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Live Cell Imaging Reveals Continuous STAT6 Nuclear Trafficking

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The STAT6 transcription factor is essential for the development of protective immunity; however, the consequences of its activity can also contribute to the pathogenesis of autoimmune disease. Tyrosine phosphorylation is known to activate STAT6 in response to cytokine stimulation, but there is a gap in our understanding of the mechanisms by which it enters the nucleus. In this study, live cell imaging was used in conjunction with photobleaching techniques to demonstrate the continual nuclear import of STAT6, independent of tyrosine phosphorylation. The protein domain required for nuclear entry includes the coiled coil region of STAT6 and functions similarly before or after cytokine stimulation. The dynamic nuclear shuttling of STAT6 seems to be mediated by the classical importin-α-importin-β1 system. Although STAT6 is imported to the nucleus continually, it accumulates in the nucleus following tyrosine phosphorylation as a result of its ability to bind DNA. These findings will impact diagnostic approaches and strategies to block the deleterious effects of STAT6 in autoimmunity. The Journal of Immunology, 2010, 185: 000–000.

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Abbreviations used in this paper: anti-pSTAT6, anti-STAT6 phosphotyrosine 641 Ab; c, control; C, cytoplasm; dl, deletion; Fl, fluorescent; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; G, GFP; hIL-4, human IL-4; impI1, importin-β1; MBP, maltose-binding protein; N, nucleus; NLS, nuclear localization signal; IP, immunoprecipitated; PS, Ponceau S; S, STAT6; SH2, Src homology 2; siRNA, short interfering RNA; STAT6-V5, STAT6 tagged with the V5 epitope; WB, Western blot; wtSTAT6, wild type STAT6.

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Materials and Methods

Cell cultures and reagents

HeLa and Cos7 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM with 8% FBS. Cells were treated with human rIL-4 (R&D Systems, Minneapolis, MN) at 10 ng/ml. DNA transfections were carried out using TransIT-LT1 transfection reagent (Mirus, Madison, WI), according to the manufacturer’s instructions. Rabbit anti-STAT6 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-STAT6 phosphotyrosine 641 Ab (anti-pSTAT6) (Cell Signaling Technology, Danvers, MA), and murine anti-GFP Ab (Roche Diagnostic Systems, Indianapolis, IN) were used for Western blotting at a 1:1000 dilution. HRP-conjugated anti-rabbit and anti-mouse Ig were used as secondary Abs for Western blotting (1:5000).

Plasmid constructs and protein purification

Full-length STAT6 cDNA and deletion mutants created by PCR were cloned into pEF1/V5-His (Invitrogen) or pMAL-c4x (New England Biolabs, Ipswich, MA) to generate V5 or maltose-binding protein (MBP) fusion proteins. A monomeric form of enhanced GFP was produced by mutating A206K, L221K, and F232R in the vector pEFGP-N1 (BD Clontech, Mountain View, CA), and it was used to generate GFP-tagged STAT6 proteins (21). Site-directed mutagenesis was performed on targeted oligonucleotides and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). All constructs were confirmed by DNA sequencing. Importin-α constructs lacking the importin-β–binding domain were generated and purified, as reported previously (20). MBP-STAT6(1–267) and MBP-STAT6(1–267 deletion [dl]136–140) proteins were prepared following the manufacturer’s instructions (New England Biolabs).

Western blot

Two days after transfection, cells were serum starved for 24 h and were treated or not with human IL-4 (hIL-4) for 30 min and lysed with cold lysis buffer (50 mM Tris [pH 8], 5 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, and 1 mM sodium selenite). Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Fizic, Rockford, IL). The proteins were detected by reacting with Abs to STAT6, STAT6 phosphotyrosine, or GFP and detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) and captured using Zeiss LSM 5 Pascal imaging software.

