Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 49, Issue of December 6, pp. 31017–31020, 1996

Interferon-γ-dependent Nuclear Import of Stat1 Is Mediated by the GTPase Activity of Ran/TC4*

(Received for publication, August 13, 1996, and in revised form, October 15, 1996)

Toshihiro Sekimoto‡, Koichi Nakajima§, Taro Tachibana‡, Toshio Hirano§, and Yoshihiro Yoneda¶

From the Department of Anatomy and Cell Biology and the §Division of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan

In response to interferon-γ (IFN-γ), Stat1 enters the nucleus, where it activates transcription. In order to better understand the mechanism of the extracellular signal-induced protein import into the nucleus, we have established an *in vivo* assay system that uses recombinant Stat1 protein as a model transport substrate. Using this system, we found that Stat1 is actively transported through the nuclear pores in an IFN-γ-dependent manner and tyrosine (Tyr^{701}) phosphorylation of Stat1 is actually required for its nuclear import. When the antibody against Ran, which was identified as an essential factor for active nuclear protein transport, was injected, the IFN-γ-dependent nuclear transport of Stat1 was completely inhibited. Furthermore, nuclear import of Stat1 was suppressed by microinjection of two mutant Ran proteins, one defective in GTP hydrolysis (G19V) and the other with little or no binding to GTP (T24N), both of which are known to act as dominant negative inhibitors of nuclear import. These results indicate that the conditional nuclear import of Stat1 requires GTP hydrolysis by Ran.

Homeostasis in cells is maintained by the exchange of molecules, such as proteins and RNAs, through the nuclear pore complex (NPC)[1] in response to intracellular changes and extracellular signals. Selective nuclear import of karyophilic proteins has been shown to be directed by short amino acid sequences termed nuclear localization signals (NLSs). This transport process into the nucleus can essentially be divided into two steps: (*a*) NLS-dependent binding to the cytoplasmic face of the nuclear pore followed by (*b*) an energy-dependent translocation through the NPC.

During the first step of nuclear import, an NLS-containing protein forms a stable complex with ~60- and 97-kDa proteins to target the nuclear pores (1–9). In subsequent steps, which require a small G protein, Ran, the NLS-containing protein is translocated into the nucleus (10, 11). Experiments using nonhydrolyzable GTP analogues in an *in vitro* transport assay with digitonin-permeabilized cells or overexpression of mutant yeast Ran, which is stabilized in GTP-bound form, indicated that hydrolysis of GTP by Ran is requisite for nuclear import (10–12). These factors have been shown to participate in the nuclear import of single (for example, SV40 large T-antigen) or bipartite (for the case of nucleoplasmin) basic type NLS containing proteins, which are constitutively transported into the nucleus immediately after their synthesis in the cytoplasm. The mechanism and the factors that are required for extracellular signal-dependent nuclear transport of proteins that preexist or are anchored in the cytoplasm, however, remain unknown.

Studies of cytokines have revealed essential components of the cytokine signaling pathway. A family of latent cytoplasmic transcription factors termed Stats (proteins acting as signal transducers and activators of transcription) are activated by a variety of cytokines and growth factors and are generally thought to play a pivotal role in the function of these factors (13–15). For example, in response to IFN-γ, a specific tyrosine residue (Tyr^{701}) of Stat1 is phosphorylated and is then translocated into the nucleus where it directly activates target genes by binding to specific promoter sequences (16–18). In contrast to the detailed understanding of the activation step and the binding of Stat1 to DNA, the NLS of Stat1 has not yet been identified, and very little is known about the translocation step from the cytoplasm to the nucleus in this signaling pathway.

This study, which focuses on Stat1 as a model protein, was undertaken in order to better understand the machinery required for extracellular signal-induced protein import into the nucleus. To understand the mechanism of IFN-γ-dependent nuclear translocation of Stat1, we established and used an *in vivo* system in which recombinant Stat1 translocates from the cytoplasm to the nucleus, after cytoplasmic injection, in response to IFN-γ. The data collected herein clearly show that tyrosine phosphorylation of Stat1 is essential for its active nuclear import, and Ran/TC4 and its GTPase activity are required for the regulated nuclear import of Stat1.

