (see next section) this protein was stimulated with a regular (not shown) or a 5-fold elevated concentration of IFNγ to ensure similar tyrosine phosphorylation and hence DNA binding. Nevertheless, the maximal bleach depth even at the high IFN concentration did not exceed the bleach depth of unstimulated cells and remained at 40%, indicating undiminished mobility (Fig. 3B). The same result was obtained for the DNA binding mutant (Fig. 3B). Contrary, the maximal bleach depth that was achieved with STAT1α increased significantly to about 60% (Fig. 3B). We consider the increased translocation to transcription complexes specifically of STAT1α as the likely cause of its reduced mobility in the nucleus of cytokine-stimulated cells. In summary, it was concluded that the transactivation domain of STAT1 has a dual role. This domain promotes the nuclear retention of tyrosine-phosphorylated STAT1 in transcription complexes. After tyrosine dephosphorylation, however, this domain facilitates the removal from the nucleus of the now transcriptionally inactive protein and its subsequent reactivation.

Nuclear Export Determines the Amplitude of STAT1 Activation—Next, we examined the physiological consequences of export modulations by asking whether the observed translocation differences resulted in differential tyrosine phosphorylation. This was done by quantitative Western blotting with an SH2 domain-specific STAT1 antibody and an antibody that reacts specifically with tyrosine-phosphorylated STAT1. The resulting signal strengths were determined by chemiluminescence imaging to calculate the specific tyrosine phosphorylation [STAT1-TyrP]/[STAT1]. At first, we confirmed that in vitro phosphorylated purified STAT1α and STAT1β reacted identically with the antibody against tyrosine-phosphorylated STAT1 (Fig. 4A). Next, extracts from cells that expressed the N-domain deletion mutants were examined. Clearly, STAT1ΔN and STAT1tc differed in the kinetics of tyrosine phosphorylation, and the rapid export of STAT1ΔN correlated with its increased tyrosine phosphorylation (Fig. 4B). However, when the rate of nuclear export was equalized by the expression of an additional transferable nuclear export signal at the C terminus (29), the specific tyrosine phosphorylation increased, and the differences in tyrosine phosphorylation disappeared (Fig. 4C). This result demonstrated that the truncation of the C-domain did not impair the recruitment to the receptor kinase complex. We then examined whether the endogenous STAT1α and STAT1β splice variants also differed in their tyrosine phosphorylation. The low basal expression level of STAT1β made the quantification of signals difficult. We therefore followed a previously described procedure to boost STAT1 expression levels by treating HeLa cells with IFNγ for 16 h (30), at which time point no residual tyrosine phosphorylation of STAT1 was detectable (Fig. 4D). After IFN withdrawal for 1 h, the cells were restimulated with IFNγ for another
hour, before the specific tyrosine phosphorylation of the endoge-
nous STAT1α and STAT1β was determined by quantitative Western
blotting. Consistent with results obtained with the N-terminal
deletion mutants, the specific tyrosine phosphorylation of STAT1α
was enhanced in comparison to the C-terminally truncated STAT1β
splice variant (Fig. 4D). In addition, artificial export equalization by
the addition of an NES leveled the extent of tyrosine phosphorylation
(Fig. 4E). We also evaluated the tyrosine dephosphorylation of the
STAT1 splice mutants, because this reaction is a major determinant
of steady-state Tyr701 phosphorylation levels. However, dephospho-
rylation differences were not observed between STAT1α and
STAT1β (supplementary Fig. S2B). We also attempted to quantify
the effect of phosphorylation of residue 727 in the transactivation
domain on the activation of STAT1. As is shown in Fig. 4F, the
mutation to alanine of residue Ser727 appeared to reduce the extent
of tyrosine phosphorylation of STAT1α. However, a statistical anal-
ysis with Student’s t test indicated that 0.1 > p > 0.05 for some time
points. Therefore, it was not possible to demonstrate statistically

