

A GTPase Distinct from Ran Is Involved in Nuclear Protein Import

Deborah J. Sweet and Larry Gerace

Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract. Signal-dependent transport of proteins into the nucleus is a multi-step process mediated by nuclear pore complexes and cytosolic transport factors. One of the cytosolic factors, Ran, is the only GTPase that has a characterized role in the nuclear import pathway. We have used a mutant form of Ran with altered nucleotide binding specificity to investigate whether any other GTPases are involved in nuclear protein import. D125N Ran (XTP-Ran) binds specifically to xanthosine triphosphate (XTP) and has a greatly reduced affinity for GTP, so it is no longer sensitive to inhibition

by nonhydrolyzable analogues of GTP such as guanosine 5'-O-(3-thiotriphosphate) (GTP γ S). Using in vitro transport assays, we have found that nuclear import supported by XTP-Ran is nevertheless inhibited by the addition of non-hydrolyzable GTP analogues. This in conjunction with the properties of the inhibitory effect indicates that at least one additional GTPase is involved in the import process. Initial characterization suggests that the inhibited GTPase plays a direct role in protein import and could be a component of the nuclear pore complex.

MOLECULAR transport between the nucleus and cytoplasm plays a fundamental role in eukaryotic cell metabolism. It takes place through nuclear pore complexes (NPCs)¹, large (~125 MD) supramolecular structures that span the nuclear envelope (for reviews see Panté and Aebi, 1993; Rout and Wentz, 1994; Davis, 1995). Ions, metabolites, and small macromolecules can cross NPCs by passive diffusion through aqueous channels (Paine et al., 1975; Peters, 1986). However, most proteins and RNAs are too large to diffuse across NPCs at physiologically significant rates, and are instead transported through a gated channel by temperature- and energy-dependent mechanisms (for reviews see Fabre and Hurt, 1994; Melchior and Gerace, 1995).

The best-characterized pathway of nucleocytoplasmic transport is protein import into the nucleus. Import is specified by nuclear localization signals (NLSs), which usually consist of short stretches of predominantly basic amino acids in a single or bipartite motif (for review see Dingwall and Laskey, 1991). The import pathway involves binding of NLS-containing proteins to the cytoplasmic

side of the NPC and then movement over a distance of >100 nm during translocation into the nucleus. It is currently envisaged as a multi-step process, which requires the activity of both cytosolic transport factors and NPC proteins (Melchior and Gerace, 1995). Significant progress has been made recently in the identification and characterization of components of the nuclear import machinery, but it is likely that many more remain to be discovered.

A number of insights into the mechanism of nuclear protein import have been obtained from in vitro transport assays (Melchior and Gerace, 1995). The most frequently used assay contains mammalian cells permeabilized with digitonin and supplemented with exogenous cytosol (Adam et al., 1990). Biochemical studies using such assays have so far led to the identification of five cytosolic transport factors that are highly conserved from yeast to mammalian cells: the NLS receptor (importin α , karyopherin α , Srp1p) (Adam and Gerace, 1991; Yano et al., 1992; Görlich et al., 1994; Imamoto et al., 1995b; Moroianu et al., 1995a; Weis et al., 1995), p97 (importin β , karyopherin β) (Adam and Gerace, 1994; Chi et al., 1995; Enekel et al., 1995; Imamoto et al., 1995a; Görlich et al., 1995; Radu et al., 1995), hsp/hsc70 (Imamoto et al., 1992; Shi and Thomas, 1992), NTF2 (p10, pp15) (Moore and Blobel, 1994; Paschal and Gerace, 1995), and the small GTPase Ran (Melchior et al., 1993, 1995a; Moore and Blobel, 1993). Characterization of the role of these cytosolic factors in transport has led to a working model of the nuclear import pathway (Melchior and Gerace, 1995). In this model, the NLS receptor interacts with NLS-containing proteins in the cytoplasm and then, in conjunction with p97, mediates their interaction

Address all correspondence to Dr. Larry Gerace, Departments of Cell and Molecular Biology, The Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037. Tel.: (619) 554-8514. Fax: (619) 554-6253.

1. *Abbreviations used in this paper:* GMPPCP, guanylyl-(β , γ -methylene)-diphosphonate; GMPPNP, 5'-guanylyl-imidodiphosphate; Gpp(NH)p, β , γ -imidoguanosine 5'-triphosphate; GTP, guanosine 5'-triphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); NPC, nuclear pore complex; XTP, xanthosine 5'-triphosphate.

with the NPC. Ran binds to the NPC at a peripheral cytoplasmic site close to the initial region of NLS-protein binding, and is thought to act as a molecular switch to commit the receptor complex to subsequent transport steps. NTF2 is not required for the initial binding step (Moore and Blobel, 1994; Paschal and Gerace, 1995), and may regulate downstream interactions with NPC proteins. Translocation into the nucleus is thought to involve stepwise movement of the receptor complex across the NPC, followed by release into the nucleoplasm.

In contrast to the cytosolic transport factors, relatively little is known about the role of NPC proteins in the import pathway. For example, the composition and function of the central gated channel remains obscure. However, one group of NPC proteins (nucleoporins) that appears to be directly involved in transport is those containing multiple dispersed FG (Phe, Gly) repeats (Panté and Aebi, 1993; Rout and Wentz, 1994; Davis, 1995). In mammalian cells these proteins are modified with O-linked *N*-acetylglucosamine and bind to the lectin wheat germ agglutinin (Finlay et al., 1987; Holt et al., 1987). The first indication that the FG repeat proteins play a role in nuclear import came from the observations that wheat germ agglutinin and specific antibodies inhibit protein import in intact cells and in vitro (Finlay et al., 1987; Yoneda et al., 1987; Dabauvalle et al., 1988a,b; Featherstone et al., 1988; Adam et al., 1990). Subsequently, FG repeat proteins have been shown to interact directly with several cytosolic transport factors, including NTF2 (Paschal and Gerace, 1995), Ran (Melchior et al., 1995a; Wu et al., 1995; Yokoyama et al., 1995), and p97 (Moroiianu et al., 1995b). FG repeat proteins are found on both the nucleoplasmic and cytoplasmic sides of the NPC (Panté and Aebi, 1994; Rout and Wentz, 1994; Davis, 1995), and the receptor complex is thought to interact sequentially with several of these proteins as it crosses the NPC (for discussion see Melchior and Gerace, 1995).

GTPases are involved in a wide variety of cellular processes, including vesicular transport in the secretory pathway, protein synthesis, and signal transduction (Bourne et al., 1991). In most cases, the complete pathway involves multiple GTPases that act at different steps. From these precedents, it seems unlikely that Ran is the only GTPase involved in nuclear protein import. Additional GTPases could take part in, for example, targeting of the receptor complex to the center of the NPC or gating of the transport channel. Nonhydrolyzable analogues of GTP strongly inhibit nuclear protein import in permeabilized cell assays (Melchior et al., 1993; Moore and Blobel, 1993). This inhibition is mediated to a large extent by Ran, and results in a block of transport at an early step (Melchior et al., 1995a). In this situation, any inhibitory effects of the nonhydrolyzable nucleotides on GTPases that act in later stages of the transport pathway would be masked by the inhibition of Ran. It would therefore be very difficult to determine whether any additional GTPases are involved using conventional nuclear import assays.

To circumvent this problem, we have made use of a mutation that has previously been shown to change the nucleotide binding specificity of a number of GTPases. The conserved G4 region of GTPase superfamily proteins has a consensus sequence of NKXD, with the D (aspartic acid) being particularly highly conserved (Bourne et al., 1991).

Mutation of this residue to asparagine in several GTPases, including Ha-Ras (Zhong et al., 1995), Ypt1p (Jones et al., 1995), FtsY (Powers and Walter, 1995), and EF-Tu (Hwang and Miller, 1987; Wiegand and Parmeggiani, 1993) leads to altered nucleotide binding specificity. These mutant proteins have a low affinity for GTP and instead bind to and hydrolyze xanthosine triphosphate (XTP). As the structure of the GTP-binding region is highly conserved, it seemed likely that an equivalent amino acid substitution in Ran (D125 to N) would also change its nucleotide binding specificity from GTP to XTP.

In this study, we have constructed an XTP-binding mutant of Ran and shown that it is functional in nuclear protein import. This mutant specifically binds to XTP rather than GTP, and is therefore no longer sensitive to nonhydrolyzable GTP analogues such as guanosine 5'-*O*-(3-thiotriphosphate) (GTP γ S). We have found that nuclear import in assays containing XTP-Ran instead of wild-type is inhibited by nonhydrolyzable analogues of GTP, demonstrating that at least one additional GTPase is involved in the nuclear import pathway. This inhibition reduces the overall rate of transport and appears to be mediated by a factor that remains associated with cells during digitonin permeabilization.

Materials and Methods

Cells and Reagents

Suspension cultures of human (HeLa) cells were grown in Joklik's modified minimal essential medium (GIBCO-BRL, Gaithersburg, MD) with 10% newborn calf serum (Hyclone Laboratories, Logan, UT). Nucleotides and nucleotide analogues were obtained from Sigma Immunochemicals (St. Louis, MO) (XTP, XDP, [8-³H]-XTP, Gpp(NH)p, ATP), Boehringer Mannheim Biochemicals (Indianapolis, IN) (GTP, GDP, GTP γ S, GMPPNP), or Amersham Corp. (Arlington Heights, IL) ([8-³H]GTP). Nucleotide stocks were prepared in 20 mM Hepes, pH 7.4, with an equimolar concentration of Mg²⁺ and stored at -80°C.

Expression and Purification of Wild-type and D125N Ran

Recombinant wild-type Ran was expressed and purified as described previously (Melchior et al., 1995b). To construct the D125N mutant, the entire Ran ORF (open reading frame) was transferred as an XbaI-BamHI fragment from pET11d-Ran (Melchior et al., 1993) to pBluescript SK- (Stratagene Corp., La Jolla, CA). Mutagenesis was carried out using the *dur⁻ung⁻* oligonucleotide-directed method (Kunkel et al., 1987), and the presence of the mutation verified by sequencing. The mutant ORF was then transferred back into a pET11d vector as an XbaI-BamHI fragment for expression in BL21(DE3) cells. The expression and purification procedures were similar to those used for wild-type Ran (Melchior et al., 1995b), the only differences being that the bacterial cells were grown at 30°C instead of 37°C to improve the solubility of the mutant protein, and XDP was added to 20 μ M during bacterial cell lysis and to 250 μ M during resolubilization of the ammonium sulfate precipitate.

