Inhibition of Nuclear Protein Import by Nonhydrolyzable Analogues of GTP and Identification of the Small GTPase Ran/TC4 as an Essential Transport Factor

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Abstract. We have investigated a possible involvement of GTPases in nuclear protein import using an in vitro transport system involving digitonin-permeabilized cells supplemented with exogenous cytosol. Transport in this system was measured with a novel ELISA-based assay that allows rapid quantitative analysis. GTP\textsubscript{\gamma}S and other nonhydrolyzable analogues of GTP were found to rapidly inhibit the rate of in vitro nuclear import. Transport inhibition by GTP\textsubscript{\gamma}S was dependent on the concentrations of permeabilized cells and cytosol, and was strongly enhanced by a cytosolic factor(s). The predominant cytosolic component responsible for this inhibition was found in a 20–30-kD fraction in molecular sieving chromatography. Furthermore, a component(s) of this 20–30-kD fraction was itself required for efficient nuclear import. Biochemical complementation with bacterially expressed protein demonstrated that this essential GTP\textsubscript{\gamma}S-sensitive transport factor was Ran/TC4, a previously described GTPase of the Ras superfamily found in both nucleus and cytoplasm. Ran/TC4 and its guanine nucleotide release protein RCC1 have previously been implicated in DNA replication, cell cycle checkpoint control, and RNA synthesis, processing and export. Our results suggest that Ran/TC4 serves to integrate nuclear protein import with these other nuclear activities.

Molecular exchanges between nucleus and cytoplasm occur continuously in eukaryotic cells and are fundamentally important for cellular metabolism. The boundary of the nucleus is formed by the nuclear envelope (NE), a double membrane system that is continuous with the endoplasmic reticulum. Transport across the NE is carried out by elaborate supramolecular structures called nuclear pore complexes (NPCs; reviewed by Nigg et al., 1991; Forbes, 1992; Gerace, 1992). The NPC has a mass of \sim{}125 \times 10^6 D (Reichelt et al., 1990), but relatively few of its polypeptides have been identified. Nevertheless, general transport properties of the NPC have been determined by functional studies. The NPC contains an aqueous channel(s) with a diameter of \sim{}10 nm, which allows rapid, nonselective diffusion of ions, metabolites and other small molecules across the NE (Paine et al., 1975; Peters, 1986).

Most proteins and RNAs are too large to diffuse through the NPC at physiologically significant rates, and instead are transported through a gated channel in the NPC by highly selective, signal-mediated mechanisms (Nigg et al., 1991; Forbes, 1992; Gerace, 1992).

Signals that specify nuclear protein import have been characterized in detail, and usually involve short basic stretches of amino acids in the transported proteins called nuclear localization sequences (NLSs; reviewed by Dingwall and Laskey, 1991). A different signal is responsible for nuclear import of small nuclear ribonucleoprotein particles containing certain U RNAs (U snRNPs) (Fischer et al., 1991; Michaud and Goldfarb, 1991), although its precise molecular nature is unknown. Furthermore, signals involved in RNA and protein export from the nucleus are just beginning to be characterized (Hamm and Mattaj, 1990; see also Schmidt-Zachmann et al., 1993).

Recent work has suggested that nuclear import can be regulated in several different ways (Nigg et al., 1991; Gerace, 1992). One type of mechanism affects the efficiency or availability of NLSs on individual proteins. For example this mechanism controls the nuclear import of certain transcription factors and protein kinases (Jans et al., 1991; Nigg et al., 1991; Moll et al., 1991; Henkel et al., 1992). A second type of mechanism appears to affect the protein import machinery rather than individual proteins (Feldherr and Akin, 1992).
Gating (Feldherr et al., 1984), which is both ATP and tem-
plasmic side of the NPC. The latter process involves channel
volves ligand “docking” at the cytoplasmic surface of the
NPC, which can occur in the absence of ATP and at 0°C
(Newmeyer and Forbes, 1988; Richardson et al., 1988). A subsequent step involves ligand translocation to the nucleo-
plasmic side of the NPC. The latter process involves channel
gating (Feldherr et al., 1984), which is both ATP and tem-
perature dependent. The precise components that mediate
these and other steps of nuclear import are unknown.