FIGURE 5. Nuclear export determines the duration of the interferon response. A and B, top, HeLa cells expressing GFP-tagged STAT1α (A), the S727A mutant (4), or STAT1β (B) were stimulated for 30 min with 20 ng/ml IFNγ, followed by interferon withdrawal, and fixation after the indicated times. Bar, 20 μm. Bottom, quantitative analysis of A. At the indicated time points, 30–40 cells expressing STAT1α or the S727A mutant were randomly selected, and the fluorescence density was determined in cytosol and nucleus. Cells were scored positive for nuclear accumulation if [nuclear fluorescence density/cytoplasmic fluorescence density] > 4. C, recombinant in vitro Tyr701-phosphorylated STAT1α, or -β, was microinjected into the cytoplasm of HeLa cells. Cells were fixed 15 min after injection and the localization of STAT1 proteins was detected by immunocytochemistry. The injection site was marked by fluorescein isothiocyanate-bovine serum albumin, and nuclei were stained with Hoechst dye. Bar, 20 μm. D, recombinant unphosphorylated STAT1α or -β was microinjected into the cytoplasm of resting HeLa cells, immediately followed by stimulation with IFNγ. After 30 min, the cells were fixed, and STAT1 was detected by immunocytochemistry. Bar, 20 μm. E, time course of IFNγ-induced specific Tyr701 phosphorylation of STAT1α and STAT1β (phosphotyrosine signal divided by STAT signal) as determined by quantitative Western blotting of whole cell extracts. STAT1α-GFP- or STAT1β-GFP-expressing HeLa cells were treated with 4 ng/ml or 20 ng/ml IFNγ, respectively, for 3 h. Subsequent-
ly, cells were kept in IFNγ-free medium containing staurosporine (0.5 μM) for the times indicated to terminate kinase activity. F, top, fluorescence analysis of fixed cells expressing GFP fusion proteins of STAT1α or -β. Cells were treated with IFNγ for 3 h before medium containing staurosporine was added for the indicated times as described in E. Bar, 20 μm. Bottom, quantitative analysis of F. This is identical to A except that cells were scored positive for nuclear accumulation if [nuclear fluorescence density/cytoplasmic fluorescence density] > 2.
significant phosphorylation differences with quantitative Western blotting (Fig. 4F).

The above data suggested that enhanced nuclear export could lower the IFN threshold for generating activated STAT1. To verify this, tyrosine phosphorylation of STAT1α and -β was determined after treatment of cells with varying concentrations of IFNγ for 45 min, at which time maximal STAT1 phosphorylation was observed. For STAT1β, detectable tyrosine phosphorylation required stimulation with 3–4 ng/ml IFNγ, whereas comparable STAT1α activation was observed already at an IFNγ dose of 0.5–1 ng/ml (Fig. 4G). Moreover, the specific tyrosine phosphorylation of STAT1β reached a plateau at about 50 ng/ml IFNγ, whereas 3 ng/ml IFNγ sufficed for STAT1α to reach this activation level, which increased even further with rising IFNγ doses (Fig. 4G). These experiments confirmed the results obtained with the endogenous STAT1 proteins (Fig. 4D). In conclusion, the above results demonstrated that nuclear export differences determined the cytokine sensitivity of STAT1 splice variants.

