The EMBO Journal Vol.16 No.23 pp.7067–7077, 1997

Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1

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In response to interferon-γ (IFN-γ), Stat1 is tyrosine phosphorylated and translocates to the nucleus where it activates transcription. In this study, we identified factors which mediate the nuclear import of Stat1. Tyrosine-phosphorylated Stat1 associated with the β subunit (a 97 kDa component) of the nuclear pore-targeting complex via the NPI-1 family, but not the Rch1 family, of α subunit (a 58 kDa component) as a result of IFN-γ stimulation. Antibodies against NPI-1 or β subunit consistently inhibited the IFN-γ-dependent nuclear import of Stat1 in living cells, although antibodies reactive to Rch1 had no effect. Solution binding assays with deletion mutants of NPI-1 showed that the Stat1-binding domain of NPI-1 was located in the carboxy-terminal region, which is clearly distinct from the SV40 large T antigen nuclear localization signal (NLS)-binding region. These results indicate that the extracellular signal-dependent nuclear transport of Stat1 is mediated by NPI-1, but not Rch1, in conjunction with β subunit, and that these factors participate in, not only constitutive, but also the conditional nuclear import of proteins.

Keywords: dominant negative/interferon-γ/NPI-1/nuclear import/Stat1

Introduction

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. In response to various cytokines, the Stat family proteins are transported to the nucleus where they activate their specific gene transcription, and thus Stat family proteins play critical roles in the signal transduction pathways for a variety of cytokine receptors (Darnell *et al.*, 1994; Ihle and Kerr, 1995; Fukada *et al.*, 1996; Nakajima *et al.*, 1996). Each cytokine activates a different Stat protein, for example interferon-γ (IFN-γ) activates Stat1 (Shuai *et al.*, 1992, 1993a), while interleukin-6 (IL-6) activates Stat3 and Stat1 (Akira *et al.*, 1994; Fujitani *et al.*, 1994; Zhong *et al.*, 1994; Nakajima *et al.*, 1995). After treatment of cells with IFN-γ, a specific tyrosine residue (Tyr701) of Stat1 is phosphorylated by receptor-associated tyrosine kinases, after which the activated Stat1 is translocated into the nucleus to activate target genes directly by binding to specific promoter sequences (Darnell *et al.*, 1994; Ihle and Kerr, 1995). In contrast to the detailed understanding of the activation step and the binding of Stat1 to DNA, very little is known about the translocation step from the cytoplasm to the nucleus in this signaling pathway.

Nuclear import of karyophilic proteins into the nucleus has been shown to be directed by short amino acid sequences, termed nuclear localization signals (NLSs). The selective protein transport process into the nucleus can essentially be divided into two steps; (i) NLS-dependent binding to the cytoplasmic face of the nuclear pore, followed by (ii) an energy-dependent translocation through the nuclear pore complex (NPC). In previous studies, we found that the first step in the nuclear import of SV40 T antigen NLS-conjugated bovine serum albumin (T-NLS–BSA) occurs via the formation of a functional complex (termed the nuclear pore-targeting complex; PTAC) in the cytoplasm, targeting the nuclear pores (Imamoto *et al.*, 1995a). It was found that in subsequent steps, a small G protein, Ran (Melchior *et al.*, 1993; Moore and Blobel, 1993) and the Ran-interacting factor p10/NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995) are required for the translocation of the NLS-containing protein into the nucleus through NPCs.

The PTAC is a stable complex, composed of a karyophilic protein and two essential components, which we have identified from mouse Ehrlich ascites tumor cells (Imamoto *et al.*, 1995b,c). One of these, a 58 kDa component of PTAC (PTAC58), functions as an NLS receptor. A variety of homologous proteins were identified almost simultaneously from various organisms, and individually named as importin α, Rch1, hSRP1α, karyopherin α and NPI-1/hSRP1 in mammals and SRP1 in yeast (Yano *et al.*, 1992; Cortes *et al.*, 1994; Cuomo *et al.*, 1994; Görlich *et al.*, 1994; Moroianu *et al.*, 1995; O'Neill and Palese, 1995; Weis *et al.*, 1995). These proteins consist of eight repeats known as the armadillo structure, which are rich in hydrophobic amino acids. From the cDNA sequences and the deduced amino acid sequences of these proteins, human homologs can be classified into two major groups, Rch1/hSRP1α and NPI-1/hSRP1 (~50% amino acid identity with each other). The 58 kDa component of PTAC which we identified previously appears to correspond to the mouse homolog of human Rch1/hSRP1α (Imamoto *et al.*, 1995b). In this report, the term 'α subunit' will be used collectively for all these molecules.

The other, 97 kDa, component of the PTAC (PTAC97) functions to target the PTAC to the NPC by binding to PTAC58 bound to a karyophile (Imamoto *et al.*, 1995c). Its homologs from various species have been isolated and are referred to as importin β, karyopherin β and p97 in

mammals and Kap95p in yeast (Chi *et al.*, 1995; Enenkel *et al.*, 1995; Görlich *et al.*, 1995; Radu *et al.*, 1995). Since these proteins have a very high amino acid identity with each other in mammals (>95%), we will refer to them as the 'β subunit' in this study. Recently, a novel receptor-mediated nuclear import pathway was demonstrated by use of a novel transport signal in the hnRNP A1, termed M9. The M9-directed nuclear import was found to be mediated by a specific interacting protein, named transportin, which is distantly related to the β subunit (Pollard *et al.*, 1996). Apart from this finding, in yeast, three additional proteins, homologous to Kap95p, were identified from the complete yeast database search. One of these, Kap104p, has been shown to act in the nuclear import of a specific class of proteins (Aitchison *et al.*, 1996).

The α and β subunits and Ran were shown to be essential factors in the nuclear import of basic-type NLS-containing proteins (Görlich *et al.*, 1994; Imamoto *et al.*, 1995b,c), which are constitutively transported to the nucleus immediately after their synthesis in the cytoplasm. On the other hand, extracellular signal-dependent nuclear protein import pathways have also been studied recently. It was shown recently that tyrosine phosphorylation of Stat1 is essential for its active nuclear import, and Ran and its GTPase activity are required for the IFN-γ-dependent nuclear translocation of Stat1 (Sekimoto *et al.*, 1996). Furthermore, Ran was found to be required for signal-dependent nuclear import of glucocorticoid receptor (Carey *et al.*, 1996). However, the mechanism and factors that participate in the first step of the signal-dependent nuclear transport of proteins which pre-exist in the cytoplasm remain unknown.

This study reports an attempt to identify the factors which mediate the targeting of Stat1 from cytoplasm to nuclear pores after cytokine stimulation. An *in vitro* solution binding assay showed that tyrosine-phosphorylated Stat1 forms a stable complex with NPI-1, but not with PTAC58 (Rch1), and the β subunit of the nuclear pore-targeting complex in response to IFN-γ. Furthermore, microinjection of anti-NPI-1, but not anti-PTAC58, antibodies into the cytoplasm inhibited the nuclear import of Stat1. These results indicate that although SV40 T antigen NLS-containing substrate is transported by both Rch1 and NPI-1, the IFN-γ-dependent nuclear import of Stat1 is mediated by NPI-1, but not by Rch1, in conjunction with β subunit. An NPI-1 deletion experiment shows that the Stat1-binding domain of NPI-1 is distinct from the conventional NLS-binding domain, which, in turn, indicates that NPI-1 binds to two different karyophiles at different domains. The data collected herein show, for the first time, that the extracellular signal-dependent nuclear import of proteins requires NPI-1 and the β subunit for targeting the nuclear pores.