EMSA

Cells were lysed with hypotonic lysis buffer (15 mM HEPES [pH 7.9], 0.2 mM spermine, 0.5 mM spermidine, 2 mM potassium-EDTA, 80 mM KCl, 1 mM NaF, and 1 mM sodium vanadate). Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Fizic, Rockford, IL). The proteins were detected by reacting with Abs to STAT6, STAT6 phosphotyrosine, or GFP and detected using the enhanced chemiluminescence system or Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

RNA interference

Short interfering RNA (siRNA) duplexes specific for human importin-β1 (Qiagen, Valencia, CA) or vimentin (control) were transfected with X-tremeGENE siRNA transfection reagent (Roche). Twenty-four hours after siRNA transfection, cells were transfected with STAT6-GFP. Cellular localization of STAT6-GFP was observed in mammalian cells by fluorescence microscopy. RNA interference was performed with SurePrep TrueTotal RNA purification kit (Fisher Scientific, Pittsburgh, PA), and cDNA was synthesized with M-MLV reverse transcriptase (Promega). RT-PCR was performed with specific primers for importin-β1 or GAPDH as an internal control. Image J software was used to estimate quantity (freely available in the National Institutes of Health public domain). Primer sequences for importin-β1 were 5′-AATCCAGGAAACGT- CAGGTTCG-3′ (forward) and 5′-AGCAGTACGCACCACATCAG-3′ (reverse) and for GAPDH were 5′-GGAGCAGGAGATGTCATCATCCTC-3′ (forward) and 5′-AGTTGGGTGTCGCTGTAGTGC-3′ (reverse).
imported to the nucleus but did not accumulate following stimulation.

Imaging results showed the double mutant, STAT6(RY)-GFP, was capable of specific DNA binding was mutated to alanine (R562A). Arginine 562 in the SH2 domain that functions to form dimers dependent of tyrosine phosphorylation, the behavior of a STAT6 protein with a double mutation was evaluated. The tyrosine 641 phosphorylated STAT6 present in nuclei prior to IL-4 treatment of fluorescence (Supplemental Fig. 1) shows that STAT6 nuclear accumulation requires tyrosine phosphorylation.

The EMSAs showed that STAT6-GFP can bind a specific DNA target sites. To determine whether the increased nuclear accumulation of STAT6 after STAT6 tyrosine phosphorylation was due to a gain in the ability to bind DNA, the behavior of a DNA-binding retains STAT6 in the nucleus.

Tyrosine phosphorylation activates STAT proteins by promoting the formation of dimers that have the ability to bind specific DNA target sites. To determine whether the increased nuclear accumulation of STAT6 seen following tyrosine phosphorylation was due to a gain in the ability to bind DNA, the behavior of a DNA-binding retains STAT6 in the nucleus.

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FIGURE 3. Live cell imaging demonstrates decreased STAT6 nuclear export following tyrosine phosphorylation. Cytoplasmic FLIP assays were performed with cells expressing STAT6-GFP not treated (upper row) or treated with IL-4 (lower row). A small region in the cytoplasm (C) was subjected to continuous high-intensity laser. Fluorescence loss was monitored with time in the cytoplasm and compared with the fluorescence loss in a region of the nucleus (N). The quantitative data of relative fluorescent (Fl) intensity with time are shown in the panels on the far right. Experiments are representative of more than three independent studies.

binding mutant was evaluated. A STAT6 DNA-binding mutant was generated based on other STAT DNA-binding mutants (23). Lysines and arginines within aa 366–374 were substituted with alanines to generate STAT6(KR). Although the STAT6(KR) mutant was accurately tyrosine phosphorylated in response to IL-4, it did not bind target DNA sequences (Fig. 4A). Microscopic imaging indicated that STAT6(KR) was imported to the nucleus with and without IL-4 stimulation, but it did not accumulate in the nucleus in response to IL-4. This indicated that DNA binding contributes to nuclear accumulation following tyrosine phosphorylation.

If DNA binding retains STAT6 in the nucleus, the mobility of tyrosine-phosphorylated STAT6 within the nucleus would be expected to be slower than unphosphorylated STAT6. A nuclear FLIP assay was used to investigate this possibility (Fig. 4B). A small region (region 1) in the nucleus of cells expressing STAT6-GFP, with or without IL-4 stimulation, was subjected to continuous laser bleaching for 5 min. The fluorescence intensity of region 1 was compared with a distinct region in the nucleus (region 2). If movement is rapid through the path of the laser, the fluorescence intensity in region 2 will decrease similarly to region 1, along with the entire nucleus. This was the case for unphosphorylated STAT6. However, following tyrosine phosphorylation in response to IL-4, STAT6 showed significantly slower movement. The fluorescence decrease in region 2 and the remainder of the nucleus was delayed considerably compared with region 1. The tyrosine-phosphorylated DNA-binding mutant, STAT6(KR), showed the same rapid nuclear movement as unphosphorylated STAT6. To establish that the DNA-binding mutant is not retained in the nucleus following IL-4 stimulation, imaging with cytoplasmic FLIP was used (Supplemental Fig. 2). The export kinetics of tyrosine-phosphorylated STAT6(KR) were similar to unphosphorylated wild type STAT6 (wtSTAT6). Together, the results support the premise that STAT6 accumulates in the nucleus only if it has a functional DNA-binding domain.