Equilibrium Binding of Nucleotides

The protocol used for nucleotide binding was based on those described by Zhong et al. (1995) and Manne et al. (1984). The binding reactions contained 0.2 μ M wild-type Ran plus 0.2 μ M [8-³H]5'-GTP (sp. act. 0.95 Ci/mmol) or 0.2 μ M D125N Ran plus 0.2 μ M [8-³H]5'-XTP (sp. act. 1.2 Ci/mmol) and unlabeled GTP, XTP, GTP γ S, or ATP at concentrations between 10⁻³ and 3 \times 10⁻⁸ M. Each assay contained 0.5 μ g Ran and all points were assayed in duplicate. The purified recombinant proteins were likely to be bound to GDP or XDP, so the assays also contain up to 0.2 μ M of these nucleotides contributed by the protein. The Ran and nucleotides were incubated at 30°C for 10 min in 50 mM Hepes, pH 8, 8 mM EDTA,

4 mM Mg(OAc)₂, 1 mM DTT, 0.1 mg/ml BSA, and 150 mM KCl. Each sample was then diluted with a 10-fold volume (1 ml) of ice-cold wash buffer (50 mM Hepes, pH 8, 150 mM KCl, 5 mM Mg(OAc)₂, and 1 mM DTT) and immediately transferred to a 0.45 μM nitrocellulose filter (HAWP 025 00; Millipore Corp., Bedford, MA). The unbound nucleotide was removed by washing with 5 ml ice-cold wash buffer. The filters were then dried and the bound nucleotide detected by liquid scintillation spectrometry using scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA).

Preparation of Ran-depleted Cytosol

HeLa cell cytosol was prepared according to the method described by Melchior et al. (1995b) and had a typical protein concentration of 10 mg/ml as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

To deplete Ran from this cytosol by column fractionation, 1.5 ml cytosol was concentrated to 600 μl using a concentrator (centricon 10, Amicon Corp., Beverly, MA) and then applied in 200-μl aliquots to either a Superose 12 or Superdex 200 column connected to an FPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The columns were equilibrated in transport buffer (20 mM Hepes, pH 7.4, 110 mM KOAc, 2 mM Mg(OAc)₂, 0.5 mM EGTA, 2 mM DTT, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin) and run at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected and those that contained Ran were identified by immunoblotting (see below). Ran was typically found in either six (Superose 12) or five (Superdex 200) of the 0.5-ml fractions. The fractions lacking Ran were combined and concentrated to 1.5 ml (equal to the original vol of cytosol) in centricon-10 concentrators. The resulting cytosol was then frozen in liquid N₂ and stored in aliquots at -80°C. This procedure removed 95–97% of the cytosolic Ran, as assayed by immunoblotting of a dilution series, and was the method used to prepare Ran-depleted cytosol unless otherwise stated. NTF2 chromatographs as a 30-kD protein on these columns, so removal of the Ran fractions also depleted significant amount of the endogenous NTF2. Supplementary recombinant NTF2 (prepared using the protocol described by Paschal and Gerace, 1995) was therefore added to transport assays containing this cytosol, to a final concentration of 12.5 μg/ml.

To affinity deplete Ran from cytosol by binding to RanBP1, 100 μl of cytosol was incubated with 50 μl of 7 mg/ml RanBP1 beads (see below) for 12 h at 4°C. The supernatant was then removed and used in transport assays. Although RanBP1 binds preferentially to Ran-GTP (Bischoff et al., 1995), which is likely to be less abundant than Ran-GDP in HeLa cytosol, incubation of the cytosol with a sufficiently high concentration of RanBP1 removed >90% of the cytosolic Ran as assayed by immunoblotting of a dilution series (Mahajan, R., and L. Gerace, unpublished observations). Purified recombinant RanBP1 was kindly provided by Dr. Rohit Mahajan (Scripps Research Institute). This protein was dialyzed against coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl) for 4 h and then combined with activated CNBr-Sepharose 4B beads (Pharmacia LKB Biotechnology Inc.) at 7 mg RanBP1 per 1 ml of beads. Coupling was carried out for 16 h at 4°C, after which no detectable protein remained unbound (Bio-Rad protein assay). The remaining sites on the beads were blocked by incubation in 0.1 M Tris-HCl, pH 8, for 2 h at room temperature. The beads were then washed in four cycles of 0.1 M NaOAc, pH 4, 0.5 M NaCl, and 0.1 M Tris, pH 8, 0.5 M NaCl, followed by four washes in transport buffer before incubation with the cytosol.

Nuclear Import Assays

Nuclear protein import was measured using the flow cytometric assay developed by Paschal and Gerace (1995). The FITC-BSA-NLS transport ligand was prepared according to the method described (Paschal and Gerace, 1995), using 25 μl of 20 mM sulfo-SMCC and 0.3 mg of NLS peptide per 1 mg of FITC-BSA.

50 ml of suspension HeLa cells (typically at a density of 5 × 10⁵/ml) were prepared for permeabilization by washing in 50 ml of ice-cold transport buffer. They were permeabilized at a density of 5 × 10⁶ cells/ml in transport buffer containing 0.006% digitonin (Calbiochem-Behring Corp., San Diego, CA) by incubation for 6 min on ice, then washed again in 50 ml of transport buffer and resuspended at ~5 × 10⁷ cells/ml for use in the transport assay. The cell concentration was determined at this stage by comparison of the A₅₅₀ of a 1/100 dilution to a standard curve, and then adjusted to give the desired number of cells per 10 μl (2–6 × 10⁵).

Transport assays (total vol 40 μl) contained 3 × 10⁵ permeabilized cells per assay (unless otherwise stated), an ATP-regenerating system (1 mM

ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase), FITC-BSA-NLS transport ligand (200–800 nM) and HeLa cell cytosol and/or recombinant transport factors. Unless otherwise stated, all assays also contained 1 mM XTP. When using 3 × 10⁵ cells per assay, a saturating level of transport was obtained with 10 μl of HeLa cytosol (final concentration 2.5 mg/ml), so an equivalent vol of Ran-depleted cytosol (determined by comparison of the vol before and after Ran depletion) was used in each case. To reconstitute transport using purified recombinant factors, hSrp1α (25 μg/ml) and NTF2 (12.5 μg/ml) were added to the permeabilized cells in the presence of wild-type or XTP Ran (25 μg/ml). In these assays, no additional stimulation of transport was obtained by adding recombinant p97 so it was not included. The recombinant NTF2 was expressed and purified as described in Paschal and Gerace (1995). Recombinant hSrp1α and p97 were prepared by Tianhua Hu using expression constructs provided by Dr. A. Lamond (University of Dundee) (hSrp1α) and Dr. S. Adam (Northwestern University School of Medicine) (p97), according to published protocols (Weis et al., 1995 for hSrp1α; Chi et al., 1995 for p97).

The assays were incubated for 30 min at 30°C (except for time courses) in 6-ml polystyrene tubes (2058; Falcon Plastics, Cockeysville, MD), and stopped by transfer to an ice water bath. The cells were washed once in 4 ml of ice-cold transport buffer and resuspended in a total vol of ~0.5 ml of the same buffer. The fluorescence of 10⁴ cells was measured for each sample using a flow cytometer (FACSort; Beckton Dickinson, San Jose, CA) and then the mean fluorescence of the cells in each sample was determined using software (CellQuest; Becton Dickinson). Due to the reproducibility of the flow cytometric method (Paschal and Gerace, 1995), assays were not normally performed in duplicate, but all the experiments shown were carried out at least three times and found to be highly reproducible. It should be noted that the mean fluorescence values obtained in different experiments are not necessarily directly comparable, as different batches and amounts of transport ligand were used and the degree of permeabilization of the cells can vary.

Gel Electrophoresis, Immunoblotting, and Immunofluorescence

SDS-PAGE and Western blotting were performed using standard protocols. Immunological detection of Ran involved preincubation of blots in PBS containing 2% dried milk powder and 0.2% Tween-20 for 1 h, probing for 1 h using anti-Ran antibodies (0.4 μg/ml) (kindly provided by Dr. F. Melchior, Scripps Research Institute) in PBS + 2% dried milk powder, washing for 10 min in PBS and then PBS + 0.2% Tween-20, incubation with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5,000, Pierce, Rockford, IL) in PBS + 2% dried milk powder for 1 h, washing as before and then detection by chemiluminescence (ECL system; Amersham Corp.). Localization of Ran by immunofluorescence was performed as described previously (Melchior et al., 1995a), using the aforementioned affinity-purified anti-Ran antibodies.

Results

D125N Ran (XTP-Ran) Has Altered Nucleotide Binding Specificity

The G4 region of Ran is located at residues 122–125, and has the sequence NKVD (Asn-Lys-Val-Asp). By analogy to other XTP-binding GTPase mutants, we predicted that replacement of Asp125 with Asn would change the nucleotide binding specificity of Ran from GTP to XTP. We therefore constructed and purified a D125N Ran mutant. To compare the nucleotide binding specificity of this mutant protein with wild-type Ran, we used competition for binding of radiolabeled nucleotide in an EDTA-based exchange assay (Fig. 1). Wild-type Ran and [³H]GTP (Fig. 1A) or D125N Ran and [³H]XTP (Fig. 1B) were incubated in the presence of a range of concentrations of unlabeled competitor GTP, GTPγS, XTP, or ATP and the labeled nucleotide bound to Ran was measured using a filter assay. The efficacy of competition by each of the unlabeled

nucleotides indicated their relative binding affinities for Ran. As shown in Fig. 1 A, the affinity of wild-type Ran for XTP is approximately three orders of magnitude lower than its affinity for GTP or GTP γ S. In contrast, the D125N Ran mutant (Fig. 1 B) has a high affinity for XTP, which is within the same order of magnitude as the affinity of wild-type Ran for GTP (compare the GTP curve in Fig. 1 A with the XTP curve in B). The D125N mutation also significantly reduces the affinity of Ran for GTP and GTP γ S, to such an extent that it no longer appears to discriminate between these nucleotides and ATP. This 1,000-fold increase in affinity for XTP, and approximately reciprocal reduction in affinity for GTP, is similar to the effect of the equivalent (D119N) mutation in Ha-Ras (Zhong et al., 1995). This single amino acid substitution therefore appears