Results

Tyrosine-phosphorylated Stat1 associates with β subunit

In order to determine whether the factors required for nuclear import of SV40 T antigen NLS-containing substrate also participate in the IFN-γ-dependent nuclear transport of Stat1, we examined the interaction between

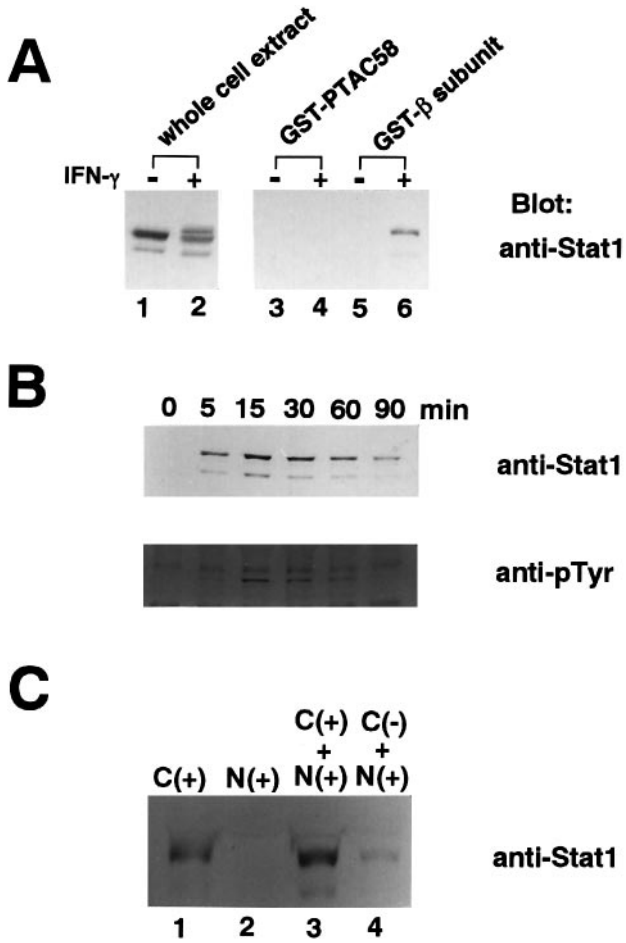


Fig. 1. The indirect interaction of β subunit with tyrosine-phosphorylated Stat1. (**A**) HeLa whole cell extract with or without IFN-γ treatment was incubated with GST–PTAC58 or GST–β subunit fusion protein absorbed to glutathione–Sepharose. Precipitates were separated on 7.5% SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-Stat1 antibody. (**B**) Time course of interaction between Stat1 and β subunit. HeLa whole cell extract treated with IFN-γ for the indicated times was incubated with GST–β subunit, and the precipitated Stat1 was detected with anti-Stat1 or anti-phosphotyrosine antibodies. (**C**) Cytosol (C) and nuclear extract (N) were prepared from HeLa cells treated with (+) or without (–) IFN-γ. Extracts were incubated with GST–β subunit, and associated Stat1 was detected as for (A). C(+) and N(+), cytosol and nuclear extract prepared from IFN-γ-treated cells, respectively; C(–), cytosol prepared from untreated cells.

Stat1 and PTAC58 or β subunit. PTAC58 and β subunit were prepared as GST fusion proteins. These GST fusions show transport activities in the *in vitro* semi-intact cell assay using SV40 T antigen NLS–BSA conjugate (T-NLS–BSA) as the substrate, and the activities are comparable with proteins which are devoid of the GST portion (Imamoto *et al.*, 1995c). HeLa whole cell extracts treated with or without IFN-γ were incubated with either GST–PTAC58 or GST–β subunit fusion proteins which were absorbed to glutathione–Sepharose. Stat1 failed to associate with GST–PTAC58 (Figure 1A, lanes 3 and 4), whereas SV40 large T antigen did associate with the GST–PTAC58 after incubation with whole cell extract of SV40-transformed human fibroblasts (Wi38 VA13) (data not shown). In contrast, the interaction of GST–β subunit with Stat1 was dependent on IFN-γ treatment (Figure 1A, lane 6). Precipitated Stat1 was tyrosine phosphorylated and the

interaction between Stat1 and GST–β subunit reached a maximum at 15 min after IFN-γ treatment, and decreased thereafter in a time-dependent manner (Figure 1B). This time course of interaction between GST–β subunit and Stat1 paralleled the tyrosine phosphorylation of Stat1 (data not shown; Shuai *et al.*, 1992). These data indicate that the β subunit associates with the activated form of Stat1.

We attempted to determine whether β subunit associates with Stat1 directly or indirectly. We precipitated Stat1 with the GST–β subunit from cytosol or nuclear extracts of HeLa cells with or without IFN-γ treatment. Although both cytosol and nuclear extract treated with IFN-γ contain tyrosine-phosphorylated Stat1 (data not shown), Stat1 was precipitated with β subunit from cytosol but not from the nuclear extract (Figure 1C, lanes 1 and 2). Stat1 was precipitated more efficiently on incubation with a mixture of both cytosol and nuclear extracts with IFN-γ treatment (Figure 1C, lane 3). Furthermore, Stat1 was also precipitated from nuclear extracts with IFN-γ treatment when cytosol without IFN-γ stimulation was added (Figure 1C, lane 4). These results indicate that the β subunit interacts with activated Stat1 indirectly and that additional factor(s) exist in the unstimulated cytosol which are required for this association.

The NPI-1 family, but not the Rch1 family, associates with tyrosine-phosphorylated Stat1

It is well known that β subunit associates with a karyophile via the α subunit (Görlich *et al.*, 1995; Imamoto *et al.*, 1995c). In humans, two types of α subunit, NPI-1 and Rch1, have already been isolated. PTAC58 is a mouse homolog of Rch1 (93% amino acid identity). Therefore, we prepared GST–NPI-1, and examined its interaction with Stat1. The GST–NPI-1 fusion protein showed *in vitro* transport activity for T-NLS–BSA in conjunction with β subunit and Ran, and this activity was comparable with that of proteins devoid of the GST portion (data not shown). Using a whole cell extract of HeLa cells treated with IFN-γ, it was found that only GST–NPI-1, but neither GST nor GST–PTAC58, associated with Stat1 (Figure 2A upper). The precipitated Stat1 was tyrosine phosphorylated (Figure 2A lower).

Since PTAC58 is a mouse protein, it is possible that human Stat1 does not interact with mouse PTAC58, but does so with its human homolog, Rch1. To clarify this problem, we attempted the same experiment using mouse NIH-3T3 cells with or without murine IFN-γ treatment. The result showed that intrinsic mouse Stat1 in NIH-3T3 cells associated with human NPI-1, but not with mouse PTAC58 (Figure 2B), which is consistent with the results obtained with HeLa cell extracts.