Several in vitro systems that yield physiologically relevant
mediated nuclear import have been developed recently
(Newmeyer et al., 1986; Adam et al., 1990), providing valu-
able approaches for analyzing transport mechanisms. A
system devised in our laboratory consists of digitinon-
permeabilized cells (which retain an intact NE) sup-
plemented with exogenous cytosol and ATP (Adam et al.,
1990). Studies with this and other systems have demon-
strated a requirement for multiple cytosolic factors in nu-
clear import (Adam et al., 1990; Newmeyer and Forbes,
1990; Moore and Blobel, 1992). One such factor is a protein
that specifically binds NLSs with high affinity and has the
properties of a functional import receptor (Adam et al.,
1989; Adam and Gerace, 1991). Other cytosolic factors
include a component that interacts with O-linked glycoproteins
of the NPC (Sterne-Marr et al., 1992), the ubiquitous cellular
protein hsc70 (Imamoto et al., 1992; Shi and Thomas,
1992), and several activities detected by fractionation of
cytosol (Newmeyer and Forbes, 1990; Moore and Blobel,
1992).

It is now evident that GTPases are regulators of a vast
diversity of cellular functions, including protein synthesis
and translocation, signal transduction, and membrane trans-
port (reviewed by Bourne et al., 1991; Pfeffer, 1992; Nuoffer
and Balch, 1993). GTPases are molecular switches that exist
in predominantly two conformations determined by the state
of bound guanine nucleotide, an active form containing GTP
and an inactive form containing GDP. The activity of
GTPases is regulated by a combination of GTPase-activating
proteins (GAPs), which stimulate the rate of GTP hydroly-
isis, and guanine nucleotide release proteins (GNRPs), which
catalyze the release of bound GDP and allow GTPases to re-
bind GTP (Bourne et al., 1991).

We previously identified a small GTP-binding protein that
is highly concentrated in the NE using photocross-linking
approaches (Seydel and Gerace, 1991). In this study we have
used an in vitro functional assay to directly investigate
whether GTPases are involved in nuclear protein import. We
found that nonhydrolyzable analogues of GTP, which often
are potent inhibitors of GTPase function (Nuoffer and Balch,
1993), strongly inhibit nuclear protein import in vitro. We
determined that the major component sensitive to nucleotide
inhibition is Ran/TC4, a previously identified member of the
Ras superfamily of GTP-binding proteins present in both nu-
cleus and cytoplasm (Drivas et al., 1990; Bischoff and Pon-
stingl, 1991a; Ren et al., 1993). In addition, we found that
Ran/TC4 is a cytosolic factor required for efficient nuclear
import. Ran/TC4 and a protein called RCC1, which is a
GNRP for Ran/TC4 (Bischoff and Ponstingl, 1991b), have
been previously implicated in a diverse range of nuclear
functions, including DNA replication, cell cycle feedback
control, and RNA processing and export (reviewed by Daso,
1993). Our results suggest that Ran/TC4 serves to integrate nucleocytoplasmic trafficking with these other nu-
clear functions.

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% FCS
(Hyclone Laboratories, Logan, UT). Nucleotides and nucleotide ana-
logs were obtained from Boehringer-Mannheim Biochemicals (Indi-
anapolis, IN). Nucleotide stock solutions (with equimolar Mg-acetate in 50
mM Hepes, pH 7.5) were aliquoted, stored in liquid nitrogen and used
within 4 wk. Digitonin (high purity), creatine phosphate and creatine phos-
phokinase were obtained from Calbiochem-Behring Corp. (San Diego,
CA). A 10% stock solution of digitonin in DMSO was stored in aliquots
at ~20°C. Stocks (10 mg/ml of Avidin (Canadian Lysozyme, Abbotsford,
Canada) and Biocytin (Sigma Immunocchemicals, St. Louis, MO) were kept
at 4°C. Purified ADP-ribosylation factor (ARF) from bovine brain was the
generous gift of Dr. Frank Peter (The Scripps Research Institute, La
Jolla, CA).