Nuclear Export Determines the Duration of the Cytokine Signal—Besides controlling the amplitude of STAT1 activation, the transactivation domain may also influence the duration of the cytokine signal by influencing the time required for STAT1 to return to its prestimulation distribution. To explore this, we determined the duration of STAT1 nuclear accumulation in response to a fixed IFNγ stimulus. A time course of the nuclear accumulation of STAT1 variant proteins is shown in Fig. 5. After stimulation of cells with IFNγ for 30 min, more than 80% of the cells expressing wild-type STAT1α or the S727A mutant displayed nuclear accumulation. Western blotting confirmed Ser727 phosphorylation of wild-type STAT1α (not shown). Four hours after IFNγ withdrawal, the proportion of wild-type STAT1-expressing cells that displayed nuclear accumulation had fallen to 30%, whereas the S727A mutant still sustained nuclear accumulation in all cells at this time point (Fig. 5A). Even after another 2 h, at which time nuclear accumulation of wild-type STAT1 was over, the IFN response persisted, as the mutant accumulated in the nucleus of about 50% of the cells. Interestingly, replacing Ser727 with the phosphate mimic aspartate measurably decreased the difference to wild-type STAT1α, although prolonged nuclear accumulation was still observed (not shown). Results obtained with inhibitors of serine kinases such as H7 were inconclusive because of a lack of specificity of these pharmacological agents (not shown). Because of its strongly reduced nuclear export, extended nuclear accumulation was also expected for STAT1β. However, this was not the case.
On the contrary, nuclear accumulation appeared delayed, but it was not prolonged in comparison to STAT1α (compare Fig. 5, A and B, panels 30′ IFNγ, and 30′ IFNγ+4h). Thus, it was examined whether differences in the nuclear import of the phosphorylated STAT1 splice variants were responsible for the observed accumulation differences. Yet, cytoplasmic microinjection of in vitro Tyr701-phosphorylated STAT1β resulted in rapid nuclear accumulation that was indistinguishable from the accumulation of in vitro Tyr701-phosphorylated STAT1α (Fig. 5C). The conclusion that identical degrees of tyrosine phosphorylation resulted in identical nuclear accumulation of the STAT1 splice variants was supported by another experiment. Here, unphosphorylated STAT1 proteins were microinjected in the cytoplasm. In this situation the cytoplasmic concentrations of both STATs are largely identical independent of replenishment from the nucleus. Now, the subsequent stimulation of the microinjected cells with IFNγ again resulted in the identical nuclear accumulation of STAT1α and STAT1β (Fig. 5D). This outcome confirmed that STAT1α and STAT1β do not differ in their receptor reactivity (Fig. 4E). Thus, according to the dose response curve of Fig. 4G, cells expressing STAT1β were treated with a 5-fold higher dose of IFNγ compared with STAT1α to achieve similar tyrosine phosphorylation (Fig. 5E). Under these conditions, both STAT1 splice variants achieved a similar degree of nuclear accumulation (Fig. 5F). To reveal the decay of nuclear accumulation it was necessary to reliably shut off further kinase activity in the cells that were treated with different concentrations of IFNγ. This was achieved by the addition of the kinase inhibitor staurosporine after 180 min, which brought the tyrosine phosphorylation of both STAT1α and -β to undetectable levels in about 30 min (Fig. 5E). As expected, the percentage of STAT1α-expressing cells with nuclear accumulation fell to 6% after 60 min in the presence of kinase inhibitor. Despite similarly reduced tyrosine phosphorylation, at this time point STAT1β showed nuclear accumulation in 41% of the cells, and even after 180 min in the presence of the kinase inhibitor nuclear accumulation was still observed in 9% of cells. These results confirmed that the prolonged nuclear accumulation of the C-terminally truncated STAT1β was caused by the retention of non-tyrosine-phosphorylated molecules.

Serine Phosphorylation-induced Export Acceleration Increases the Transcriptional Activity of STAT1—The above results implied that Ser727 phosphorylation could enhance transcription in two independent ways: by increased cofactor recruitment (20, 31), and by accelerating nuclear export. To assess the contributions of recruitment and transport, we tested transcriptional activation under conditions where nucleocytoplasmic translocation plays a minimal or no role. In vitro transcription reactions with purified RNA polymerase II and a DNA template without or with two canonical STAT1 binding sites upstream of the HIV-1 core promoter were performed with unphosphorylated, Tyr701-, Ser727-, or doubly phosphorylated STAT1α. As expected, only tyrosine-phosphorylated STAT1α was capable of transcription initiation in vitro (Fig. 6A), but its activity was not affected by the additional phosphorylation of Ser727 (Fig. 6A). Whereas gene induction on chromatin templates is likely to require STAT1α serine phosphorylation (18, 31), these data show for the first time that it does not facilitate STAT1α-induced transcription on DNA. Therefore, we made use of a transiently transfected reporter gene, which is unlikely to acquire higher order levels of chromatin structure (32). This assay was used to compare the influence of the S727A mutation on wild-type STAT1 and an N-domain point mutant (F77A). Because of defective tyrosine dephosphorylation, the mutant displays virtually irreversible IFNγ-dependent nuclear accumulation of transcriptionally active molecules (33), thus obliterating the need for repeated nuclear export. Consequently, this STAT1 variant achieved 3–4-fold increased gene induction of an IFNγ-responsive reporter gene compared with wild type (Fig. 6B). As expected, mutation of Ser727 diminished the transcriptional activity in response to IFNγ both of wild-type STAT1 and the F77A mutant (Fig. 6B). However, the single S727A mutant retained only 20% activity, whereas the double mutant was less severely affected and remained 36% active (Fig. 6C). Based on these results, we estimate that facilitated nuclear export accounts for about one-fifth of the stimulatory influence of Ser727 phosphorylation on IFNγ-induced STAT1 activity.

**DISCUSSION**

The continuous nucleocytoplasmic cycling of STAT transcription factors has been identified as a basic principle of cytokine signaling.
Moreover, it was shown that nucleocytoplasmic shuttling of signal transducers can couple the duration of their activity in the nucleus with the receptor activation at the cell membrane (7, 34, 35). Here, we provide evidence that nucleocytoplasmic cycling can play an additional role in the modulation of the signal that emanates from the cytokine receptor. We identified a crucial role for nuclear export in the activation of STAT1 splice variants. This occurred despite their identical ability to recognize the receptor kinase complex. Hence, the regulation of constitutive nucleocytoplasmic transport can constitute another specificity determinant in extracellular signaling to the nucleus. For the STATs, it therefore becomes clear that both the cytokine-independent constitutive nucleocytoplasmic cycling and the cytokine-induced nuclear translocation are intimately intertwined to regulate the expression of cytokine responsive genes.