Since these results were obtained using an *in vitro* solution binding assay employing recombinant proteins, in order to confirm whether native NPI-1 and β subunit interact with Stat1 *in vivo*, we transiently expressed His_6-tagged Stat1 in human 293T cells. After IFN-γ stimulation, the expressed His_6-Stat1 was precipitated with Ni-NTA–agarose and the bound proteins were analyzed by immunoblotting with anti-NPI-1 antibodies, or anti-PTAC58 antibodies which specifically recognize the human counterpart, Rch1. The results showed that the expressed His_6-Stat1 interacted with NPI-1, but not with Rch1, in an IFN-γ-dependent manner (Figure 3). In this

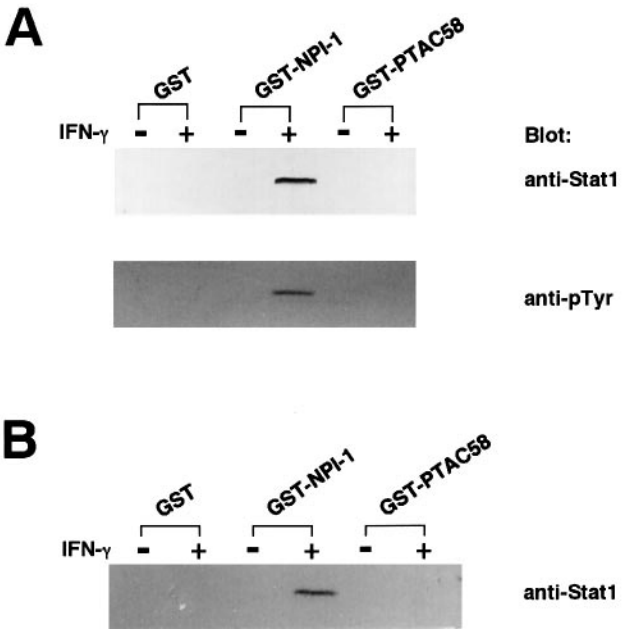


Fig. 2. The interaction of NPI-1 with tyrosine-phosphorylated Stat1. (**A**) HeLa whole cell extract with or without IFN-γ treatment was incubated with GST, GST–NPI-1 or GST–PTAC58 adsorbed to glutathione–Sepharose. Precipitates were separated on 7.5% SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-Stat1 and anti-phosphotyrosine antibodies. (**B**) NIH-3T3 whole cell extract with or without murine IFN-γ treatment was used as for (A) instead of HeLa cells.

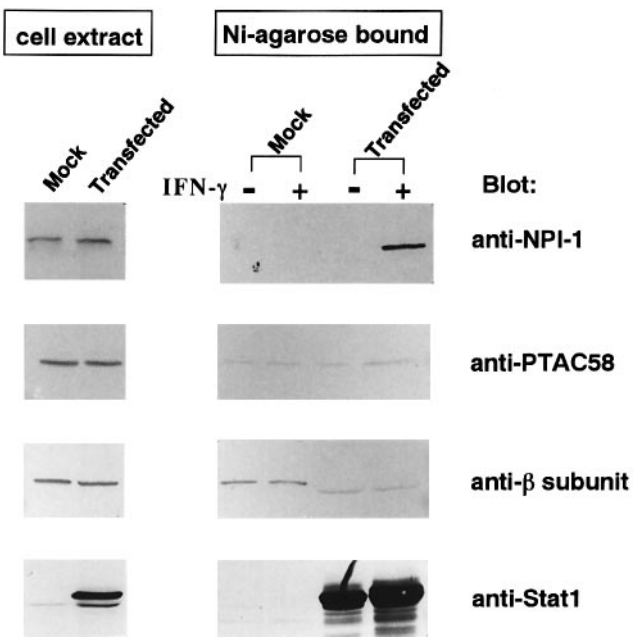


Fig. 3. The interaction of endogenous NPI-1 with expressed Stat1. His_6-tagged Stat1 was transiently expressed in human 293T cells and the cells were treated with or without IFN-γ. Ni-NTA–agarose-bound proteins from the cell extract were separated on SDS–PAGE and immunoblotted with anti-NPI-1, anti-PTAC58, anti-β subunit and anti-Stat1 antibodies. As a control experiment, the cell extract was prepared from mock-transfected 293T cells.

series of experiments, β subunit was not detected by immunoblotting, probably as a result of a large amount of co-migrating His_6-Stat1 in SDS–PAGE. These results

indicate that activated Stat1 associates with NPI-1, but not with Rch1 *in vivo*.

To confirm further that NPI-1 and β subunit participate in the transport of Stat1 from cytoplasm to the nucleus *in vivo*, affinity-purified polyclonal anti-NPI-1 and anti-β subunit antibodies were used. Both antibodies recognized one band when a whole cell extract of HeLa cells was analyzed by immunoblotting (Figure 4A). When recombinant NPI-1 and PTAC58 were resolved, anti-NPI-1 antibodies had no cross-reactivity with PTAC58, although anti-PTAC58 antibodies cross-reacted slightly with NPI-1 (data not shown). First, by using these antibodies, we checked ability of these antibodies to inhibit the nuclear import of T-NLS–BSA. Microinjected anti-PTAC58 and anti-β subunit antibodies inhibited the nuclear import of T-NLS–BSA effectively, but anti-NPI-1 antibodies did so only slightly (Figure 4B). To assess the effects of these antibodies on signal-dependent nuclear import of Stat1, we prepared hemagglutinin (HA)-tagged Stat1 to detect intracellular localization of injected Stat1 by indirect immunofluorescence with anti-HA antibodies, as described previously (Sekimoto *et al.*, 1996). Stat1, when microinjected into the cytoplasm of HeLa cells, was translocated into the nucleus dependent on IFN-γ stimulation (Figure 4C). Microinjected anti-PTAC58 antibodies, which have already been shown to strongly inhibit the nuclear import of T-NLS–BSA (Imamoto *et al.*, 1995b; Figure 4B), had no effect on the IFN-γ-dependent nuclear import of Stat1 (Figure 4D). In contrast, affinity-purified anti-NPI-1 antibodies inhibited the IFN-γ-dependent nuclear import of Stat1 (Figure 4D). Furthermore, affinity-purified anti-β subunit antibodies strongly inhibited the nuclear import of Stat1 when injected 30 min prior to IFN-γ stimulation (Figure 4D). It is also noteworthy that, unlike anti-Ran (Sekimoto *et al.*, 1996) or anti-β subunit antibodies, cytoplasmic injection of anti-NPI-1 antibodies, but not anti-PTAC58 antibodies caused the distinct aggregation of Stat1 in the cytoplasm. A similar aggregation pattern was observed when T-NLS–BSA was co-injected with anti-PTAC58 antibodies (Figure 4B), and this aggregation was supposed to be the result of precipitation of T-NLS–BSA with Rch1 by the antibodies (Imamoto *et al.*, 1995b). These results confirm that NPI-1, but not Rch1, and β subunit are involved in the IFN-γ-dependent nuclear translocation of Stat1.