Identification of amino acids in STAT6 that are required for nuclear import

Nuclear import of large molecules, such as STAT6, requires an amino acid sequence or structure that serves as an NLS. To identify amino acids that function to facilitate STAT6 nuclear import, a series of deletion mutations were generated, and the cellular localization of the truncated proteins was evaluated with or without IL-4 stimulation. Small proteins were tagged with two GFP molecules to ensure that they did not passively diffuse into the nucleus; a diagram of some of the truncations is shown in Fig. 5. The cellular localization of these truncations indicated that a region in the coiled coil domain is needed for nuclear import. STAT6(1–267) containing the N terminus and the coiled coil domain of STAT6 was imported to the nucleus. However, STAT6(268–847) containing the DNA-binding domain, SH2 domain, and transactivation domain remained in the cytoplasm with or without IL-4 stimulation. Deletions within the coiled coil domain identified a region required for STAT6 nuclear import. STAT6(136–847) was imported and accumulated in the nucleus following tyrosine phosphorylation, whereas STAT6(141–847) remained in the cytoplasm with or without tyrosine phosphorylation. Western blotting with Abs to STAT6 phosphotyrosine 641 confirmed that the deletions were accurately phosphorylated in response to IL-4 (Fig. 5).

The studies with STAT6 truncations identified a sequence between aa 136–140 (RLQHR) that is required for nuclear import. To determine the effect of a specific deletion or substitution of these amino acids in otherwise full-length STAT6, we evaluated the localization of two mutants linked to GFP (Fig. 6A). STAT6 dl 136–140 or STAT6 containing a substitution of 135–140 aa with alanine residues (sub6A) were expressed in cells stimulated or not with IL-4. The cellular localization of both mutants was restricted to the cytoplasm, indicating a deficiency in nuclear import. These mutants were accurately tyrosine phosphorylated in response to

FIGURE 4. DNA binding promotes nuclear accumulation of tyrosine-phosphorylated STAT6. A, The DNA-binding mutant STAT6(KR)-GFP was expressed in cells and examined by fluorescence microscopy before (−) or after (+) treatment with IL-4 (original magnification ×100). Western blot (lower panels) was performed with anti-pSTAT6 or anti-GFP Abs. EMSA (right panel) was performed with the IL-4R target oligonucleotide and lysates from cells expressing STAT6-GFP (wt) or STAT6(KR)-GFP (KR) without (−) or with (+) IL-4 treatment. Abs to STAT6 (S), GFP (G), or MOPC control (c) were added to the binding reactions. B, Live cell imaging was used with nuclear FLIP to evaluate STAT6-GFP and STAT6(KR)-GFP mobility within the nucleus. A small region in the nucleus (region 1) was subjected to continuous high-intensity laser. Fluorescence loss was monitored with time in this region and a distinct region in the nucleus (region 2). Quantitative data of relative fluorescent (Fl) intensity with time are shown in the panels on the far right. Experiments are representative of more than three independent studies.
IL-4, indicating that the internal deletion and substitution did not disrupt STAT6 activation. To evaluate the influence of specific residues in this region, each amino acid was mutated in the context of full-length STAT6. However, the individual point mutants behaved as wtSTAT6 (Supplemental Fig. 3). Together, these results indicate that aa 136–140 are required for STAT6 nuclear import, but they may function within the context of a conformational NLS.

Transcriptional regulation is the primary function of STAT6, and for this reason we evaluated the ability of STAT6 mutants to induce gene expression. Mutants defective in nuclear localization, STAT6(dll136–140), or DNA binding, STAT6(KR), were tested for their competence to induce the characterized promoter of the IL-4R gene (22). Transient transfections clearly demonstrated the ability of wtSTAT6 to induce the IL-4R reporter in response to IL-4,

whereas STAT6(dll136–140) and STAT6(KR) did not induce the gene (Fig. 6B).