Preparation of Transport Substrate

Synthetic peptides containing the SV-40 large T antigen wild-type NLS
(CGPGPKKKRKVED) or a transport deficient "reverse" NLS peptide
(CGGDKKEVRKKKKK) were obtained from the Core Facility of TSRI and
further purified by HPLC. Biotinylated BSA (B-BSA; Pierce Chemical Co.,
Rockford, IL) was activated with Sulfo-SMCC (Pierce Chemical Co.) and
mixed with a 50-fold excess of peptide. After overnight incubation at 4°C
the B-BSA-peptide conjugate was separated from free peptide using a PD10
column (Pharmacia Biotech Inc., Piscatway, NJ) and subsequent dialysis
(25-kD molecular weight cut-off). The concentration of the conjugate was
determined by absorbance at 280 nm. Aliquots were stored at ~80°C.
The number of peptides conjugated to the protein was estimated by mobility
shift on SDS-polyacrylamide gels to be ~5-15 peptides per B-BSA molecule.

Preparation of HeLa Cell Cytosol

Exponentially growing cultures of HeLa cells were collected by centrifuga-
tion at 250 × g for 10 min and washed twice with ice cold PBS by resuspension
and centrifugation. The cells were then washed with 10 mM Hepes, pH 7.3,
110 mM potassium acetate, 2 mM magnesium acetate, and 2 mM DTT. The
cell pellet was resuspended in 1 vol of lysis buffer (5 mM Hepes, pH 7.3,
10 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.1 mM
PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin, and swelled
for 10 min on ice. The cells were then lysed by five strokes in a tight fitting
stainless steel dounce homogenizer. The resulting homogenates were cen-
trifuged at 1,500 × g for 15 min to remove nuclei and cell debris. The superna-
tant was then sequentially centrifuged at 15,000 × g for 20 min and 100,000 × g
for 30 min. The final supernatant was dialyzed for 3-4 h with a collodion
membrane apparatus (molecular weight cut-off of 10 kD; Schleicher &
Schuell, Inc., Keene, NH) against transport buffer (20 mM Hepes pH 7.3,
110 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM
DTT, and 1 μg/ml of aprotinin, leupeptin, and pepstatin) with 0.1
mM PMSF and frozen in aliquots in liquid nitrogen before storage at
~80°C. The protein concentration of the cytosol was 10-12 mg/ml as deter-
mined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Preparation of ELISA Plates

Rabbit anti-BSA antibody preparations were obtained from Calbiochem-
Behring Corp. and IgGs were purified by chromatography on protein G
Sepharose. A solution of 4 μg/ml IgG in 50 mM Hepes, pH 7.6 was plated onto immunomodule strips (Nunc Maxisorp F8 microwell im-
munomodules; Thomas Scientific, Swedesboro, NJ). Plates were incubated
for 3 h at 37°C or overnight at 4°C, washed three times in PBS, and then
incubated in blocking buffer (0.05% Tween 20 and 0.05% Gelatin in PBS)
for 1 h at 37°C. Plates were stored in blocking buffer at 4°C and used within 2 wk. Immediately before using, the strips were washed three times in PBS.

**Quantitative In Vitro Transport Assay**

**Cell Permeabilization.** HeLa suspension cells were harvested by centrifugation, washed once in transport buffer and resuspended to a density of 0.5 × 10^6 cells/ml. Digitonin was added to a final concentration of 0.008% and the cells were allowed to permeabilize for 7 min on ice. The suspension was diluted 10-fold with transport buffer, the cells were harvested and finally resuspended in the desired volume (normally 2-4 × 10^5 cells/10 μl).

**In Vitro Transport.** Transport mixtures (final volume 40 μl) contained cytosol (usually 2-5 mg/ml), an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, 20 U/ml creatine phosphokinase), transport substrate (100 mM biotinylated BSA-peptide), and permeabilized cells (2-8 × 10^5 cells) in transport buffer. The samples were mixed on ice in wells of Nunc microwell immunomodules, medium binding (Thomas Scientific). The transport reaction was started by transfer into a 30°C water bath and stopped, normally after 30 min, by dilution with 200 μl of ice cold transport buffer and transfer of plates to ice. All experiments were carried out in triplicate to allow accurate quantitation.