MATERIALS AND METHODS

Preparation of Recombinant Proteins—Full-length cDNA of human Stat1 was inserted into pGEX-5X-3 (Pharmacia Biotech Inc.) in frame with a tag encoding an influenza virus hemagglutinin (HA) epitope, YPYDVPDYA, at the N-terminal end. Recombinant Stat1 was expressed by 0.1 mM IPTG for 20 h at 20 °C in *Escherichia coli* strain BL21(DE3) as GST-fusion proteins and purified with glutathione-Sepharose (Pharmacia) following the manufacturer's recommendations. The GST portion was cleaved with Factor Xa, and Stat1 was further purified by gel chromatography. Mutant Stat1s were expressed and purified as wild type.

pET-3d expression constructs of wild type and mutant Ran proteins were introduced into *E. coli* strain BL21(DE3). Recombinant Ran proteins were expressed by 1 mM IPTG for 6 h at 37 °C (wild type) or for 12 h at 20 °C (mutants) and purified essentially as described previously (19). 25 mM EDTA and 2 mM GTP (for GTP-Ran and GTP-Ran-G19V) or GDP (for GDP-Ran, GDP-Ran-G19V, and GDP-Ran-T24N) were added to Ran, and after incubation for 1 h on ice, $MgCl_2$ was added to a final

* This work was supported by a grant-in-aid for scientific research on priority areas, a grant-in-aid for scientific research, and a grant-in-aid for COE research from the Japanese Ministry of Education, Science, Sports and Culture, the Nissan Science Foundation, and the Naito Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Research Fellow of the Japanese Society for the Promotion of Science.

¶ To whom correspondence should be addressed. Tel: 81-6-879-3211; Fax: 81-6-879-3219.

[1] The abbreviations used are: NPC, nuclear pore complex; IFN-γ, interferon-γ; NLS, nuclear localization signal; HA, hemagglutinin; IPTG, isopropyl-β-D-thiogalactopyranoside; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; WGA, wheat germ agglutinin.

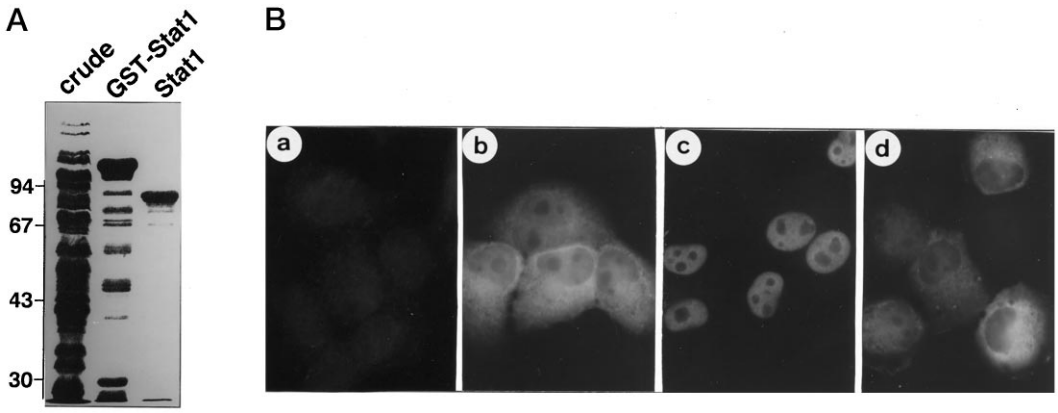

FIG. 1. **Purification of recombinant Stat1 from *E. coli* and IFN-γ-dependent nuclear translocation of purified Stat1.** *A*, HA-tagged human Stat1 was expressed in *E. coli* as a GST fusion protein and purified to homogeneity. *Left lane*, crude extract; *center lane*, GST-Stat1 fusion protein purified on glutathione-Sepharose; *right lane*, purified recombinant Stat1. Proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. *B*, purified recombinant Stat1 (3 mg/ml) was microinjected into the cytoplasm of human FL cells, and the cells were then treated with (*c* and *d*) or without (*b*) recombinant human IFN-γ at 50 ng/ml for 30 min at 37 °C. Cells were fixed with 3.7% formaldehyde, and localization of Stat1 was visualized by indirect immunofluorescence with anti-HA tag. WGA (0.5 mg/ml) was co-injected with Stat1 (*d*). *a*, uninjected cells were stained with anti-HA antibodies, as a control.

concentration of 50 mM. The samples were then subjected to a Superose 12 column (Pharmacia), equilibrated with transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, and 1.5 mM 2-mercaptoethanol). The nucleotides bound to Ran were determined by HPLC (20).

Mutations were introduced by the method using polymerase chain reaction (21) with appropriate oligonucleotides. All mutants were verified by DNA sequencing.