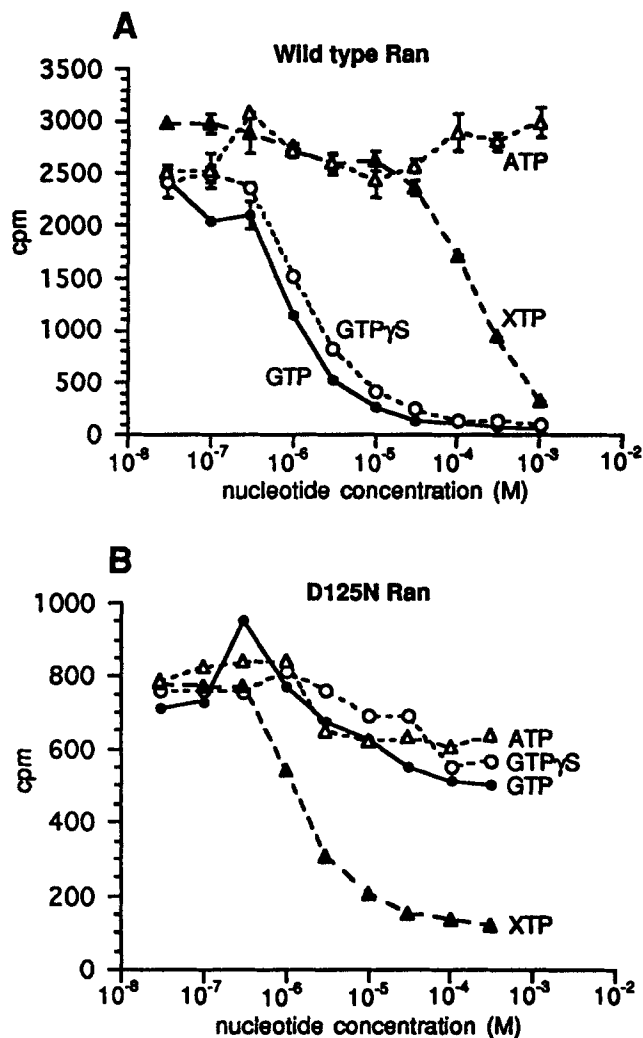


Figure 1. Nucleotide binding specificity of wild-type and D125N Ran. This was determined by competition for binding of labeled nucleotide to wild-type (A) and D125N Ran (B). 0.2 μ M wild-type Ran and [³H]GTP (A) or D125N Ran and [³H]XTP (B) were incubated with a range of concentrations of unlabeled competitor GTP, GTP γ S, XTP, or ATP in an EDTA-based exchange assay (see Materials and Methods). The Ran-associated nucleotide was collected by filter binding and detected by scintillation counting. All points represent an average of the counts from two duplicate assays. —●—, GTP; —▲—, XTP; —△—, ATP; —○—, GTP γ S.

to change effectively the nucleotide binding specificity of Ran, and results in a protein that binds specifically to XTP.

Having established that D125N Ran (XTP-Ran) has altered nucleotide binding specificity, we then tested its activity in nuclear protein import. In permeabilized cell nuclear import assays, Ran is a major rate-limiting cytosolic transport factor (Melchior et al., 1993). As shown in Fig. 2 A, addition of Ran to assays containing a subsaturating concentration of cytosol results in a strong stimulation of transport. We were therefore able to test the transport activity of XTP-Ran by examining its ability to stimulate nuclear protein import. Addition of XTP-Ran stimulates nuclear import in an XTP-dependent manner (see Fig. 2 B). Maximal stimulation of transport was obtained with 1 mM XTP. The concentrations of wild-type and XTP-Ran required to give a maximal stimulation of transport (in the presence of XTP) were the same (25 μ g/ml, data not shown), indicating that they are of equal activity in the import assay. In a comparison of the time course of transport stimulation by wild-type and XTP-Ran (Fig. 2 C), we found that the activity of the mutant protein, in the presence of XTP, was essentially indistinguishable from that of wild-type Ran. Previous studies have shown that GTP hydrolysis by Ran is required for nuclear protein import (Melchior et al., 1993, 1995a; Moore and Blobel, 1993). We therefore concluded that XTP-Ran can both bind to and hydrolyze XTP and, at least in the context of these in vitro transport assays, perform the same function as wild-type Ran.

Nuclear Import Supported by XTP-Ran Is Inhibited by Nonhydrolyzable Analogues of GTP

Ran behaves as a cytosolic factor in permeabilized cell nuclear import assays, and is efficiently released from HeLa cells during digitonin permeabilization. We were therefore able to construct assays in which nuclear import is supported by XTP-Ran by substituting it for the endogenous cytosolic Ran. We used three different methods to generate transport assays lacking endogenous Ran: removal of Ran from HeLa cytosol by either column fractionation or affinity depletion, and partial reconstitution of cytosol using purified recombinant transport factors. The column fractionation procedure involved separation of cytosolic proteins using a molecular sieving column and then recombination of the fractions that did not contain Ran. Affinity depletion of Ran was accomplished by incubating cytosol with Sepharose-immobilized RanBP1, a small Ran-binding protein of as yet uncharacterized function (Coutavas et al., 1993; Bischoff et al., 1995). Partial reconstitution of transport was achieved by adding a subset of the currently identified cytosolic transport factors (hSrp1 α , NTF2, and Ran) to the permeabilized cells. In all three cases, the Ran activity necessary for efficient nuclear import was provided by addition of purified recombinant wild-type or XTP-Ran.

Using these three different means of replacing Ran with the XTP-Ran mutant, we were able to test whether nuclear import supported by XTP-Ran is affected by nonhydrolyzable analogues of GTP (see Fig. 3). Transport assays containing either column fractionated cytosol (Fig. 3 A), RanBP1-depleted cytosol (Fig. 3 B), or hSrp1 α and NTF2 (Fig. 3 C) showed only a low level of transport unless ei-

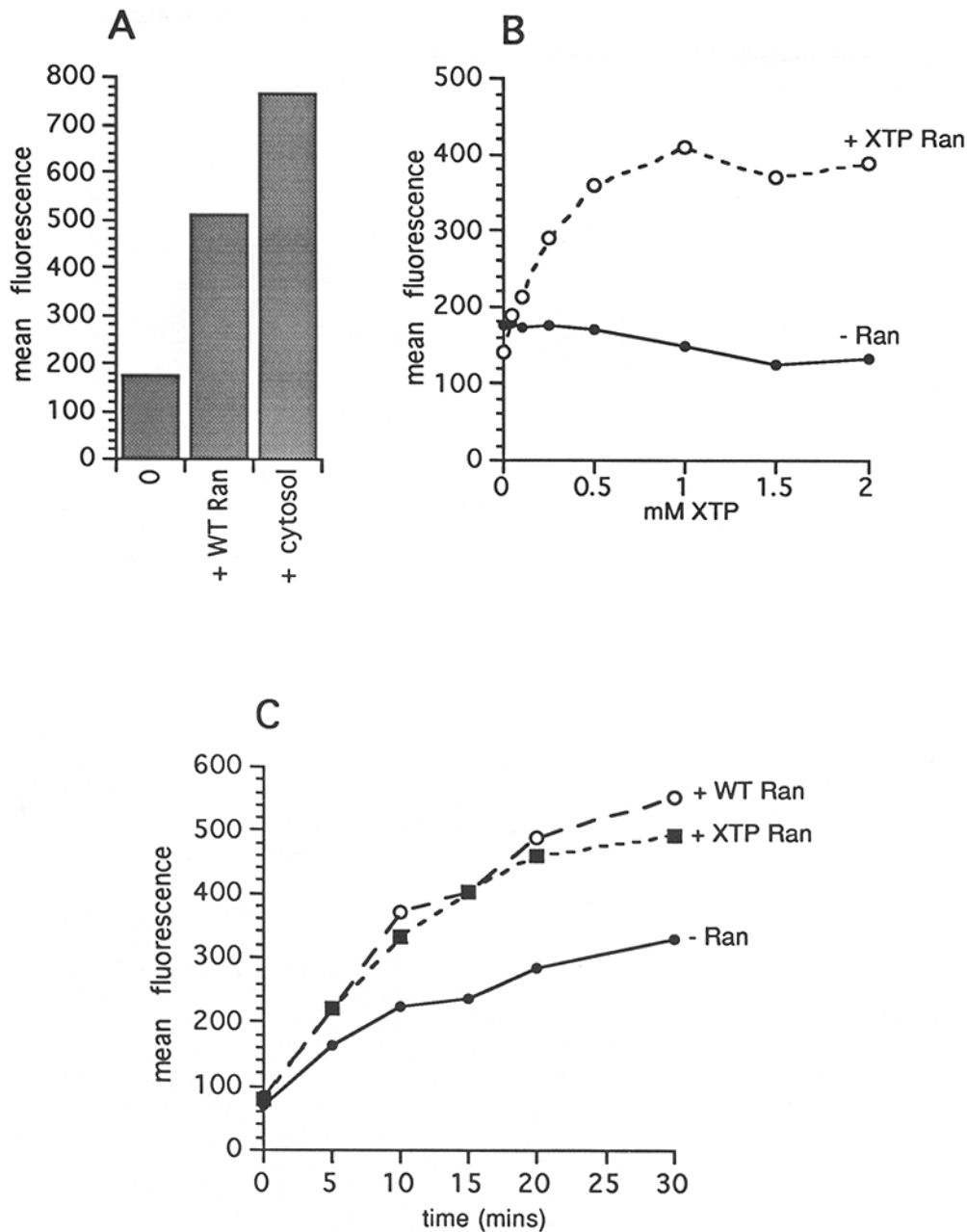


Figure 2. XTP-Ran is functional in nuclear import in the presence of XTP. Nuclear import was measured using the flow cytometric method developed by Paschal and Gerace (1995) (see also Materials and Methods). (A) Stimulation of transport by wild-type Ran. Transport assays containing a subsaturating concentration of cytosol (0.9 mg/ml) were stimulated by adding either 25 μ g/ml wild-type Ran (+WT Ran) or cytosol to a final concentration of 2.4 mg/ml (+cytosol). These assays did not contain added XTP. (B) XTP dependence of stimulation by XTP-Ran. This shows a titration of addition of XTP to transport assays containing a subsaturating concentration of cytosol (0.9 mg/ml) plus 25 μ g/ml XTP-Ran, compared with controls that lack added Ran. (C) Time course of stimulation by wild-type and XTP-Ran. Transport assays containing a subsaturating concentration of cytosol (1.5 mg/ml) plus 25 μ g/ml wild-type or XTP-Ran, or no added Ran, were incubated for the indicated times. All the assays in C contained 1 mM XTP. The concentration of XTP-Ran required to give maximal stimulation was determined in separate experiments (data not shown) and found to be the same as for wild-type Ran.

ther wild-type or XTP-Ran was added. As expected, addition of the nonhydrolyzable GTP analogue GTP γ S to assays containing wild-type Ran strongly inhibited nuclear import (see Fig. 3, A–C). This is consistent with the inhibition observed with whole cytosol (Melchior et al., 1993). In all three cases, addition of the same concentration of GTP γ S to assays containing XTP-Ran also resulted in a substantial inhibition of transport, to ~50–60% of the control level. Thus, using three distinct methods of replacing wild-type Ran with XTP-Ran, we found that transport supported by XTP-Ran is still sensitive to inhibition by GTP γ S. The nuclear import pathway therefore seems to involve at least one other GTPase in addition to Ran.