It has been proposed that tyrosine-phosphorylated Stat1 forms a homodimer between phosphotyrosine and the SH2 domain of each Stat1, and the dimerization is required for its DNA binding (Shuai *et al.*, 1994). It is known that the DNA binding of Stat1 is inhibited in the presence of phosphotyrosine, but not phosphoserine and phosphothreonine, which means that free phosphotyrosine disrupts the homodimerization of Stat1 (Sadowski *et al.*, 1993). We confirmed that increasing amounts of free phosphotyrosine caused inhibition of DNA binding of Stat1 (Figure 5A). To determine whether tyrosine-phosphorylated Stat1 binds to NPI-1 as a homodimer, we assessed the effect of free phosphotyrosine, phosphoserine or phosphothreonine on the interaction between Stat1 and NPI-1. We found that phosphotyrosine, but not phosphoserine or phosphothreonine, inhibited the interaction of Stat1 with NPI-1 in a dose-dependent manner (Figure 5B, upper panel), whereas these phosphorylated amino acids had no effects on the interaction between NPI-1 and SV40 T antigen (Figure 5B, lower panel). These results clearly indicate that homodimerization of Stat1 is required for its interaction with NPI-1.

Stat1 does not have a conventional NLS

Since the NLSs previously identified consist of a cluster of basic amino acid residues in the primary structure (Dingwall and Laskey, 1991) and NPI-1 is known to recognize the NLSs, we examined whether the primary structure of Stat1 contains a functional NLS belonging to the NLSs identified previously. To determine the region of Stat1 which is critical for IFN-γ-dependent nuclear import, we constructed several deletion mutants of Stat1 and expressed them transiently in human FL cells. Unlike wild-type Stat1, however, none of these mutants was translocated to the nucleus, even in the presence of IFN-γ (data not shown). Therefore, since human Stat1 contains three regions of basic amino acid clusters in its deduced amino acid sequence (Figure 6A), we introduced mutations into these regions by site-directed mutagenesis, in order to assess their contribution to the nuclear import of Stat1. Because the substitution of three or four amino acids caused low expression and instability of the mutant Stat1, we constructed nine mutants containing single or double mutations, expressed them in *Escherichia coli* and purified them to homogeneity. When microinjected, all the recombinant mutant proteins were transported into the nucleus in a manner similar to the wild-type, although the R88L mutant showed a slightly reduced nuclear import activity (Figure 6B). However, when the region in the vicinity of Arg88, ^{78}LLQHNIRKSKRNLQ^{91}, was inserted into the GST–green fluorescence protein (GFP) fusion, this protein was not transported into the nucleus in the presence or absence of IFN-γ, although the insertion of the NLS of SV40 T antigen showed efficient import activity of the fusion protein (data not shown). These data indicate that Stat1 does not contain a classical NLS, as defined by the basic amino acid cluster.

The Stat1-binding domain of NPI-1 is located at its C-terminus

Since it was found that NPI-1 is capable of interacting with Stat1 as well as the SV40 T antigen NLS substrate, we tried to determine if Stat1 and SV40 T antigen NLS interact with the same domain of NPI-1. For this purpose, we prepared several deletion mutants of NPI-1 and assessed their binding ability with Stat1 and SV40 large T antigen. These deletion mutants of NPI-1 were expressed and purified as GST fusion proteins (Figure 7A). We first checked the binding activity of these mutants with SV40 large T antigen by using Wi38 VA13 cell extracts. Four mutants (294–538, 338–538, 404–538 and 425–538) failed to bind to the SV40 large T antigen. In contrast, deletion mutants of the C-terminal region (1–492, 1–475 and 78–475) bound to the SV40 large T antigen to the same extent as the full-length NPI-1 (Figure 7B, upper). The same results were obtained when the bipartite-type NLS of nucleoplasmin-conjugated BSA was used as substrate (data not shown), suggesting that the binding domain of NPI-1 for the conventional basic-type NLSs is located in the armadillo repeats. Using HeLa cell extracts treated with IFN-γ, all the N-terminal deletion mutants of NPI-1

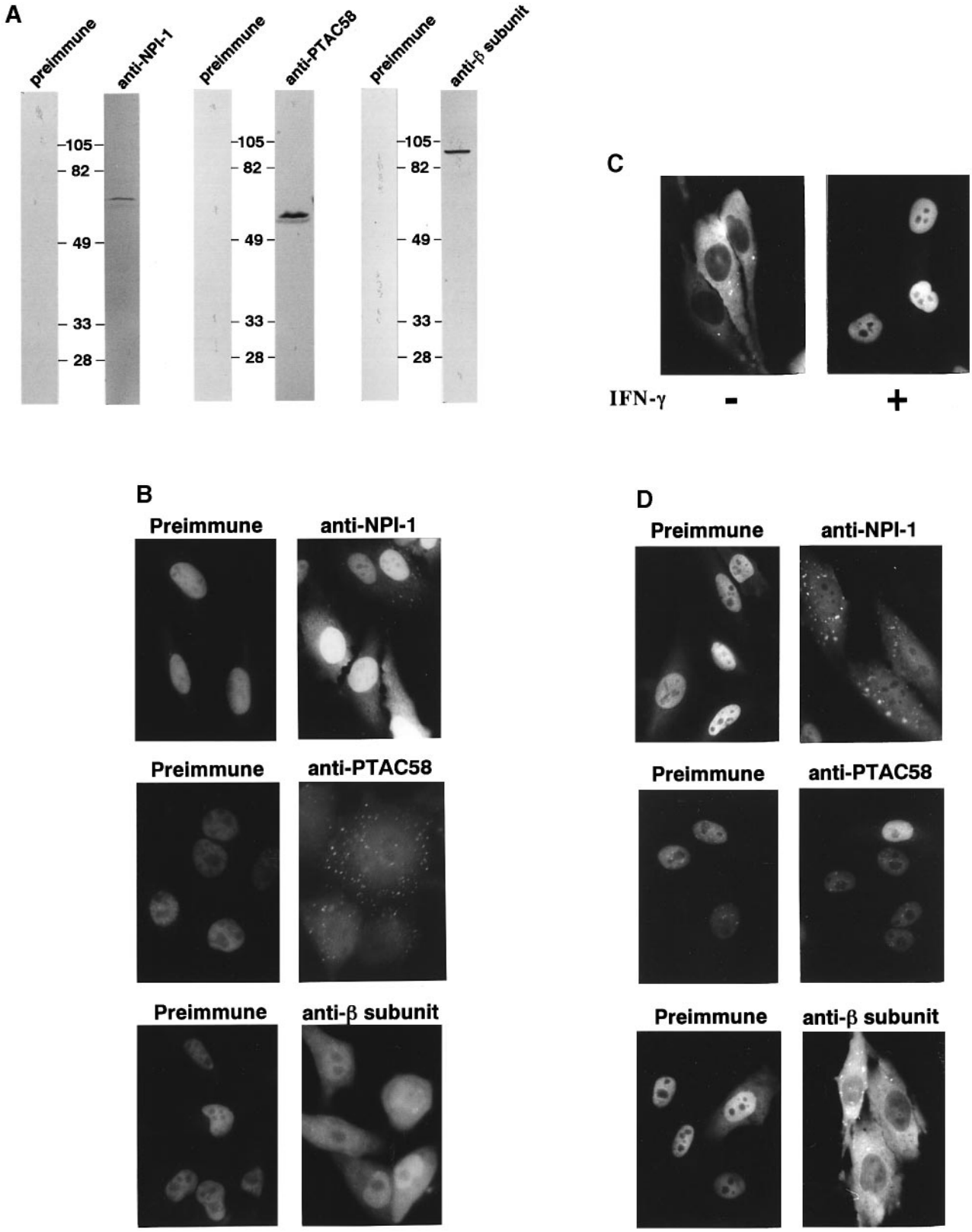


Fig. 4. Effect of affinity-purified antibodies against nuclear import factors on the nuclear import of Stat1. (**A**) HeLa whole cell extracts were resolved by SDS–PAGE and immunoblotted with anti-NPI-1, anti-PTAC58, anti-β subunit or their pre-immune IgG. (**B**) Pre-immune IgG or affinity-purified anti-NPI-1, anti-PTAC58 or anti-β subunit antibodies (25 mg/ml) were co-injected with FITC-labeled T-NLS–BSA and, after 30 min incubation, the cells were fixed. (**C**) Purified HA-tagged Stat1 protein was microinjected into the cytoplasm of HeLa cells and the cells were treated with IFN-γ (50 ng/ml) for 30 min. After fixation of the cells, the intracellular localization of Stat1 was examined by indirect immunofluorescence with anti-HA antibody. (**D**) Pre-immune IgG or affinity-purified anti-NPI-1, anti-PTAC58 or anti-β subunit antibodies (25 mg/ml) were microinjected with Stat1 into the cytoplasm of HeLa cells. Thirty minutes later, the cells were stimulated with IFN-γ (50 ng/ml) for 30 min.