Evidence supporting a role of importin-α/β1 in STAT6 nuclear import

Active transport of large molecules through the nuclear pore complex usually requires facilitation by carrier proteins of the karyopherin-β family. Importin-β1 is a primary karyopherin-β transporter that can bind directly to NLS-containing proteins or indirectly via the family of importin-α adapters. Importin-α adapters bind directly to the NLS. In vitro binding assays were performed to evaluate whether one or more of the importin-αs can recognize

![Diagram](http://www.jimmunol.org/)
STAT6 (Fig. 7A), STAT6-V5 was expressed in mammalian cells and immunoprecipitated from cell lysates with V5 Ab and protein G agarose beads. GST-tagged importin-α was expressed in bacteria and added to the STAT6-V5 immunocomplexes collected on beads. Interaction of importins with STAT6 was detected by Western blot with Ab to GST. The results indicated that STAT6 is recognized primarily by importin-α3 and -α6. Similar results were obtained with STAT6 isolated from untreated cells or IL-4–stimulated cells, indicating that binding is independent of tyrosine phosphorylation. Because importin-α6 is restricted to the testes, importin-α3 seems to be the primary import adapter (24, 25).

Because aa 136–140 in the coiled coil region of STAT6 are critical for nuclear import, we determined whether this sequence was required for direct interaction with importin-α3. We expressed fragments of STAT6 tagged with MBP in bacteria corresponding to STAT6 1–267 aa or 1–267 containing the 136–140 deletion, MBP-STAT6(1–267) and MBP-STAT6(dll136–140) were incubated with bacterially expressed GST–importin-α3 or GST–importin-α1 as a control and evaluated for binding (Fig. 7B). The results showed that STAT6(1–267) can bind importin-α3 specifically, but the deletion mutant cannot. These data suggest aa 136–140 are required for STAT6 binding to importin-α3 and nuclear import in vivo.

Given that the importin-α/β1 system may mediate STAT6 nuclear import, we evaluated the effect of RNA interference on the inhibition of expression of importin-β1 (Fig. 7C). siRNA duplexes corresponding to importin-β1 or to vimentin as a control were transfected into cells with STAT6-GFP, and the localization of STAT6-GFP was visualized microscopically. The behavior of STAT6-GFP was notably different in the cells treated with importin-β1 siRNA. Approximately 10% of cells showed STAT6 restricted to the cytoplasmic compartment, often with punctate cytoplasmic fluorescence. Because the siRNA may not completely inhibit importin-β1 expression in all cells expressing STAT6-GFP, the effect seems to be significant. To evaluate the effectiveness of the importin-β1 siRNA complexes, mRNA levels in cells treated with control or importin-β1 siRNA were assayed by RT-PCR. The siRNA to importin-β1 reduced endogenous mRNA by ~70%. Together, the results suggest that importin-α/importin-β1 may mediate STAT6 nuclear import.

Discussion

Nuclear trafficking of STAT6 is integral to its function as a signal transducer and activator of transcription. By attaching a fluorescent probe to STAT6 we were able to study its intracellular dynamics with microscopy in real time. The advantage of live cell imaging is that it avoids fixation techniques that can influence cellular architecture. Cell fractionation has been used to evaluate cellular localization; however, the technique is limited in interpreting in vivo protein localization, particularly if the protein is actively imported and exported from the nucleus. Our studies indicated that STAT6 moves continually within the cytoplasm; additionally, it is transported continually into and out of the nucleus, independent of tyrosine phosphorylation.

Specific phosphorylation of tyrosine 641 promotes STAT6 dimerization and its ability to bind DNA target sites. In addition to this activating modification, other modifications have been reported that include serine phosphorylation of the carboxyl transactivation domain, which may influence DNA binding (26–28), and acetylation, which may contribute to induction of gene expression (12, 29). Methylation of arginine 27 was reported to be required for STAT6 tyrosine phosphorylation, nuclear translocation, and DNA binding (30). However, our studies indicate that arginine 27 is not necessary for tyrosine phosphorylation, nuclear translocation, or DNA binding. STAT6 that completely lacks 135 aa from the N terminus is imported to the nucleus, is tyrosine phosphorylated in response to IL-4, and can bind DNA (Fig. 5) (H.C. Chen and N.C. Reich, unpublished observations).