As an initial characterization of this inhibition, we examined the dose–response profile of inhibition by GTP γ S and the effect of other nonhydrolyzable GTP analogues

(Fig. 4). We found that for assays containing either wild-type or XTP-Ran maximal inhibition was achieved by adding 50–100 μ M GTP γ S. The maximum inhibition obtained in XTP-Ran assays was to ~50% of control transport, while with wild-type Ran transport was inhibited to a greater extent. The concentrations required to give half-maximal inhibition were within the same order of magnitude (2.5 μ M with wild-type Ran and 5 μ M with XTP-Ran) (see Fig. 4 A), suggesting that the GTP γ S inhibition in XTP-Ran assays is mediated by a high affinity interaction. We obtained similar results, i.e., approximately parallel concentration dependence but a lower maximal level of inhibition with XTP-Ran versus wild-type, from titrations of GMPPNP, Gpp (NH)p, and GMPPCP (data not shown). Fig. 4, B and C shows a comparison of the inhibition obtained with maximally inhibiting concentrations of GTP γ S, GMPPNP, and Gpp

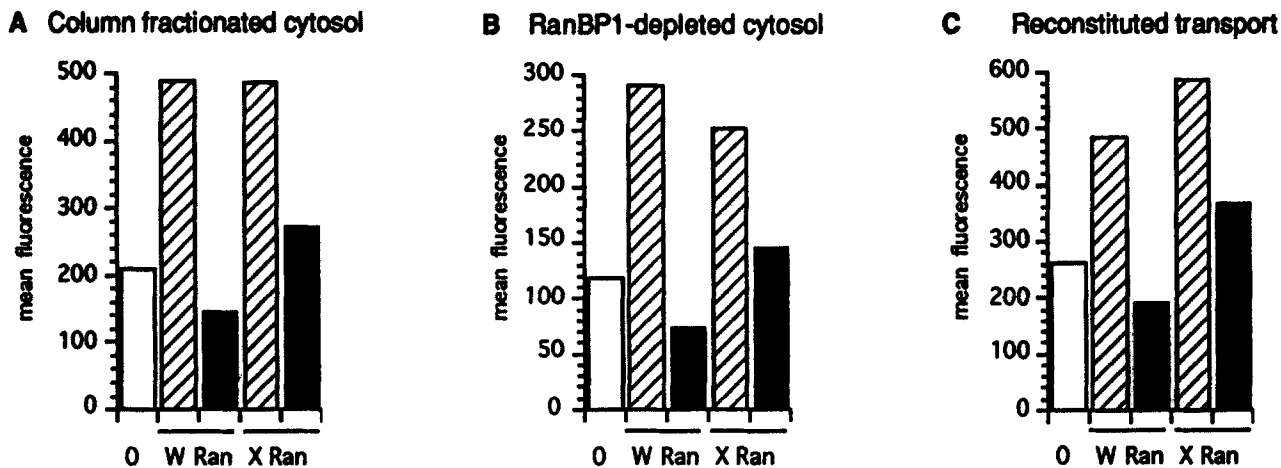


Figure 3. GTP γ S inhibits nuclear import supported by XTP-Ran. Transport assays depleted of wild-type Ran contained a saturation equivalent (see Materials and Methods) of Ran-depleted HeLa cell cytosol, prepared by (A) column fractionation and (B) affinity depletion, or (C) purified transport factors (25 μ g/ml hSrp1 α and 12.5 μ g/ml NTF2) instead of HeLa cell cytosol. Transport was measured in the absence of added Ran (white bars), in the presence of 25 μ g/ml wild type or XTP-Ran (hatched bars) and in the presence of 25 μ g/ml wild type or XTP-Ran plus 200 μ M GTP γ S (black bars). Note that the experiments shown in parts A, B, and C were not performed simultaneously, so the relative mean fluorescence values do not necessarily reflect differences in the overall level of transport.

(NH)p in assays containing either wild-type (B) or XTP-Ran (C). All three of these nonhydrolyzable GTP analogues gave a similar level of inhibition in XTP-Ran assays (Fig. 4 C), demonstrating that the inhibitory effect is not restricted to GTP γ S.

Inhibition Is Not Mediated by Ran

Although the endogenous Ran is efficiently removed from the XTP-Ran assays, a trace amount remains in the permeabilized cells (Melchior et al., 1995) and depleted cytosol. We found that wild-type Ran has a dominant negative effect on GTP γ S-inhibited XTP-Ran assays, i.e., addition of 25 μ g/ml wild-type Ran increases the inhibition to the same level as observed in assays containing wild-type Ran alone (illustrated in Fig. 5). This raised the possibility that the inhibition observed in the XTP-Ran assays is caused by the residual wild-type Ran. It was also formally possible that the inhibition was mediated by the XTP-Ran binding inefficiently to the nonhydrolyzable GTP analogues, although this seemed unlikely from the nucleotide binding data (Fig. 1) and the GTP γ S dose-response curve (Fig. 4 A). We therefore decided to test both of these hypotheses directly by altering the ratio between wild-type and XTP-Ran in the assays.

If the intermediate level of inhibition is due to competition between residual wild-type Ran (which binds to the inhibitor) and the XTP-Ran, one would expect that increasing the concentration of XTP-Ran would reduce the level of inhibition. Conversely, if it is caused by inefficient binding of GTP γ S by XTP-Ran, raising the concentration of XTP-Ran would increase the overall amount of inhibited Ran present and, as inhibited (wild-type) Ran has a dominant effect, might well increase the level of inhibition. We therefore carried out transport assays containing a range of different concentrations of XTP-Ran (12.5–62.5 μ g/ml) in the presence or absence of GTP γ S (Fig. 5 A). To eliminate residual cytosolic Ran from consideration in these

experiments, we used the partially reconstituted system containing purified transport factors (see Fig. 3 C). As shown in Fig. 5 A, increasing the concentration of XTP-Ran over a fivefold range had no effect on the degree of inhibition observed in the assay. A control assay containing added wild-type Ran showed a greater degree of inhibition, as would be expected. Thus, the results of this titration suggest that neither the residual wild-type Ran nor the XTP-Ran mediates the GTP γ S inhibition observed in the XTP-Ran assays.

To further confirm that the residual Ran is not responsible for the GTP γ S-dependent inhibition, we also carried out a titration assay involving addition of wild-type Ran to XTP-Ran assays. Having previously observed that wild-type Ran has a dominant inhibitory effect, we wished to investigate how much exogenous wild-type Ran is required to increase the level of inhibition and compare this to the amount of Ran that remains in the cells. We reasoned that if the amount of wild-type Ran that needs to be added to increase the inhibition is significantly greater than the amount present in the cells, this would provide strong evidence that the inhibition is not caused by the residual Ran. We used purified recombinant wild-type Ran for these experiments, which we have found by titration and immunoblotting to have similar activity in nuclear import assays to the endogenous cytosolic Ran (data not shown). Fig. 5 B shows the results of a titration of wild-type Ran in GTP γ S-inhibited XTP-Ran assays. As for Fig. 5 A, to eliminate residual cytosolic Ran from consideration we used purified transport factors to reconstitute transport. We found that addition of 0.125 μ g (1/8) or less of wild-type Ran had no effect on the level of inhibition, while addition of 0.2 μ g (1/5) or more increased it. Immunoblotting of the Ran dilutions and comparison to the amount of Ran present in the permeabilized cells (Fig. 5 C) showed that the cells contained <0.0625 μ g (1/16) of Ran. Thus, addition of more than twice as much wild-type Ran as was present in the permeabilized cells had no effect on the level of in-

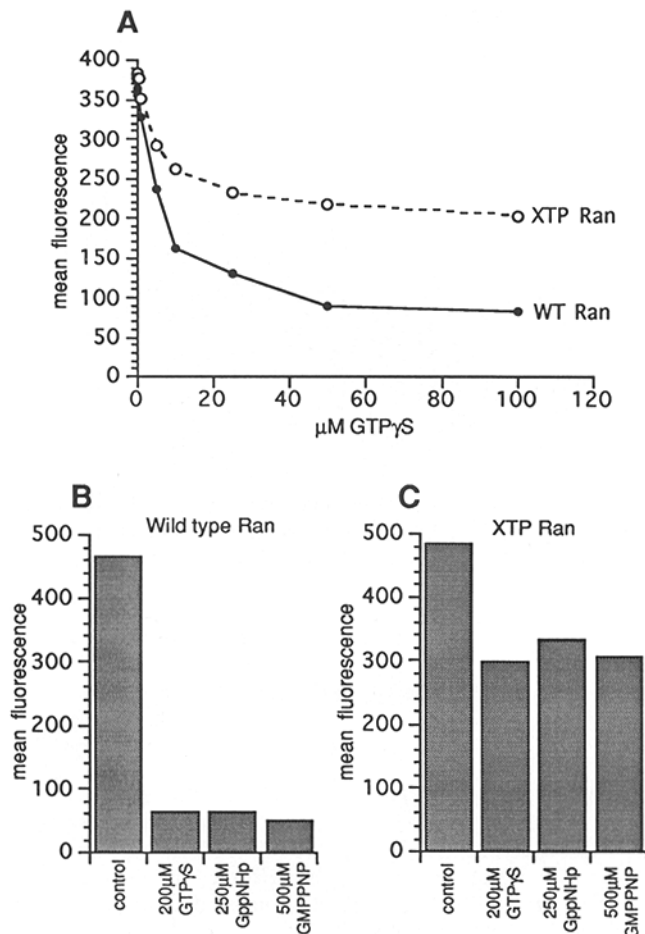


Figure 4. Titration of GTP γ S and inhibition of transport by GMPPNP and Gpp(NH)p in XTP-Ran transport assays. (A) Titration of GTP γ S. Between 0 and 100 μ M GTP γ S was added to transport assays containing cytosol depleted of Ran by column fractionation and 25 μ g/ml wild-type or XTP-Ran. (B and C) Comparison of inhibition by GTP γ S, GMPPNP, and Gpp(NH)p. These assays contained cytosol depleted of Ran by column fractionation, 25 μ g/ml of either wild-type (B) or XTP-Ran (C) and 200 μ M GTP γ S, 250 μ M Gpp(NH)p, 500 μ M GMPPNP, or no GTP analogue as indicated.

hibition. In fact, to double the level of inhibition it was necessary to add over 10-fold more wild-type Ran than was present in the cells. Titration experiments performed with column fractionated cytosol instead of purified transport factors gave very similar results (data not shown). Therefore, although wild-type Ran can have a dominant competitive effect, it is highly unlikely that competition by residual Ran is responsible for the GTP γ S inhibition observed in the XTP-Ran transport assays.