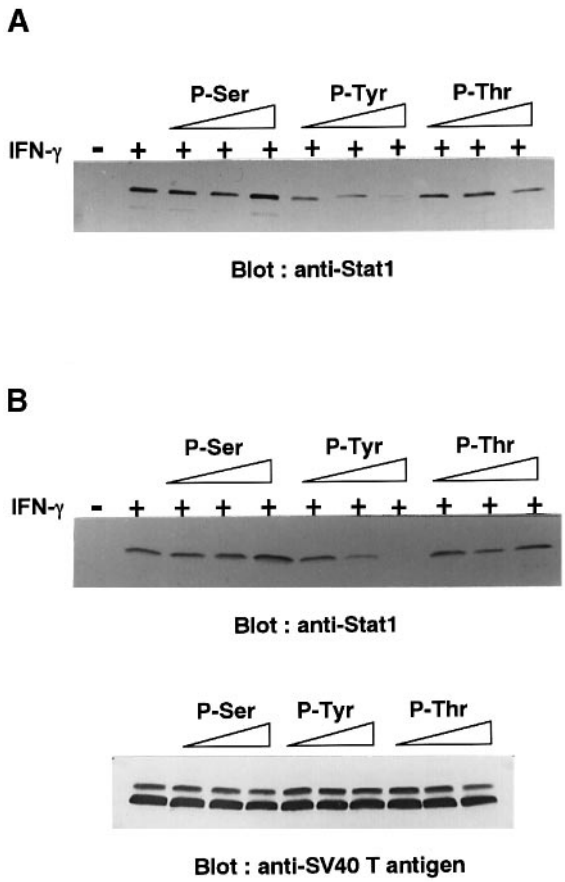
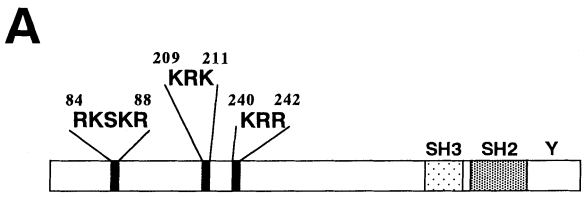


Fig. 5. Effects of phosphotyrosine on the binding of Stat1 to DNA or NPI-1. (**A**) HeLa whole cell extract with or without IFN-γ treatment was incubated with high affinity SIE of c-*fos* gene-conjugated Sepharose in the presence of increasing amounts of phosphoserine, phosphotyrosine or phosphothreonine (2.5, 5 and 20 mM). Gels were washed extensively, and bound proteins were detected by immunoblotting using anti-Stat1. (**B**) HeLa whole cell extract with or without IFN-γ treatment, or whole cell extract of SV40-transformed human fibroblasts (Wi38 VA13 cells) was incubated with GST–NPI-1 in the presence of increasing amounts of phosphoserine, phosphotyrosine or phosphothreonine (2.5, 5 and 20 mM). The bound proteins were detected by immunoblotting with anti-Stat1 or anti-SV40 T antigen antibodies.



B

Mutants	Localization
R84L/K85T	N
K87T	N
R88L	C/N
K209T	N
R210L	N
K211T	N
K240T	N
R241L	N
R242L	N

Fig. 6. Nuclear import activity of amino acid substitution mutants of basic amino acid clusters of Stat1. (**A**) Three basic regions exist in the deduced amino acid sequence of human Stat1. (**B**) Mutants in these basic regions were purified to homogeneity and microinjected into the cytoplasm of human FL cells. The intracellular localization of injected mutants after stimulation with IFN-γ is shown. N, nucleus; C, cytoplasm.

were found to associate with Stat1, whereas the C-terminal deletions abolished the Stat1-binding activity of NPI-1 (Figure 7B, lower). Although attempts were made to prepare a shorter form of NPI-1, to define the essential Stat1-binding domain, these short mutant constructs were not expressed as complete entities in *E.coli*.

In order to determine whether NPI-1 binds to two cargo proteins (Stat1 and T-NLS–BSA) simultaneously, competition experiments were done. The interaction between SV40 large T antigen and NPI-1 was competed with wild-type T-NLS–BSA, but not reverse type T-NLS–BSA (Figure 7C, upper). In contrast, the presence of T-NLS–BSA did not interfere with the interaction between Stat1 and NPI-1 (Figure 7C, lower). Addition of wild-type SV40 T antigen NLS peptides (0.1 mM) caused complete inhibition of the interaction between SV40 T antigen and NPI-1, but NLS peptide had no effect on the interaction between Stat1 and NPI-1 even at 1 mM (data not shown). These results indicate that the binding site of Stat1 is distinct from that of the conventional basic-type NLS and, thus, that NPI-1 contains at least two binding sites for karyophiles, and Stat1 and conventional NLS-containing substrate can bind to NPI-1 simultaneously.

In vivo inhibition of nuclear import of Stat1 and SV40 T-NLS substrate by distinct dominant-negative forms of NPI-1

It is well known that the amino-terminal region of NPI-1 is essential for β subunit binding, and deletion of this region causes loss of import activity. As expected, NPI-1 mutants 294–538 and 78–475 bind to Stat1 and SV40 T antigen NLS, respectively (Figure 7B), but not to β subunit (data not shown). Therefore, we examined whether they can inhibit the nuclear protein import in a dominant-negative manner. When 294–538 was microinjected with Stat1 or T-NLS–BSA into the cytoplasm of HeLa cells, the IFN-γ-dependent nuclear import of Stat1 was strongly inhibited, but not the nuclear import of T-NLS–BSA (Figure 8). In contrast, co-injection of 78–475 only inhibited the nuclear import of T-NLS–BSA. Injection of GST or GST–NPI-1 (full-length) had no effect on the nuclear import of either Stat1 or T-NLS–BSA (Figure 8). These results clearly indicate that these two mutants (294–538 and 78–475) act as quite specific dominant-negative inhibitors for the nuclear import of Stat1 and SV40 large T antigen, respectively, and confirm that Stat1 is transported into the nucleus through the specific interaction with the C-terminal domain of NPI-1 *in vivo*.