To prepare SV40 T-antigen NLS conjugated BSA (SV40 T-NLS-BSA), the synthetic peptides (CYGGPKKKRKVEDP) were chemically conjugated with BSA as described previously (22).

Antibodies—Rabbit anti-Ran antibodies were prepared by subcutaneous injection of purified recombinant Ran and affinity purified by purified full-length Ran-conjugated Sepharose 4B. For microinjection experiments, preimmune IgG and affinity purified anti-Ran antibodies were concentrated by ultrafiltration using Centricon 30 (Amicon). The affinity purified anti-Ran antibodies were found to inhibit the nuclear import of SV40 T-NLS-BSA *in vivo*.[2] Anti-HA (12CA5) antibody was purchased from Boehringer Mannheim, and monoclonal anti-Stat1 antibody was purchased from Transduction Laboratory.

Microinjection and Indirect Immunofluorescence—Cells were grown on coverslips in Dulbecco's modified Eagle's minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Purified recombinant Stat1 (3 mg/ml) was microinjected into the cytoplasm, and cells were then treated with IFN-γ (50 ng/ml, Genzyme) for 30 min at 37 °C. Cells were fixed with 3.7% formaldehyde for 10 min at room temperature and permeabilized with 0.5% Triton X-100 for 5 min. After blocking with 3% skimmed milk in phosphate buffered saline, subcellular localization of Stat1 was detected by indirect immunofluorescence using anti-HA and fluorescein isothiocyanate-labeled goat anti-mouse IgG. WGA (0.5 mg/ml), antibodies (18 mg/ml) or purified Ran proteins (3 mg/ml) were co-injected with Stat1 and then cells were treated with IFN-γ. For quantitation, photographic negatives were analyzed by Adobe Photoshop, and fluorescence intensities of cytoplasm and nuclei were calculated by integrating the pixel intensities of the fluorescence image using MacScope.

RESULTS AND DISCUSSION

IFN-γ-dependent Nuclear Translocation of Recombinant Stat1—In order to investigate the mechanism of IFN-γ-dependent nuclear translocation of Stat1, HA-tagged human Stat1 was expressed in *E. coli* and then purified to homogeneity (Fig. 1*A*). The tyrosine residue (Tyr^{701}) of recombinant Stat1 was phosphorylated by the cytosol from human 293T cells expressing human Jak1, and the phosphorylated form showed DNA binding activity *in vitro* (data not shown). When the recombinant Stat1 was microinjected into the cytoplasm of human FL cells, it remained predominantly in the cytoplasm in the absence of IFN-γ stimulation (Fig. 1*B*, *b*). However, after 30 min of treatment with IFN-γ, Stat1 localized in the nucleus in an extracellular signal-dependent manner (Fig. 1*B*, *c*). Endogenous Stat1 translocates into the nucleus in response to IFN-γ within same time frame, as evidenced by indirect immunofluorescence with anti-Stat1 antibody (data not shown). These findings indicate that recombinant Stat1 is functionally active and, thus, the transport system developed herein is suitable for examining the intracellular behavior of Stat1, as well as for the assay of transport activity of a variety of mutant Stat1s, which are exogenously injected.

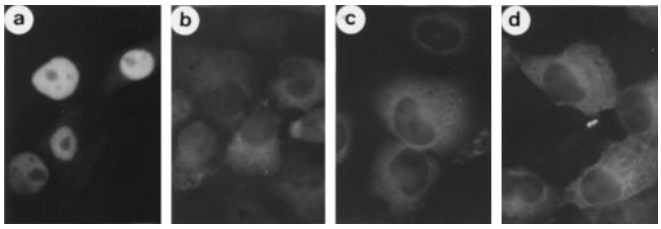

FIG. 2. **Tyrosine phosphorylation is required for the nuclear import of Stat1.** *a*, recombinant Stat1 was injected to the cytoplasm of FL cells, and then the cells were treated with IFN-γ. *b*, FL cells were treated with 0.5 μM staurosporine for 10 min prior to microinjection of wild type Stat1. After injection, the cells were then treated with IFN-γ for 30 min at 37 °C in the presence of staurosporine. *c* and *d*, Y701F or Y701E mutant Stat1 (3 mg/ml) was microinjected to FL cells and stimulated with IFN-γ.