Inhibition of Transport Does Not Lead to Accumulation of Ran at the Nuclear Envelope

A characteristic feature of inhibition of nuclear import by nonhydrolyzable GTP analogues is accumulation of Ran on the cytoplasmic side of the NPC, close to the site of initial NLS-protein binding (Melchior et al., 1995a). This and other observations led to the conclusion that hydrolysis of GTP by Ran is involved in a relatively early step of the

transport process. Localization of XTP-Ran under inhibition conditions could potentially be equally informative about the mechanism of inhibition in XTP-Ran assays. As an initial investigation of this, we carried out immunofluorescence localization of XTP-Ran in transport assays inhibited by the addition of GMPPNP. We chose GMPPNP for this experiment because it has previously been used in studying the accumulation of Ran at the nuclear envelope (Melchior et al., 1995a) and it is an effective inhibitor in XTP-Ran assays (see Fig. 4 C). For comparison, we also carried out localization of wild-type Ran under the same conditions. As shown in Fig. 6 d, wild-type Ran accumulates at the nuclear envelope in the presence of GMPPNP. However, in XTP-Ran assays GMPPNP did not lead to accumulation of Ran at the nuclear envelope (Fig. 6 h). The localization of Ran in these assays was predominantly nuclear, and similar to the control (compare Fig. 6, h and f). As observed in the flow cytometric transport assays, the inhibition of transport in assays supported by XTP-Ran was not as extensive as with wild-type Ran. Reduced transport inhibition per se does not affect Ran accumulation, as a half-maximally inhibiting concentration of GMPPNP (25 μ M) still caused strong accumulation of wild-type Ran at the nuclear envelope (data not shown). We did not observe increased binding of transport ligand at the nuclear envelope in any of the inhibited assays, and immunofluorescence localization of NTF2, hSrp1 α (NLS receptor), and p97 showed no obvious changes in their distribution (data not shown). This localization of Ran represents a clear difference between the inhibition of transport by nonhydrolyzable GTP analogues in assays containing wild-type versus XTP-Ran. It therefore provides further evidence that the inhibition of transport observed in XTP-Ran assays is mediated by a different GTPase.

Inhibition Reduces the Rate of Transport and Is Not Dependent on the Ratio between Cells and Cytosol

One reason for obtaining a maximum of 50% inhibition in the XTP-Ran assays might be that the nonhydrolyzable GTP analogues have only an indirect effect on the import pathway. If this were the case, it might well be reflected in a delay of the onset of inhibition during the transport assay. To investigate whether any lag period exists, we examined the time course of GTP γ S inhibition in transport assays containing XTP-Ran (Fig. 7 A) or, for comparison, wild-type Ran (Fig. 7 B). As has been demonstrated previously (Melchior et al., 1993), GTP γ S inhibition of wild-type Ran resulted in a reduction in the rate of transport over the entire course of the 30-min assay (Fig. 7 B). Fig. 7 A shows that the same was also true for assays supported by XTP-Ran, i.e., that in both the presence and absence of inhibitor transport was linear over the 30-min time period but in the presence of inhibitor the overall rate of transport was reduced. The inhibitory effect of GTP γ S in these assays was manifest even at the earliest time point, and it continuously affected the rate of transport over the entire course of the assay. This consistent reduction in the overall rate of transport suggests that the GTP γ S has a direct effect on one or more components of the nuclear import machinery, rather than acting *via* an indirect mechanism.

Using this *in vitro* assay system, nonhydrolyzable GTP

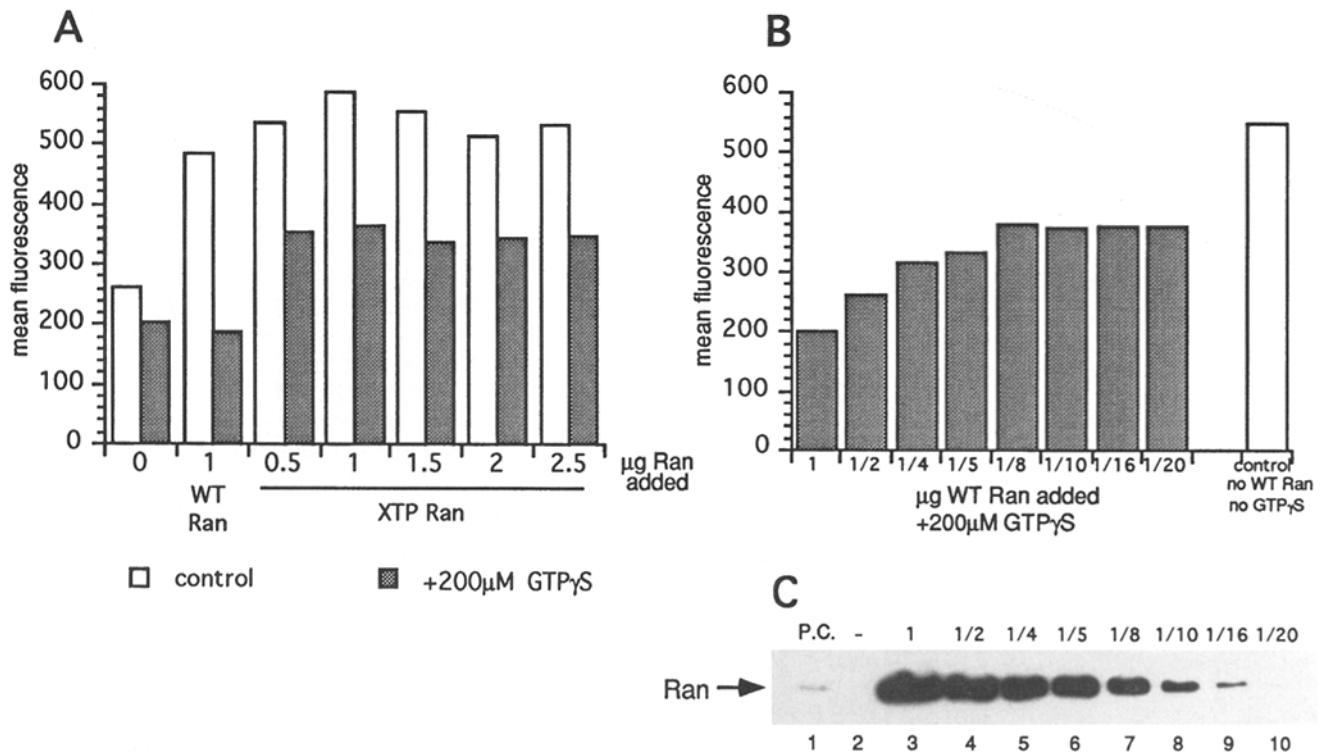


Figure 5. Inhibition of transport in XTP-Ran assays is not mediated by Ran. Varying amounts of wild-type and XTP-Ran were added to transport assays containing purified transport factors (25 μ g/ml hSrp1 α and 12.5 μ g/ml NTF2) as indicated; the shaded bars represent the assays that contained 200 μ M GTP γ S. (A) Titration of XTP-Ran. Transport assays containing 0.5–2.5 μ g of XTP-Ran per assay (final concentrations 12.5–62.5 μ g/ml), in comparison to either no Ran (0) or 1 μ g of wild-type Ran, were performed in the absence or presence of 200 μ M GTP γ S. (B) Titration of wild-type Ran addition. Different amounts of wild-type Ran, forming a dilution series from 1 μ g (1) to 0.05 μ g (1/20), were added to assays containing 1 μ g of XTP-Ran (25 μ g/ml) and 200 μ M GTP γ S (shaded bars). The white bar represents a control assay containing only 1 μ g of XTP-Ran. (C) Immunoblot of the wild-type Ran titration. Permeabilized cells (P.C., lane 1) and samples of the wild-type Ran dilution series used in B (lanes 3–10) were immunoblotted using anti-Ran antibody. Each lane contains 0.5 \times the amount present in a transport reaction.

analogues do not give an irreversible inhibition of transport (Melchior et al., 1993; data not shown), so it is not possible to determine whether inhibited factors are cell or cytosol associated by preincubation with inhibitor. As an alternative approach to addressing this issue, we investigated whether the degree of inhibition in XTP-Ran assays is affected by the ratio between cells and cytosol. We chose this method because one of the initial indications that GTP γ S inhibition was mediated by a cytosolic factor was the observation that the ratio between cells and cytosol strongly affected the level of inhibition (Melchior et al., 1993). We carried out a titration of cytosol addition (using cytosol depleted of Ran by column fractionation) in XTP-Ran transport assays containing either 2×10^5 or 6×10^5 permeabilized cells (Fig. 8). Note that this flow cytometric assay measures the mean fluorescence per cell, so it gives a similar maximal level of transport regardless of the number of cells in the assay. As might be expected, a higher concentration of cytosol was required to give maximal transport in the assays containing more cells (Fig. 8 B). However, unlike for inhibition of wild-type Ran (Melchior et al., 1993; data not shown), the number of cells in the assay had no effect on the overall level of inhibition obtained. In fact, we found that GTP γ S inhibition seemed to reduce the maximum level of transport that could be reached by the cells, irrespective of the concentration of cytosol present in

the assay. This result was clearest in the assays containing 6×10^5 cells (Fig. 8 B) where, although a threefold increase in cytosol concentration (compare 0.31 and 0.94 mg/ml) gave a substantial increase in the control level of transport, in the presence of GTP γ S there was virtually no increase at all. Thus, in XTP-Ran assays, GTP γ S seemed to restrict the level of transport that could be attained by the permeabilized cells in a way that was neither overcome nor exacerbated by additional cytosol. This observation represents another difference between GTP γ S inhibition in XTP-Ran assays and inhibition mediated by wild-type Ran. It also suggests that the inhibited component of the transport machinery is a cell-associated rather than cytosolic factor.