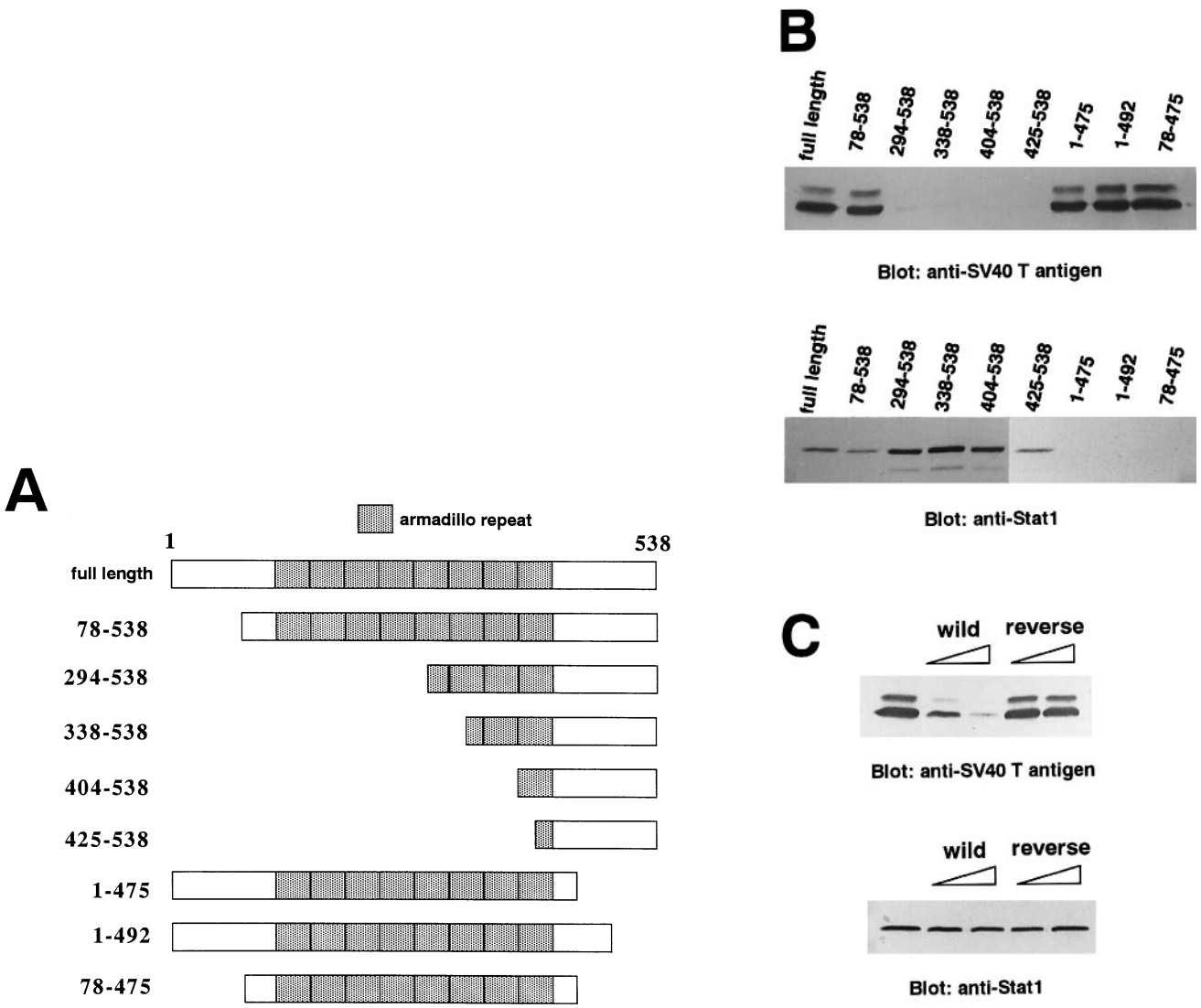


Fig. 7. Mapping of the NPI-1 region which interacts with Stat1. (**A**) Deletion mutants of NPI-1 used in this study. All mutants were expressed as GST fusion protein in *E.coli* and purified using glutathione–Sepharose. (**B**) The ability of these mutants to bind to SV40 T antigen or Stat1 is shown. Wi38 VA13 or HeLa whole cell extracts treated with IFN-γ were incubated with various deletion mutants of NPI-1, and bound proteins were detected by immunoblotting with anti-SV40 T antigen or anti-Stat1 antibodies. (**C**) Effects of T-NLS–BSA on the binding of NPI-1 to SV40 T antigen or Stat1. Wi38 VA13 or HeLa whole cell extracts treated with IFN-γ were incubated with GST–NPI-1 in the presence of wild-type or reverse type T-NLS–BSA (0.1 and 1 μM), and bound proteins were detected by immunoblotting with anti-SV40 T antigen or anti-Stat1 antibodies.

Discussion

The factors required for nuclear import of proteins that are transported constitutively after their synthesis have been identified, and findings relating to their function in nuclear transport processes have been accumulating for several years. Although it has been shown that Ran and its GTPase activity are essential for extracellular signal-dependent nuclear import of glucocorticoid receptor and Stat1 (Carey *et al.*, 1996; Sekimoto *et al.*, 1996), the factors that mediate the targeting of the proteins from cytoplasm to the nuclear pores are still unknown. The present study revealed the factors which participate in IFN-γ-dependent nuclear pore targeting of Stat1.

It has been shown that in vertebrates, the α subunit of the nuclear pore-targeting complex appears to consist of diverse, but related proteins, based on the deduced amino acid sequences of cloned genes. The NPI-1/hSRP1 family and Rch1/hSRP1α/PTAC58 family of α subunit have ~50% amino acid identity with each other, and these family members were found to participate in the nuclear import of both single and bipartite-type conventional NLS-containing proteins. Although these members were found to co-exist in single cell populations, it remains unknown why such divergence in the cell is required, and how these molecules recognize different classes of NLSs in the cell cytoplasm. Moreover, as far as we know, there is no evidence that a class of NLS is recognized specifically by only one α subunit family. In this study, we found that NPI-1, but not Rch1 (PTAC58), interacts with activated Stat1 *in vitro* and *in vivo* (Figures 2 and 3), and that microinjected affinity-purified polyclonal antibodies to NPI-1, but not the antibodies to PTAC58, inhibited IFN-γ-dependent nuclear import of Stat1 (Figure 4D). Thus, this is the first *in vivo* evidence indicating that different α subunit families function differently from each other at the recognition step of the transport in the cytoplasm.