Two different assays were used to investigate the nuclear export of STAT1. First, the microinjection of recombinant STAT proteins into the nuclei of unstimulated HeLa cells resulted in a pancellular localization for STAT1α after 2 h, whereas STAT1β was still nuclear at this time and a pancellular localization was only achieved after another 2 h (Ref. 4 and data not shown). Second, we introduced a novel assay to examine the nuclear export of STAT1 during the cytokine stimulation of cells. Here, we used N-domain truncation mutants of STAT1 to prevent both the nuclear entry of activated molecules as well as their inactivation, thus precluding the accumulation of STAT1 in the nucleus and revealing the ongoing nuclear export. In this assay, cytokine treatment of cells induced the clearance of STAT1 from the nucleus instead. The nuclear concentration of N-terminally truncated STAT1α was reduced to one-half in about 80 min, whereas the respective derivative of STAT1β did not achieve this level of reduction in 180 min. Mutation of the conserved phosphorylation site serine 727 to alanine also reduced nuclear export, albeit much less severely, and the exchange for aspartate was even less destructive in terms of nuclear export. These translocation rates may appear low. However, the recordings were performed with STAT1-GFP fusion proteins, and the addition of GFP reduces the carrier-independent export of STAT1 considerably.4 In addition, the translocation assay of Fig. 2 determined the clearance from the nucleus, which is the result of nuclear export minus the continued, albeit gradually decreasing import of unphosphorylated molecules. Therefore, the assay underestimated the actual rate of export.

Remarkably, it was the expression of the transactivation domain that facilitated the nuclear export of STAT1. Therefore, this domain appears to have the opposite effect on nuclear retention of latent and activated STAT1. Best known is the recruitment of transcription cofactors to activated STAT1. In fact, STAT1α showed reduced mobility in the nucleus of cytokine-stimulated cells, whereas the C-terminally truncated splice variant STAT1β or a DNA binding mutant both did not. Nevertheless, we report here that STAT1 variants that express the transactivation by STAT1, whereas the duration of the cytokine signal was minimized. In contrast, the nuclear export of latent STAT1β, the splice variant that lacks the TAD, was reduced. Despite that, the nuclear mobility of activated STAT1β was increased, presumably because of weaker tethering to transcription complexes. Consequently, latent STAT1β preferentially accumulates in the nucleus, and despite the unimpaired receptor recognition of STAT1β, this resulted in its lower cytokine sensitivity in comparison to STAT1α.

FIGURE 7. A model depicting the dual role of the C-terminal TAD and its serine phosphorylation in controlling the activation of STAT1 splice variants. A, TAD of latent STAT1α facilitated CRM1-independent nuclear export that was further enhanced by serine phosphorylation of the TAD. Therefore, the access to membrane-bound Jak kinases was enhanced during the cytokine stimulation of cells. This, in turn, resulted in the maximal sensitivity of the pathway to extracellular stimulation. The TAD of activated dimers of STAT1α, on the other hand, promoted the tethering to transcription cofactors on DNA, resulting in the increased retention of activated molecules at the promoters in the nucleus. Together, the activities of the TAD of latent and activated STAT1α increased the transactivation by STAT1, whereas the duration of the cytokine signal was minimized. B, contrary, the nuclear export of latent STAT1β, the splice variant that lacks the TAD, was reduced. Despite that, the nuclear mobility of activated STAT1β was increased, presumably because of weaker tethering to transcription complexes. Consequently, latent STAT1β preferentially accumulates in the nucleus, and despite the unimpaired receptor recognition of STAT1β, this resulted in its lower cytokine sensitivity in comparison to STAT1α.

4 U. Vinkemeier, unpublished observation.
for reactivation (7). The IFN-induced activation of STAT1 is an affinity-driven process (39), which therefore is sensitive to the concentrations of the reactants, phosphorylated receptor chain and latent STAT1. Because phosphorylation of STAT1 will deplete the cytoplasmic pool of latent STAT1, this will reduce the rate of association of STAT1 with the receptor and hence STAT activation. Cytoplasmic depletion of STAT1 can be counteracted by exporting latent STAT1 from the nucleus. However, STAT1 variant proteins differ in their rates of nuclear export (Fig. 2). Therefore, the variants suffer from different degrees of cytoplasmic depletion during cytokine stimulation, with slow export resulting in increased cytoplasmic depletion and hence reduced activation. Quantitative data indicating that the STAT1/IFN receptor dissociation equilibrium (~50–140 nM, Refs. 39 and 40) is well above the STAT1 concentration (~40 nM in HeLa cells) are in agreement with this reasoning.