Discussion

An XTP-binding Mutant of Ran

We have constructed and purified a D125N mutant of Ran, and demonstrated that this mutant protein has altered nucleotide binding properties. As predicted, the D125N mutation changes the nucleotide binding specificity of Ran from GTP to XTP and results in a protein that is only functional in nuclear protein import in the presence of XTP. In fact, in the absence of XTP, D125N Ran (XTP-

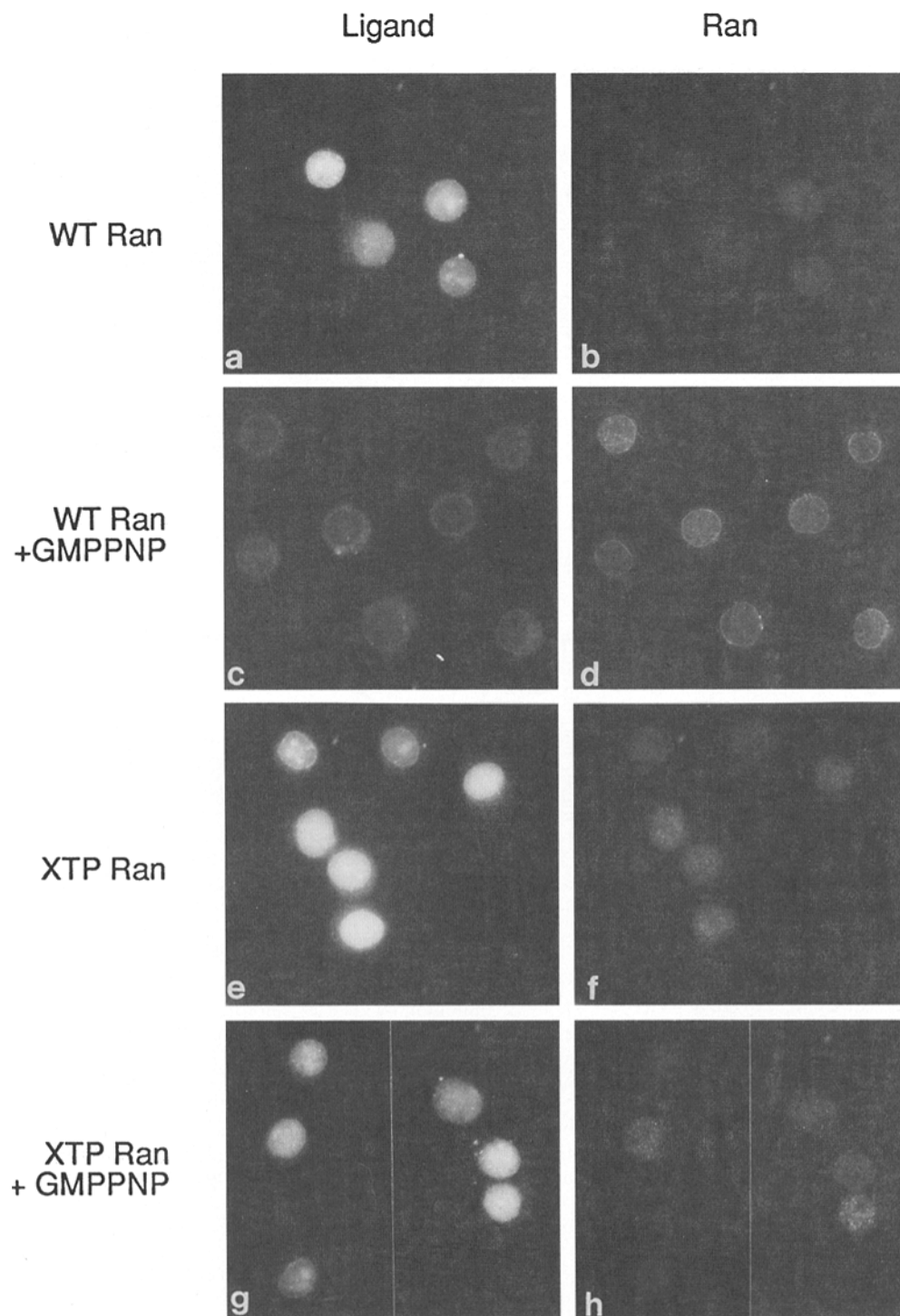


Figure 6. Inhibition of transport by GMPPNP does not lead to accumulation of Ran at the nuclear envelope in XTP-Ran assays. Transport assays containing cytosol depleted of Ran by column fractionation and 25 $\mu\text{g/ml}$ wild-type or XTP-Ran were incubated for 30 min at 30°C in the presence or absence of 500 μM GMPPNP. The cells were then fixed and permeabilized, and the Ran was detected by immunofluorescence microscopy. Left panels show the localization of the FITC-BSA-NLS transport ligand and right panels show the Ran.

Ran) acts as an inhibitor of nuclear protein import (data not shown). The reason for this is not clear, but one possibility is that, like D124N Ypt1p (Jones et al., 1995), XTP-Ran sequesters its cognate guanine nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991; Dasso, 1993) and thereby inhibits nucleotide exchange on wild-type Ran. In the presence of XTP, XTP-Ran appears to function in an equivalent manner to wild-type Ran in permeabilized cell nuclear import assays.

The diversity of GTPases in which a D to N mutation in the G4 domain produces an effective change in nucleotide

binding specificity suggests that this mutation may be widely applicable within the GTPase superfamily (Hwang and Miller, 1987; Weijland and Parmeggiani, 1993; Jones et al., 1995; Kang et al., 1995; Powers and Walter, 1995; Zhong et al., 1995). In previous studies, XTP-binding mutants have been used to specifically examine the role of nucleotide hydrolysis by the GTPase in question (Weijland and Parmeggiani, 1993; Powers and Walter, 1995). We have not focused on the role of nucleotide hydrolysis by Ran here, but XTP-Ran may well be useful for such studies in the future, particularly when more purified as-

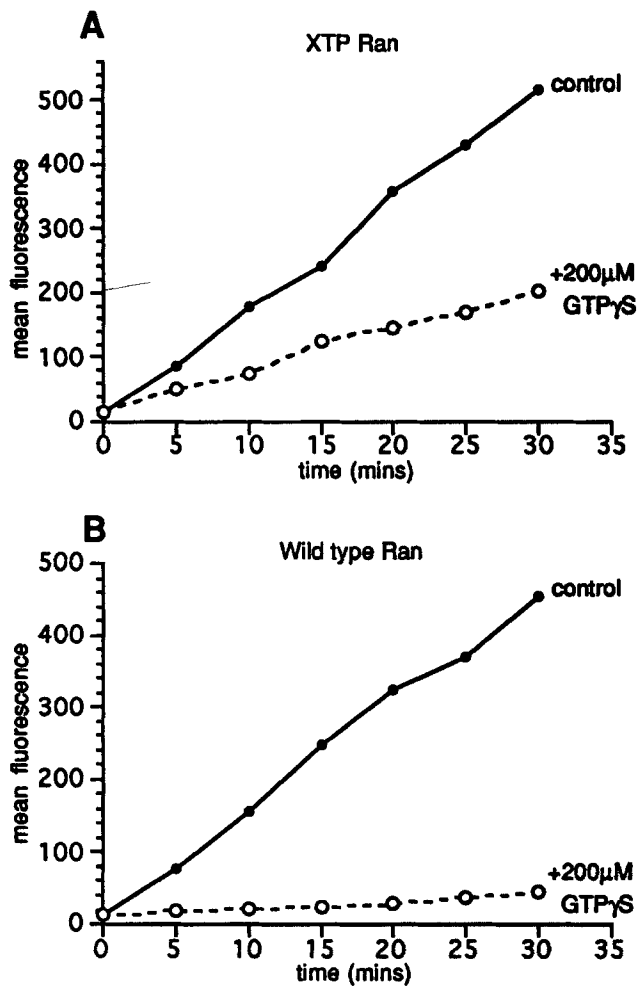


Figure 7. GTP γ S inhibition reduces the rate of nuclear import in XTP-Ran assays. This shows a time course of inhibited nuclear import in assays supported by (A) XTP-Ran or (B), for comparison, wild-type Ran. Transport assays containing cytosol depleted of Ran by column fractionation and 25 μ g/ml Ran were incubated at 30°C for the indicated times then stopped by transfer to an ice-water bath and dilution with ice-cold transport buffer (see Materials and Methods). The open circles represent assays that contained 200 μ M GTP γ S, and the closed circles represent controls that did not.

says that represent either part or all of the nuclear import pathway are developed. In this study we have instead used the XTP-binding mutant in a novel way, to “bypass” Ran-mediated inhibition of nuclear import. This approach is of potentially general application, and could be used in characterization of other intracellular pathways in which GTPases are involved.