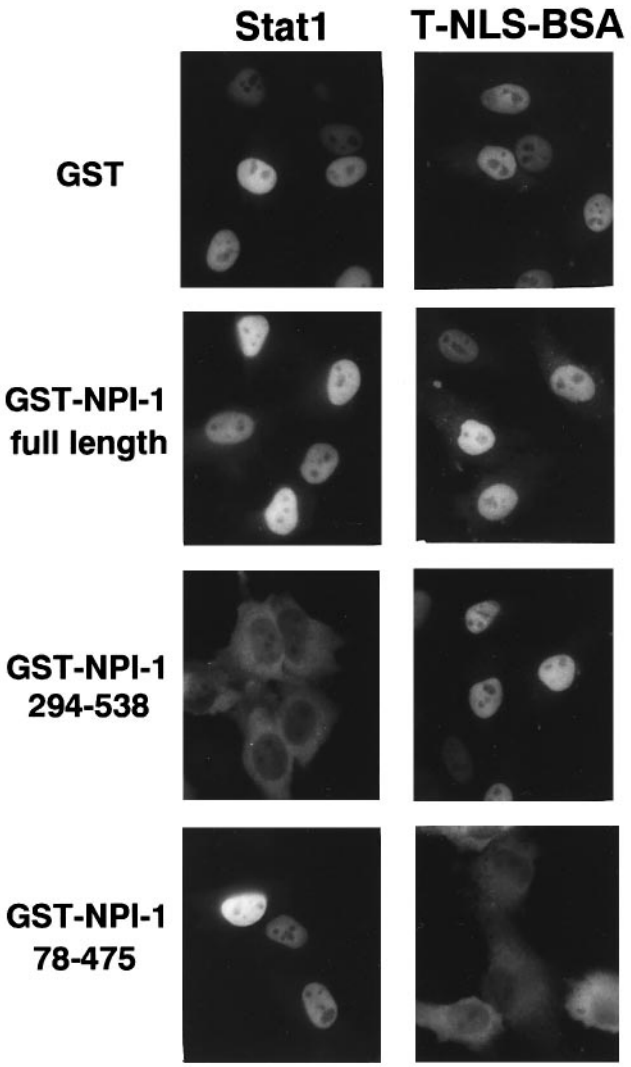


Fig. 8. *In vivo* effect of deletion mutants of NPI-1 on nuclear translocation of Stat1 or T-NLS–BSA. GST, GST–NPI-1 (full length), GST–NPI-1 (294–538) or GST–NPI-1 (78–475) (each 60 μM) was microinjected with Stat1 or FITC-labeled T-NLS–BSA (10 μM) into the cytoplasm of HeLa cells, and the cells were treated with IFN-γ. After 30 min at 37°C, the cells were fixed with formaldehyde, and the intracellular localization of Stat1 was examined by indirect immunofluorescence with anti-HA antibody.

How does a distinct family of α subunit show different binding affinity for Stat1? The NPI-1 gene deletion experiment showed that the Stat1-binding site is different from the conventional NLS-binding site; the conventional NLS binds to armadillo repeats and Stat1 binds to the C-terminal non-armadillo region of NPI-1 including a C-terminal portion of the eighth armadillo repeat (Figure 7B). The NPI-1 and Rch1 families have a high degree of similarity at the N-terminal region (known as the β subunit-binding domain; Görlich *et al.*, 1996; Weis *et al.*, 1996) and throughout the armadillo repeats. The amino acid sequences of the C-terminal non-armadillo region of these family members have a low degree of similarity, and thus it is likely that the non-conserved amino acid sequences of this region provide the functional divergence of each family member. The competition experiments indicated the possibility that NPI-1 binds to Stat1 and SV40 T antigen NLS-containing protein simultaneously (Figure 7C), and thus it is possible that a complex of NPI-1 and β subunit can transport both Stat1 and SV40 T antigen NLS-containing protein at the same time. Furthermore, consistent with the results of the solution binding assays, we found that distinct deletion mutants of NPI-1, 294–538 and 78–475, can function to inhibit the nuclear import of Stat1 and SV40 T NLS-containing protein, respectively, in a dominant-negative manner *in vivo* (Figure 8). These dominant negatives will be useful as specific inhibitors of each nuclear transport pathway.

Although Moroianu *et al.* (1996) showed that the SV40 T antigen NLS-binding site of human karyopherin α1 (identical to NPI-1 or hSRP1) was located in the C-terminal domain, our data show that both single and bipartite-type NLSs bind to the armadillo repeats of NPI-1, and PTAC58 also binds to NLS directly through the armadillo repeats (Y.Miyamoto and Y.Yoneda, unpublished data). Similar results were obtained by other groups, indicating that the α subunit NLS-binding site is located in the armadillo repeats (Cortes *et al.*, 1994; Görlich and Mattaj, 1996). At the moment, the reason for this discrepancy is unclear, and further studies will be required for a complete understanding.

Both NPI-1 and Rch1 were cloned from HeLa cells. When affinity-purified anti-PTAC58 antibodies were microinjected into HeLa cells, the nuclear import of T-NLS–BSA was strongly, but not completely inhibited (Figure 4B). A similar result was obtained when human embryonic lung cells were used (Imamoto *et al.*, 1995b). Furthermore, affinity-purified anti-NPI-1 antibodies inhibited the nuclear import of T-NLS–BSA only slightly (Figure 4B). These results could be explained by the fact that SV40 T antigen NLS is recognized by both the NPI-1 and Rch1 family, and the population of Rch1 is estimated to be several times larger than that of NPI-1 in HeLa cells (data not shown). Thus, since affinity-purified antibodies against NPI-1 inhibited the IFN-γ-dependent nuclear import of Stat1 strongly, but not completely (Figure 4D), it seems reasonable to presume that other Stat1 recognition factor(s) apart from NPI-1 and Rch1 may exist, which are capable of mediating the IFN-γ-dependent nuclear pore targeting of Stat1, although we must consider the possibility that there are differences in the quality of antibodies used.

Two types of NLS, single and bipartite, have been identified previously in many karyophilic proteins, and it has been shown that both types consist of basic amino acid residues (Dingwall and Laskey, 1991). In the case of Stat1, it has been shown that tyrosine phosphorylation (Tyr701) is required for both nuclear accumulation and transcriptional activity, using wild-type and mutant Stat1-expressing cell lines (Shuai *et al.*, 1993a). Staurosporine, a kinase inhibitor, was found to inhibit the extracellular signal-dependent nuclear import of Stat1, and the substitution of Tyr701 by phenylalanine or glutamic acid caused a defect in nuclear import activity (Sekimoto *et al.*, 1996). It thus appears that phosphorylation of Tyr701, but not negative charge at this position, is essential for the extracellular signal-dependent nuclear import of Stat1. However, the NLS of Stat1 has not yet been identified. Since the tyrosine-phosphorylated active form of Stat1 was found to be recognized specifically by NPI-1 protein which interacts with the basic-type NLSs, such as that of

SV40 T antigen, it was hypothesized that the basic amino acid cluster region in Stat1 might function as its NLS. Unexpectedly, nine mutant Stat1s which have single or double mutations at the basic amino acid cluster region were found to be transported to the nucleus as effectively as wild-type in response to IFN-γ (Figure 6), and the Stat1-binding domain of NPI-1 is different from the SV40 T antigen NLS-binding region (Figure 7B). It has been proposed that tyrosine-phosphorylated Stat1 forms a homodimer through an interaction between phosphorylated tyrosine and the SH2 domain of another Stat1 (Shuai *et al.*, 1994; Gupta *et al.*, 1996). Moreover, we showed that homodimerization of Stat1 is essential for the interaction with NPI-1 as well as the DNA binding (Figure 5). Although we have not yet identified the NLS of Stat1, since it was found that a domain of NPI-1 different from the SV40 T antigen NLS-binding region is involved in the interaction with Stat1 homodimer, there is the possibility that the NLS of Stat1 may be quite novel.

The β subunits isolated from various species have a great deal of similarity in their amino acid sequences (>95% identity). Consistent with the finding that a monoclonal antibody to p97 inhibits the nuclear transport of SV40 T antigen NLS-conjugated allophycocyanin completely *in vitro* (Chi *et al.*, 1995), our affinity-purified polyclonal antibodies to β subunit strongly inhibited the nuclear transport of Stat1 in living cells (Figure 4D). Furthermore, tyrosine-phosphorylated Stat1 interacted with the β subunit (Figure 1). These results clearly indicate that NPI-1, a class of α subunit, and β subunit participate in the nuclear import of Stat1.