By studying the cellular localization of various STAT6 deletions, we identified a region within the coiled coil domain required for STAT6 nuclear import (Fig. 5). STAT6(136–847) was imported to the nucleus constitutively, whereas STAT6(141–847) was not imported. Deletion or substitution of the small region between aa 135–140 eliminated the ability of otherwise full-length STAT6 to be imported to the nucleus, although the proteins were still tyrosine phosphorylated accurately (Fig. 6). The best-characterized classical NLS sequences contain one or two stretches of basic amino acids, particularly lysines (31). Although the sequence 135–140 (RLLQHR) contains arginine residues, site-directed mutation of individual amino acids within this region was not sufficient to block nuclear import (Supplemental Fig. 3). This finding suggests that a noncanonical NLS may be functional within 136–267. Other STAT molecules seem to use noncanonical NLSs to drive import, whether they are constitutive or conditional (18).

Although the STATs do not display classical NLSs, they seem to use the importin-α–importin-β1 receptors. Importin-α5 binds to STAT1 when it is in the conformation of a tyrosine-phosphorylated dimer and facilitates its nuclear import (19, 32, 33), whereas importin-α3 and -α6 bind constitutively to STAT3 (20). In this study, we found that importin-α3 and -α6 also bind constitutively to STAT6; additionally, downmodulation of importin-β1 by RNA interference notably reduces STAT6 nuclear import. The results suggest that STAT6 is imported by importin-α–importin-β1 receptors (Fig. 7). It is challenging to determine specific importin-α recognition of a particular NLS outside the framework of the native protein, because recognition depends on the NLS sequence, as well as the protein context (34). The crystal structure of STAT6 remains to be solved. However, the identity of the importin-α that binds a particular protein may be significant because the importin-α proteins display specific expression in tissues and during differentiation (25, 35). It was reported that a Rac GTPase-activating protein is responsible for nuclear import of activated STAT proteins and that the dominant negative N17Rac1 protein can block nuclear import of the STATs (36). For this reason, we tested the effect of N17 Rac1 on STAT6 nuclear import but did not detect any effect (Supplemental Fig. 4).

Latent-unphosphorylated and tyrosine-phosphorylated STAT6 are imported to the nucleus. The difference is that STAT6 accumulates in the nucleus when it is tyrosine phosphorylated (Fig. 1). Live cell imaging with photobleaching techniques provides a more quantitative and temporal measure of protein mobility and localization (37–39). By using the technique of nuclear FRAP, the transport of STAT6-GFP into the nucleus was observed to be similar for unphosphorylated or tyrosine-phosphorylated STAT6-GFP (Fig. 2). However, the average fluorescence intensity of phosphorylated STAT6-GFP becomes significantly greater in the nucleus than in the cytoplasm. The nuclear accumulation is the consequence of decreased nuclear export. This was demonstrated with cytoplasmic FLIP (Fig. 3). Repeated photobleaching of one small region in the cytoplasm resulted in the loss of total cytoplasmic fluorescence, independent of STAT6 phosphorylation. For unphosphorylated STAT6-GFP, this was followed by a gradual loss of fluorescent signal from the nucleus, indicating continuous export. In contrast, nuclear fluorescence of tyrosine-phosphorylated STAT6-GFP did not decrease during the experiment. Therefore, the increase in STAT6 nuclear accumulation following tyrosine phosphorylation is a result of decreased nuclear export.

The mechanism of STAT6 nuclear export remains to be determined; nonetheless, it seems that DNA binding is responsible for STAT6 nuclear accumulation. A STAT6 DNA-binding mutant was
shown to behave like unphosphorylated STAT6 and did not accumulate in the nucleus following phosphorylation (Fig. 4). In addition, nuclear FLIP analyses determined that DNA binding dramatically reduced STAT6 movement within the nucleus. These observations indicate that nuclear accumulation of tyrosine-phosphorylated STAT6 is due to retention by association with DNA. DNA binding may be a general cause for observed nuclear accumulation of STAT proteins (23, 38, 40, 41).

Accurate cellular localization is essential for the effective function of transcription factors, such as STAT6. The constitutive nuclear import and export of latent STAT6 may provide an advantage for the rapid response to cytokine-stimulated tyrosine phosphorylation, or it may enable an activating response to nuclear kinases. Alternatively, because there is precedence for the function of unphosphorylated STATs contributing to gene expression, unphosphorylated STAT6 may have an undiscovered function in the nucleus (42). Understanding the mechanisms that regulate STAT6 nuclear trafficking will support means to manipulate its activity in health and disease.

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Disclosures

The authors have no financial conflicts of interest.

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