A Second GTPase in Nuclear Protein Import

The overall aim of this study was to use the XTP-Ran mutant to investigate whether any additional GTPases are involved in nuclear protein import. This was carried out by replacing wild-type Ran in *in vitro* assays with XTP-Ran, which, as a result of its altered nucleotide binding specificity, is no longer sensitive to inhibition by nonhydrolyzable GTP analogues such as GTP γ S. Using these XTP-Ran as-

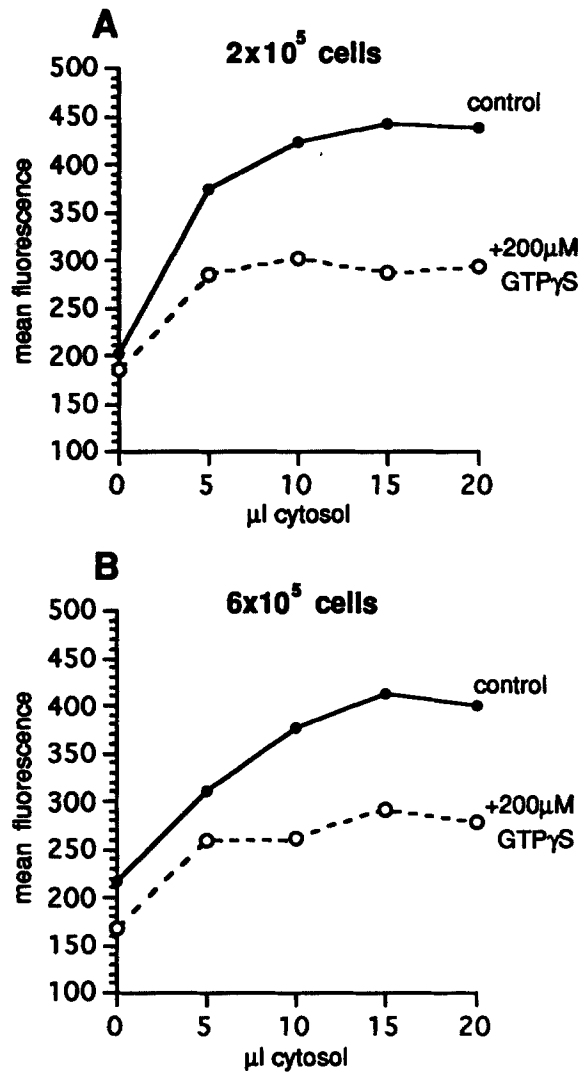


Figure 8. Inhibition by GTP γ S is not influenced by the ratio between permeabilized cells and cytosol in XTP-Ran assays. This shows a comparison of the effect of GTP γ S on transport assays containing either 2×10^5 (A) or 6×10^5 (B) permeabilized cells. 1 μ g of XTP-Ran (25 μ g/ml), between 0 and 1.25 mg/ml cytosol depleted of Ran by column fractionation and the appropriate number of permeabilized cells were added to each assay (final vol 40 μ l) in the presence or absence of 200 μ M GTP γ S.

says, we were able to test whether any components of the nuclear import machinery other than Ran are sensitive to inhibition by GTP analogues. We found that nonhydrolyzable analogues of GTP, such as GTP γ S and GMPPNP, inhibit nuclear import in XTP-Ran assays by \sim 50%. We therefore conclude that at least one additional GTPase is involved in the nuclear import pathway.

Blocking GTP hydrolysis by Ran has a potent inhibitory effect on nuclear import (Melchior et al., 1993, 1995a; Moore and Blobel, 1993; this study), so it was important to clearly establish that the inhibition in XTP-Ran assays is not in fact due to Ran. There are two potential sources of Ran-mediated inhibition: the XTP Ran itself and the trace amount of wild-type Ran that remains in the permeabilized cells and depleted cytosol. Several experiments in this

study (detailed below) gave results indicating that neither the XTP-Ran nor residual wild-type Ran are responsible for the inhibition observed.

If the inhibition were mediated by XTP-Ran, it would involve unexpected binding of the GTP analogues by the mutant protein. In EDTA-based exchange assays, XTP-Ran has a much higher affinity for XTP than GTP, GTP γ S, or ATP and is therefore unlikely to bind to GTP γ S in the transport assays, which contain 1 mM XTP and ATP. The inhibitor dose–response curves suggest that the inhibition involves a high affinity interaction with a protein that shows similar sensitivity to the GTP analogues as wild-type Ran. From the nucleotide binding studies, this does not seem to be the case for XTP-Ran. Increasing the concentration of XTP-Ran did not increase the level of inhibition which, bearing in mind the dominant effect of inhibited Ran, might be expected to occur if the XTP-Ran bound to the GTP analogues. Inhibitor-bound XTP-Ran would also be expected to accumulate at the nuclear envelope like wild-type Ran, but no accumulation of Ran was detected in the XTP-Ran assays. Finally, the ratio between cells and cytosol did not affect the level of inhibition in the XTP-Ran assays, whereas Ran-mediated inhibition in conventional transport assays (Melchior et al., 1993) or assays containing recombinant wild-type Ran (data not shown) was substantially reduced by increasing the number of cells in the assay. Taken together, these results provide strong evidence that GTP γ S inhibition of nuclear import in XTP-Ran assays is not mediated by XTP-Ran.

The lack of Ran accumulation at the nuclear envelope and independence from the cell/cytosol ratio also provide evidence that the residual wild-type Ran is not responsible for the inhibition observed, as they represent clear differences between the inhibition in XTP-Ran assays and that mediated by wild-type Ran. In addition, if the intermediate level of inhibition in XTP-Ran assays were due to a competition between XTP-Ran and residual wild-type Ran bound to inhibitor, increasing the concentration of XTP-Ran would be expected to relieve the inhibition, but this did not occur. As wild-type Ran has a dominant inhibitory effect in these *in vitro* assays, we were able to test directly whether the residual wild-type Ran could account for the inhibition using a titration of wild-type Ran addition. This experiment showed that the amount of Ran that needed to be added to increase the level of inhibition was significantly greater than the amount already present, demonstrating that the residual Ran present is not sufficient to cause the inhibition observed. Thus, it is highly unlikely that inhibition of nuclear import in XTP-Ran assays by nonhydrolyzable GTP analogues is caused by the trace amount of wild-type Ran that remains in the assay.

As well as substantiating the evidence that the inhibition is mediated by a factor other than Ran, the characteristics of the inhibitory effect provide some initial insights into the mechanism involved. The consistent reduction in the rate of transport over the course of the 30-min assays suggests that the inhibited component is directly involved in the nuclear import pathway. Increasing the concentration of cytosol in the assay was not able to overcome the inhibition, also arguing that it does not result from a defect in recycling of cytosolic transport factors. Also, the similarity of the dose–response curve to that of many GTPases, in-

cluding Ran (this study; Melchior et al., 1993), suggests that the inhibited factor is a protein which specifically binds to GTP, i.e., a GTPase, rather than, for example, a kinase with relatively promiscuous nucleotide binding properties such as casein kinase II (Allende and Allende, 1995).

An obvious difference between the wild-type and XTP-Ran assays is in the overall level of inhibition caused by GTP γ S: a maximum of 50–60% in the XTP-Ran assays and 80–90% with wild-type Ran (Melchior et al., 1993; this study). Obtaining only ~50% inhibition by nonhydrolyzable GTP analogues in GTPase-dependent processes is not unusual, and has been observed in a number of previous studies, e.g., GTP γ S inhibition of vacuolar inheritance (Conradt et al., 1994), endosome fusion (Lenhard et al., 1992; Spiro et al., 1995), and coated pit invagination and budding (Carter et al., 1993). The reason for the partial inhibition obtained in this study is not yet clear, but there are a number of possible explanations. One of the most straightforward could be that the second GTPase plays an indirect role in the import process. However, as discussed above, the rapid onset of the inhibition suggests that this is not the case. Alternatively, there could be an exchange factor acting on the GTPase in the transport assays, making the inhibition to some extent reversible. If the activity of the GTPase in transport is not normally rate limiting, this could lead to an incomplete level of inhibition. Another possibility is that there are two pathways for nuclear import downstream of Ran, only one of which is inhibited by nonhydrolyzable GTP analogues. Clarification of the significance of this result will require more detailed characterization of both the nuclear import pathway and more specifically the role of GTPases.

Several lines of evidence point towards the inhibited GTPase being cell associated rather than cytosolic. Perhaps the strongest of these is that we observed a similar degree of inhibition in transport assays reconstituted with purified transport factors as in those that contained Ran-depleted cytosol. If the inhibition were due to a soluble component one would expect it to be present in cytosol and to affect the level of inhibition when added. A second indication comes from the observation that the degree of inhibition was not influenced by the ratio between cells and cytosol in the assay. In this experiment, it seemed that GTP γ S reduced the level of transport that could be reached by the permeabilized cells irrespective of the concentration of cytosolic factors present. This suggests that, under GTP γ S inhibition conditions, the rate-limiting step in transport in XTP-Ran assays is defined by a cell-associated component of the transport machinery. Fractionation of cytosol by molecular sieving and ion exchange chromatography has also not revealed any cytosolic factors other than Ran that specifically modulate GTP γ S inhibition in XTP-Ran assays (Sweet, D.J., and L. Gerace, unpublished observations).

A cell-associated GTPase that is directly involved in nuclear import would most likely be a component of the NPC. None of the nucleoporins that have been characterized so far contain a GTP-binding domain (Rout and Wentz, 1994; Davis, 1995), although the 60- and 20–30-kD nuclear envelope-associated GTP-binding proteins identified by GTP overlay and photoaffinity labeling could be NPC proteins (Rubins et al., 1990; Seydel and Gerace,

1991). The NPC is a large and unique structure, so it is difficult to make comparisons between nuclear import and other translocation processes. Nevertheless, it is interesting that GTPases are involved in the transport of proteins across at least two more intracellular membranes. The role of GTP hydrolysis in protein translocation into the ER has been well documented, and shown to involve the activity of several soluble and membrane-associated GTPases (Walter and Johnson, 1994). In this case, the membrane-associated GTPases (SRP receptor) are thought to act catalytically in targeting and formation of the translocation complex rather than being involved in translocation itself. Chloroplasts also contain GTPases that are localized to the protein translocation site (Kessler et al., 1994; Seedorf et al., 1995). The functions of these proteins have not been characterized in detail, but they are thought to form part of the translocation pore (Schnell et al., 1994; Seedorf et al., 1995) and may also be involved in targeting of translocated proteins to the chloroplast surface (Schnell, 1995).

What might be the role of an NPC-associated GTPase in nuclear import? One model is that it would act in an analogous way to Ran, and promote movement of the receptor complex along a later part of the transport pathway (Melchior et al., 1995). However, it could also have a somewhat different function, such as regulating the opening of the central gated channel. GTPases are generally viewed as having two conformational states, GTP and GDP bound, with interconversion between the two states determining the activity and interactions of the protein (Bourne et al., 1991; Nuoffer and Balch, 1994). A conformational change induced by GTP hydrolysis (or GTP/GDP exchange) could act as a trigger for opening of the gated channel and thereby facilitate passage of the receptor complex into the nucleus. Clarification of the potential role of GTP hydrolysis in central channel gating, and regulation of other transport steps, will require more detailed characterization of both the structure of the nuclear pore complex and the mechanism of transport across it.

In summary, we have made use of a mutant form of Ran that binds to XTP instead of GTP to investigate whether any additional GTPases are involved in the nuclear import pathway. Our results have shown that the transport machinery does indeed contain a second GTPase, which we speculate may be associated with the nuclear pore complex. Purification and characterization of the GTPase that mediates this inhibitory effect will shed more light on its relationship to the NPC and its role in the nuclear import process.

We are grateful to Frauke Melchior for providing anti-Ran antibodies, Rohit Mahajan for purified RanBP1, and Tianhua Hu for purified p97 and hSrp1 α . We would also like to thank Frauke Melchior and Bryce Paschal for helpful comments on the manuscript, and all the members of the Gerace laboratory for stimulating discussions.