It has been proposed that karyophiles first form a complex with an NLS receptor (α subunit) and a 97 kDa protein (β subunit) in the cytoplasm and then target to the nuclear pore complex in an energy-independent manner (Melchior and Gerace, 1995; Görlich and Mattaj, 1996). The present findings indicate that Stat1 binds to the NPI-1 family of α subunit (previously identified as NLS receptor) and β subunit which is required for the targeting of the karyophile to the nuclear pore. Although the NLS of Stat1 has not yet been determined, it seems reasonable that different types of NLSs may be recognized by different classes of α subunit, and that the β subunit may bind to various classes of α subunit. Thus, the initial step of nuclear import, recognition of the structure corresponding to the NLS, of Stat1 may be distinct from that of SV40 T antigen NLS-containing substrate, but subsequent steps may share a common pathway, requiring the β subunit and Ran. We propose that, in response to IFN-γ, Stat1 is tyrosine phosphorylated, dimerizes and forms a complex with the NPI-1 family of α subunit and the β subunit to target the nuclear pore and then, as described previously (Sekimoto *et al.*, 1996), translocates through the nuclear pores in a Ran-mediated manner.

Materials and methods

Cell culture and antibodies

HeLa, NIH-3T3, Wi38 VA13 and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum. To prepare anti-PTAC58, anti-NPI-1 and anti-β subunit antibodies, full-length recombinant GST–PTAC58, GST–NPI-1 or GST–β subunit fusion protein was used to immunize rabbits, and recombinant protein, without the GST moiety, conjugated to Sepharose was used for affinity purification of each antibody from serum. Monoclonal anti-Stat1 and anti-Stat3 antibodies were purchased from Transduction Laboratory. Monoclonal anti-phosphotyrosine (4G10) antibody and anti-SV40 T antigen antibody were purchased from UBI and Oncogene Science, respectively. Human or murine IFN-γ (20 ng/ml, purchased from Genzyme and Gibco BRL, respectively) treatment of cells was for 15 min at 37°C unless otherwise indicated.

Expression and purification of recombinant proteins

Recombinant Stat1 and mutant Stat1s with an HA tag at the N-terminal end were expressed and purified as described previously (Sekimoto *et al.*, 1996). Mutations were introduced using PCR (Higuchi, 1989) with appropriate oligonucleotides. All mutants were verified by DNA sequencing. NPI-1 was cloned from a HeLa cDNA library using PCR to pGEX-4T-3 (Pharmacia), and verified by DNA sequencing. Recombinant GST–PTAC58, GST–β subunit and GST–NPI-1 were expressed as described previously (Imamoto *et al.*, 1995a,b) and purified to homogeneity using glutathione–Sepharose (Pharmacia) following the manufacturer's recommendations. GST-fused deletion mutants of NPI-1 were prepared using the appropriate restriction enzyme or PCR with appropriate oligonucleotides, and verified by DNA sequencing. All deletion mutants were expressed and purified as for full-length NPI-1.

Preparation of extract

To prepare whole cell extract, 10^7 cells were resuspended in 300 μl of ice-cold extraction buffer [20 mM HEPES pH 7.8, 0.5% NP-40, 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM 2-mercaptoethanol, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each aprotinin, leupeptin and pepstatin] and gently rocked at 4°C for 10 min. The mixture was sonicated briefly and centrifuged at 16 000 *g* for 10 min. The supernatant was then diluted to 1.5 ml with transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 1.5 mM magnesium acetate, 1 mM EGTA, 1.5 mM 2-mercaptoethanol with phosphatase and protease inhibitors). To prepare cytosol and nuclear extract, 2×10^7 cells were resuspended in three packed cell volumes of transport buffer without potassium acetate and swollen for 10 min at 4°C. The cells were then homogenized and nuclei were pelleted. The supernatant was centrifuged further at 16 000 *g* (cytosol). Nuclei were extracted with high salt buffer (20 mM HEPES pH 7.8, 420 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1.5 mM 2-mercaptoethanol with phosphatase and protease inhibitors) for 30 min at 4°C and centrifuged (nuclear extract). Extracts were applied to a PD-10 column (Pharmacia) equilibrated with transport buffer.

Solution binding assay

HeLa whole cell extract with or without IFN-γ treatment (20 ng/ml for 15 min) was pre-incubated with glutathione–Sepharose at 4°C for 1 h and then incubated with various GST fusion proteins (1 nmol) absorbed to glutathione–Sepharose at 4°C for 2 h. Gels were washed extensively with transport buffer, and bound proteins were eluted with sample buffer for SDS–PAGE. Eluted proteins were separated on 7.5% SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with antibodies. Cytosol and nuclear extract were prepared from HeLa cells treated with or without IFN-γ at 20 ng/ml for 15 min. Extracts were incubated with GST–β subunit, and bound Stat1 was detected as described above.

For the DNA-binding assay, ligated oligonucleotide of the high-affinity SIE of the c-*fos* gene, CATTTCCCGTAAATC, was conjugated with CNBr-activated Sepharose according to the manufacturer's instructions and incubated with HeLa whole cell extract in transport buffer at 4°C for 1 h. After extensive washing with transport buffer, bound proteins were analyzed by immunoblotting with anti-Stat1 antibodies.

Transfection experiments

Full-length Stat1 cDNA with His_6 at the N-terminal end was inserted into a pCAGGS expression vector, and used to transfect human 293T cells by the standard calcium phosphate method. After 36 h of transfection, the cells were treated with IFN-γ (20 ng/ml) for 15 min at 37°C. Whole cell extract was prepared as described above, and the His_6-tagged Stat1 was precipitated with Ni-NTA–agarose. Gels were washed extensively with 100 mM imidazole in transport buffer. Bound proteins were eluted with SDS sample buffer supplemented with 600 mM imidazole and analyzed by immunoblotting.

Microinjection

HeLa cells were grown on cover slips in DMEM supplemented with 10% fetal bovine serum. Purified HA-tagged Stat1 protein (3 mg/ml)

was microinjected into the cell cytoplasm and the cells were then treated with IFN-γ (50 ng/ml, Genzyme) for 30 min at 37°C. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 10 min at room temperature. The subcellular localization of Stat1 was detected by indirect immunofluorescence using anti-HA antibody (Sekimoto *et al.*, 1996). Antibodies (25 mg/ml) were microinjected with Stat1 into the cytoplasm and the cells were treated with IFN-γ. For detection of the subcellular localization of T-NLS–BSA, fluoresecein isothiocyanate-labeled T-NLS–BSA was used.

Acknowledgements

We thank Dr Taro Tachibana for a gift of FITC-labeled T-NLS–BSA. This work was supported by Grant-in-Aid for Scientific Research on Priority Areas (No. 07282103), Grant-in-Aid for Scientific Research (B) (No. 08458229) and Grant-in-Aid for COE Research (No. 07CE2006) from the Japanese Ministry of Education, Science, Sports and Culture, the Nissan Science Foundation and the Naito Foundation. T.S. is a Research Fellow of the Japanese Society for the Promotion of Science.

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Received on July 11, 1997; revised on September 16, 1997