It is well known that certain WGA-reactive nuclear pore proteins appear to play an important role in nuclear transport (23–25) and that WGA inhibits NLS-mediated active nuclear import (26–28). When WGA was co-injected with the recombinant Stat1, the IFN-γ-dependent nuclear import of Stat1 was inhibited (Fig. 1*B*, *d*), and endogenous native Stat1 was also inhibited by WGA injecton (data not shown), indicating that Stat1 is actively but not passively transported into the nucleus through the NPC.

IFN-γ stimulation causes phosphorylation of proteins (such as Jak kinases), and staurosporine, a kinase inhibitor, which is known to prevent the activation of Stat1 (16). Moreover, using cell lines that express wild type and mutant Stat1, it has been demonstrated that phosphorylation of Tyr^{701} is required for both nuclear accumulation of Stat1 as well as the activation of specific genes (18). In this study, we found that the IFN-γ-dependent nuclear translocation of Stat1 was inhibited in cells

[2] T. Tachibana and Y. Yoneda, unpublished data.

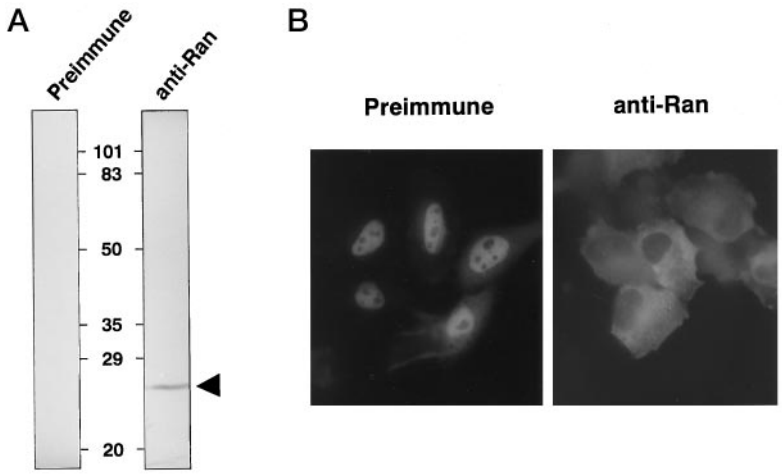

FIG. 3. **Effects of anti-Ran antibodies on nuclear import of Stat1.** *A*, whole cell extracts of FL cells were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with preimmune IgG or affinity purified anti-Ran antibodies. *B*, preimmune IgG or affinity purified anti-Ran antibodies (18 mg/ml) were microinjected with Stat1, and the cells were treated with IFN-γ.

treated with staurosporine (Fig. 2*b*). In additional experiments, tyrosine 701 was substituted with phenylalanine or glutamic acid by site-directed mutagenesis, and these mutant Stat1s were purified to homogeneity. Neither of the Stat1 mutants translocated into the nucleus, even in the presence of IFN-γ (Fig. 2, *c* and *d*), indicating that phosphorylation, but not negative charge at this position, is essential for the signal-dependent nuclear import of Stat1. It has been proposed that tyrosine phosphorylated Stat1 forms a homodimer through an interaction between phosphorylated tyrosine and the SH2 domain of another Stat1 (29). The data presented here are consistent with this proposal and suggest that tyrosine unphosphorylated Stat1 localizes in the cytoplasm as a monomer prior to cytokine stimulation, and when the tyrosine residue is phosphorylated after stimulation, Stat1 forms a homodimer that then actively translocates into the nucleus.

Ran Is Involved in Extracellular Signal-dependent Nuclear Import of Stat1—It is well known that a small G protein, Ran, is essential for SV40 T antigen-NLS containing substrates to translocate through the nuclear pore *in vitro* (10, 11). In order to determine if Ran also mediates IFN-γ-dependent nuclear import of Stat1, we prepared polyclonal anti-Ran antibodies that specifically recognized endogenous human Ran of FL cells (Fig. 3*A*). When the anti-Ran antibodies were injected with Stat1, IFN-γ-induced nuclear import of Stat1 was strongly inhibited, whereas preimmune IgG had no effect on the process (Fig. 3*B*). Nuclear import of native Stat1 was similarly inhibited by injection of the antibodies (data not shown). These results indicate that Ran participates in both constitutive and extracellular signal-dependent, conditional nuclear import of proteins.