This study was supported by grants from the National Institutes of Health and Human Frontiers Science Program (to L. Gerace) and a SERC-NATO (United Kingdom) postdoctoral fellowship (to D. Sweet).

Received for publication 1 December 1995 and in revised form 21 February 1996.

References

Allende, J.E., and C.C. Allende. 1995. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J.* 9:313-323.

- Adam, S.A., R.E. Sterne-Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807-816.
- Adam, S.A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell.* 66:837-847.
- Adam, E.J.H., and S.A. Adam. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* 125:547-555.
- Bischoff, F.R., and H. Ponstingl. 1991. Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature (Lond.)* 354:80-82.
- Bischoff, F.R., H. Krebber, E. Smirnova, W. Dong, and H. Ponstingl. 1995. Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:705-715.
- Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.)* 349:117-127.
- Carter, L.L., T.E. Redelmeier, L.A. Woolenweber, and S.L. Schmid. 1993. Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. *J. Cell Biol.* 120:37-45.
- Chi, N.C., E.J.H. Adam, and S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* 130:265-274.
- Conradt, B., A. Haas, and W. Wickner. 1994. Determination of four biochemically distinct sequential stages during vacuole inheritance in vitro. *J. Cell Biol.* 126:99-110.
- Coutavas, E., M. Ren, J.D. Oppenheim, P.D. D'Eustachio, and M.G. Rush. 1993. Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature (Lond.)* 366:585-587.
- Dabauvalle, M.-C., R. Benavente, and N. Chaly. 1988a. Monoclonal antibodies to a M, 68,000 pore complex glycoprotein interfere with nuclear protein uptake in *Xenopus* oocytes. *Chromosoma (Berl.)* 97:193-197.
- Dabauvalle, M.-C., B. Schulz, U. Scheer, and R. Peters. 1988b. Inhibition of nuclear accumulation of karyophilic proteins in living cells by microinjection of the lectin wheat germ agglutinin. *Exp. Cell Res.* 174:291-296.
- Dasso, M. 1993. RCC1 in cell cycle: the regulator of chromosome condensation takes on new roles. *Trends Biochem. Sci.* 18:96-101.
- Davis, L.I. 1995. The nuclear pore complex. *Annu. Rev. Cell Biol.* 64:865-896.
- Dingwall, C., and R. Laskey. 1991. Nuclear targeting signals—a consensus? *Trends Biochem. Sci.* 16:478-481.
- Enkel, C., G. Blobel, and M. Rexach. 1995. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.* 270:16499-16502.
- Fabre, E., and E. Hurt. 1994. Nuclear transport. *Curr. Opin. Cell Biol.* 6:335-342.
- Featherstone, C.M., M.K. Darby, and L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of both protein and RNA in vivo. *J. Cell Biol.* 107:1289-1297.
- Finlay, D.R., D.D. Newmeyer, T.M. Price, and D.J. Forbes. 1987. Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. *J. Cell Biol.* 104:189-200.
- Görlich, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell.* 79:767-778.
- Görlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* 5:383-392.
- Holt, G.D., C.M. Snow, A. Senior, R.S. Haltiwanger, and L. Gerace. 1987. Nuclear pore complex glycoproteins containing cytoplasmically disposed O-linked N-acetylglucosamine. *J. Cell Biol.* 104:291-308.
- Hwang, Y.-W., and D. Miller. 1987. A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. *J. Biol. Chem.* 262:13081-13085.
- Imamoto, N., Y. Matsuoka, T. Kurihara, K. Kohno, M. Miyagi, F. Sakiyama, Y. Okada, S. Tsunasawa, and Y. Yoneda. 1992. Antibodies against 70-kD heat shock cognate protein inhibit mediated nuclear import of karyophilic proteins. *J. Cell Biol.* 119:1047-1061.
- Imamoto, N., T. Shimamoto, S. Kose, T. Takao, T. Tachibana, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda. 1995a. The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile. *FEBS LETT.* 368:415-419.
- Imamoto, N., T. Shimamoto, T. Takeo, T. Tachibana, S. Kose, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda. 1995b. In vivo evidence for involvement of a 58 kDa component of the nuclear-pore targeting complex in nuclear protein import. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:3617-3626.
- Jones, S., R.J. Litt, C.J. Richardson, and N. Segev. 1995. Requirement of nucleotide exchange factor for Ypt1 GTPase mediated protein transport. *J. Cell Biol.* 130:1051-1061.
- Kang, C., N. Sun, R.B. Hontzatzko, and H.J. Fromm. 1995. Replacement of Asp333 with Asn by site-directed mutagenesis changes the substrate specificity of *Escherichia coli* adenylosuccinate synthetase from guanosine 5'-triphosphate to xanthosine 5'-triphosphate. *J. Biol. Chem.* 269:24046-24049.
- Kessler, F., G. Blobel, H.A. Patel, and D.J. Schnell. 1994. Identification of two GTP-binding proteins in the chloroplast protein import machinery. *Science (Wash. DC)* 266:1035-1039.
- Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:

- Lenhard, J.M., R.A. Kahn, and P.D. Stahl. 1992. Evidence for ADP-ribosylation factor (ARF) as a regulator of in vitro endosome-endosome fusion. *J. Biol. Chem.* 267:13047-13052.
- Manne, V., S. Yamazaki, and H-F. Kung. 1984. Guanosine nucleotide binding by highly purified Ha-ras-encoded p21 protein produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 81:6953-6957.
- Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* 123:1649-1659.
- Melchior, F., and L. Gerace. 1995. Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.* 7:310-318.
- Melchior, F., T. Guan, N. Yokoyama, T. Nishimoto, and L. Gerace. 1995a. GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.* 131:571-581.
- Melchior, F., D.J. Sweet, and L. Gerace. 1995b. Analysis of Ran/TC4 function in nuclear protein import. *Methods Enzymol.* 257:279-291.
- Moore, M.S., and G. Blobel. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature (Lond.)*. 365:661-663.
- Moore, M.S., and G. Blobel. 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA.* 91:10212-10216.
- Moroianu, J., G. Blobel, and A. Radu. 1995a. Previously identified protein of uncertain function is karyopherin α and together with karyopherin β docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 92:2008-2011.
- Moroianu, J., M. Hijikata, G. Blobel, and A. Radu. 1995b. Mammalian karyopherin $\alpha 1b$ and $\alpha 2b$ heterodimers: $\alpha 1$ and $\alpha 2$ subunit binds nuclear localization signal and β subunit interacts with peptide repeat-containing nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:6352-6356.
- Nuoffer, C., and W. Balch. 1994. GTPases: multifunctional molecular switches regulating vesicular traffic. *Annu. Rev. Biochem.* 63:949-990.
- Paine, P.L., L.C. Moore, and S.B. Horowitz. 1975. Nuclear envelope permeability. *Nature (Lond.)*. 254:109-114.
- Panté, N., and U. Aebi. 1994. Toward the molecular details of the nuclear pore complex. *J. Struct. Biol.* 113:179-189.
- Paschal, B.M., and L. Gerace. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* 129:925-937.
- Peters, R. 1986. Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta.* 864:304-369.
- Powers, T., and P. Walter. 1995. Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. *Science (Wash. DC)*. 269:1422-1424.
- Radu, A., G. Blobel, and M.S. Moore. 1995. Identification of a protein complex that is required for nuclear protein import and mediated docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:1769-1773.
- Rout, M.P., and S.R. Wente. 1994. Pores for thought: nuclear pore complex proteins. *Trends Cell Biol.* 4:357-365.
- Rubins, J.B., J.O. Benditt, B.F. Dickey, and N. Riedel. 1990. GTP-binding proteins in rat liver nuclear envelopes. *Proc. Natl. Acad. Sci. USA.* 87:7080-7084.
- Schnell, D.J. 1995. Shedding light on the chloroplast protein import machinery. *Cell.* 84:521-524.
- Schnell D.J., F. Kessler, and G. Blobel. 1994. Isolation of components of the chloroplast protein import machinery. *Science (Wash. DC)*. 266:1007-1012.
- Seedorf, M., K. Waegemann, and J. Soll. 1995. A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J.* 7:401-411.
- Seydel, U., and L. Gerace. 1991. A 28,000-Da GDP/GTP-binding protein specific to the nuclear envelope. *J. Biol. Chem.* 266:7602-7608.
- Shi, Y., and J.O. Thomas. 1992. The transport of proteins into the nucleus requires 70 kDa heat shock protein or its cytosolic cognate. *Mol. Cell. Biol.* 12:2186-2192.
- Spiro, D.J., T.C. Taylor, P. Melancon, and M. Wessling-Resnick. 1995. Cytosolic ADP-ribosylation factors are not required for endosome-endosome fusion but are necessary for GTPS inhibition of fusion. *J. Biol. Chem.* 270:13693-13697.
- Walter P., and A.E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10:87-119.
- Weis, K., I.W. Mattaj, and A.I. Lamond. 1995. Identification of hSrp1 α as a functional receptor for nuclear localization sequences. *Science (Wash. DC)*. 268:1049-1053.
- Wiejland, A., and A. Parmeggiani. 1993. Toward a model of the interaction between elongation factor Tu and the ribosome. *Science (Wash. DC)*. 259:1311-1314.
- Wu, J., M.J. Matunis, D. Kraemer, G. Blobel, and E. Coutavas. 1995. Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.* 270:14209-14213.
- Yano, R., M. Oakes, M. Yamagashi, J.A. Dodd, and M. Nomura. 1992. Cloning and characterization of SRP1, a suppressor of temperature sensitive RNA polymerase I mutations, in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 12:5640-5651.
- Yokoyama, N., N. Hiyashi, T. Seki, N. Pante, T. Ohba, K. Nishii, K. Kuma, T. Hayashida, T. Miyata, U. Aebi et al. 1995. RanBP2, a gigantic nuclear pore complex protein that has Zn²⁺ fingers, a cyclophilin domain and binds Ran/TC4. *Nature (Lond.)*. 376:184-188.
- Yoneda, Y., N. Imamoto-Sonobe, M. Yamaizumi, and T. Uchida. 1987. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into tissue culture cells. *Exp. Cell. Res.* 173:586-595.
- Zhong, J.-M., M.-C. Chen-Hwang, and Y.-W. Hwang. 1995. Switching nucleotide specificity of Ha-Ras p21 by a single amino acid substitution at aspartate 119. *J. Biol. Chem.* 270:10002-10007.