The signal-dependent serine phosphorylation of the transactivation domain also enhanced nuclear export. Accordingly, mutation of the phosphorylation site Ser727 measurably prolonged the nuclear accumulation phase during the cytokine stimulation. We attempted to assess the contribution of serine phosphorylation-induced export acceleration to the activation of STAT1. Significantly reduced tyrosine phosphorylation could not be demonstrated by quantitative Western blotting. Nevertheless, it is demonstrated here that mutation of Ser727 reduced the transcriptional activity of STAT1 much more severely in a situation where constant reactivation, and thus nuclear export was required. Under conditions where nuclear export plays no role, the transcriptional activity of STAT1 remained considerably higher after the mutation of Ser727, indicating a stimulatory role for Ser727 phosphorylation for the activation of STAT1. The analysis of IFN-dependent gene expression in macrophages derived from mice expressing STAT1 with a S727A mutation demonstrated that all STAT1 target genes analyzed were negatively affected, but to a variable extent (31). Promoters with “weak” STAT1 binding sites may be particularly sensitive to variations in the concentration of activated STATs. Here, low stimulation intensities may not suffice to yield enough STAT1 DNA binding activity in order to ensure promoter occupancy. The concomitant exposure of cells to stimuli such as lipopolysaccharides or TNFa, which exclusively boost the serine phosphorylation of STAT1, might increase the availability of STAT1 in the cytoplasm, and thus push the activation above a critical threshold level. Under high stimulation intensities, on the other hand, these effects may be negligible.

Much needs to be learned about the molecular mechanisms of export acceleration. Clearly, the C terminus did not function as a transferable NES, nor did inhibition of the exportin CRM1 abolish the export differences. Notably, the cytoplasmic injection of C-terminally truncated STAT1ctc did not result in the nuclear accumulation despite its reduced nuclear export, but the net import ceased when an even nucleocytoplasmic distribution was reached (4). This and the FRAP data (Fig. 3) argue against the possibility that reduced export is caused by decreased dissociation from nuclear binding sites. Rather, this behavior can be explained by carrier-independent facilitated diffusion, whereby the permeability of STAT1 through the nuclear envelope is regulated and rate-limiting. We propose that at least two distinct regions of STAT1 can contact the nuclear pore proteins: one the linker domain (4) and the other likely to be the transactivation domain. The combination of two nucleoporin-contacting domains to accelerate carrier-free translocation was also demonstrated for importin β (41).

The generating by alternative splicing of STAT1β forms that possess an altered transactivation domain has been described for STAT1, -3, -4, and -5 (10, 42–44). In addition, proteolytic processing of STAT proteins results in the generation of C-terminally truncated variants that lack the transactivation domain (45). At present, it is not known whether these modifications control permeability through the nuclear pore of STATs other than STAT1, but export of C-terminally truncated STAT3 and STAT5 is slow and comparable to truncated STAT1 (4). Alternative splicing has also been implicated in tyrosine phosphorylation and the stability of the STAT molecule, although the molecular mechanisms are incompletely understood (33, 44, 46, 47). Of note, serine phosphorylation of an unrelated site of STATα from Dictyostelium increases nuclear export, again in the absence of NES activity of the phosphorylated peptide (48).

Whereas nuclear import has traditionally received almost exclusive attention when the amplification of inducible nuclear functions was considered, this work demonstrated for STAT1 a crucial stimulatory role for nuclear export. Continuous nucleocytoplasmic shuttling is a hallmark not only of STATs in cytokine signal transduction, but also of other key signaling molecules, such as the SMADs and the mitogen-activated protein kinases in growth factor signaling. Therefore, the importance of facilitated nuclear export for maximal activity of signal transducers may prove to be a common theme in extracellular signaling to the nucleus.

Acknowledgments—We thank Drs. M. Beyermann, J. Han, F. Horn, M. Kaleffe, E. Krause, and A. Nebreda for generously providing reagents or technical expertise. The technical assistance of J. Eichhorst, M. Kummerow, and S. Meyer is gratefully acknowledged. Drs. J. E. Darnell and K. Weber provided critical comments on the manuscript.

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