Ran has GTPase activity and its activity is known to be essential for translocation of SV40 T-NLS-BSA (10–12). In order to determine if GTP hydrolysis on Ran is required for IFN-γ-dependent nuclear import of Stat1, we prepared a mutant Ran protein, Ran-G19V, that was deficient in GTP hydrolysis. The guanine nucleotides binding to wild type and Ran-G19V loaded with GTP (GTP-Ran and GTP-Ran-G19V, respectively) were estimated by HPLC to be 70% GTP and 30% GDP and with GDP (GDP-Ran and GDP-Ran-G19V, respectively) were determined to be nearly 100% GDP. When buffer, wild type GTP- or GDP-Ran was microinjected with Stat1, Stat1 was transported in a normal fashion to the nucleus. In contrast, injection of GDP or GTP bound form of Ran-G19V strongly inhibited IFN-γ-dependent nuclear import of Stat1 (Fig. 4). These results indicate that Ran GTPase is required for IFN-γ-dependent nuclear import of Stat1. The mutant Ran protein also inhibited nuclear import of SV40 T-NLS-BSA (data not shown), which is consistent with the recent report that injection of another GTPase-deficient mutant Ran-Q69L blocked the nuclear import of SV40 T antigen *in vivo* (30) and that of SV40 T-NLS conjugated BSA *in vitro* (31). More recently, similar results were reported on the nuclear import of snRNP and glucocorticoid receptor using Ran-Q69L or Ran-G19V (31, 32). These findings with the Ran-G19V and Ran-Q69L mutant proteins, which are insensitive to GAP activity, implicate that Ran acts as a common key factor for constitutive and extracellular signal-dependent nuclear import of proteins and snRNP import.

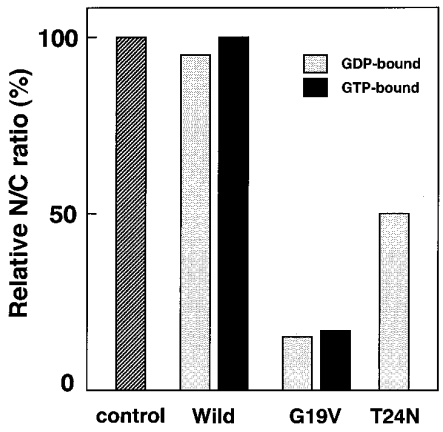

FIG. 4. **Effects of microinjected recombinant Ran proteins on nuclear accumulation of Stat1.** Stat1 was microinjected with buffer (*control*) or recombinant Ran proteins (3 mg/ml) in transport buffer, and the cells were treated with IFN-γ. Relative nucleus to cytoplasm (*N/C*) ratio (average values for 20 cells) was determined as described under "Materials and Methods."

The loss of activity of chromatin-associated protein RCC1, which mediates the GDP-GTP exchange of Ran (33), resulting in the accumulation of GDP-bound Ran, leads to suppression of nuclear import of NLS-BSA (34). To better understand the effect of GDP-Ran on nuclear import of Stat1, we prepared another mutant Ran protein, Ran-T24N, which has little or no binding affinity to GTP but has GTPase activity and thus accumulates in the GDP-bound form and binds tightly to RCC1 (35). The guanine nucleotide binding to Ran-T24N loaded with GDP (GDP-Ran-T24N) was determined to be nearly 100% GDP. When wild type GDP-Ran was microinjected with Stat1, Stat1 was transported in a normal fashion to the nucleus as described above. In contrast, injection of the GDP-Ran-T24N blocked nuclear import of Stat1 (Fig. 4) as well as SV40 T-NLS-BSA (data not shown) *in vivo*, although the inhibition was less

effective than that by GTP-Ran-G19V. This is consistent with recent data that show that nuclear import of SV40 T antigen-NLS conjugated substrate and snRNP was inhibited by Ran-T24N *in vitro* (31). These results suggest that GDP-bound mutant Ran reduces both constitutive and conditional nuclear import of proteins, although further experiments are required to understand the exact mechanism by which GDP-bound mutant Ran suppresses the nuclear import.

In this study, we established an *in vivo* system that is useful for understanding extracellular signal-dependent nuclear import of proteins. The experimental evidences presented here indicate that Stat1 is actively transported in response to IFN-γ through the nuclear pore depending on the phosphorylation of a single tyrosine residue (Tyr^{701}). Although the NLS of Stat1 has not yet been determined, IFN-γ-dependent nuclear import of Stat1 is mediated by Ran and requires its GTPase activity, indicating that extracellular signal-dependent active nuclear import of Stat1 shares at least one common key factor, Ran, which was first identified to support the translocation step of constitutive nuclear import of SV40 T-NLS-bearing substrate.

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