**Wash and Lysis Conditions.** To remove and block remaining extracellular transport substrate, the wells were spun for 3 min at 300 rpm in a Beckmann J6-B centrifuge. 200 μl of the supernatant were removed and 100 μl of avidin in transport buffer (0.1 mg/ml) was added to the cells. After 30 min at 4°C, 100 μl bovine in transport buffer (0.2 mg/ml) was added and the samples were allowed to sit for another 15 min. The samples were centrifuged again, 200 μl supernatant were removed, and 200 μl of lysis buffer (1.0% Triton X-100 in PBS) was added. The cells were thoroughly resuspended and allowed to lyse for at least 2 h or overnight at 4°C. ELISA-based Detection of Unblocked Transport Ligand. 5 μl of the cell lysates were added to 200 μl of Triton buffer (0.2% Triton X-100 and 0.05% Gelatin in PBS) in immunomodule strips previously coated with rabbit anti-BSA antibodies. After incubation for 3 h at 37°C or overnight at 4°C, strips were washed three times with PBS, incubated for 5 min in Triton buffer and washed three times in PBS (this is referred to as a wash cycle). To each well 200 μl of streptavidin-HRP (Boehringer-Mannheim Biochemica), diluted 1:5,000 in blocking buffer was then added. After 1 h at room temperature the strips were washed as before. 200 μl substrate solution (10 mg o-phenylenediamine, 10 μl H2O2 in 25 ml of 50 mM Na2HPO4, 27 mM citrate, pH 5) was added to each well and the incubation allowed to proceed until sufficient color was developed, typically 1-3 min. The reaction was terminated by addition of 50 μl of 1 M H2SO4. The A405 was read on an ELISA plate reader (Bio-Rad Laboratories) and corrected for the background signal. SDS-PAGE was carried out as described previously (Adam and Gerace, 1991). Detection of Ran/TC4 on immunoblots was performed as described (Ren et al., 1993) using antibodies kindly provided by Dr. Peter D'Eustachio.

**Results**

An Improved Assay for Quantitative Analysis of In Vitro Nuclear Import

We adapted the in vitro assay for nuclear protein import developed by Adam et al. (1990) to include an ELISA-based detection system to allow rapid quantitative analysis of a large number of samples (see Materials and Methods). The transport ligand in this assay was B-BSA conjugated with synthetic NLS peptides. This new assay fulfills numerous criteria for physiologically relevant nuclear import. Transport depends on the presence of cytosol (Fig. 1A), saturating at a cytosolic protein concentration of 2-3 mg/ml, and is dependent on temperature and ATP (Fig. 1C).

The accumulation of B-BSA-NLS in the nucleus is linear over at least 25 min. A representative experiment is shown in Fig. 1B, where 45 ng of ligand were transported during 25 min in an assay containing 6 × 10^5 cells. This is equivalent to roughly 6.6 × 10^6 molecules per cell. Assuming there are 2 × 10^4 active NPCs per nucleus (Gerace, 1992), this would reflect an average rate of about 1 molecule per NPC per 4 s. This in vitro transport rate obtained with our standard subsaturating concentration of ligand is in the same range as rates determined for in vivo nuclear import in Xenopus oocytes (Goldfarb et al., 1986).

Nonhydrolyzable Analogues of GTP Specifically Inhibit Nuclear Protein Import

We found that addition of nonhydrolyzable GTP analogues to our in vitro nuclear import system resulted in virtually complete inhibition of transport under standard cytosol and cell concentrations (see below). The three analogues that we tested, GMP-PNP, GMP-PCP, and GTPγS were all effective.
Figure 1. Characterization of an improved assay for quantitative analysis of in vitro nuclear import. Transport assays and measurement of transported ligand using an ELISA method were carried out as described in Materials and Methods. Each point represents the average of triplicate transport assays and error bars represent standard deviations. (A) Cytosol dependence of transport. The accumulation of biotinylated BSA-NLS (B-BSA-NLS) in the nucleus as a function of cytosol concentration in the reaction mix was determined after incubation of $4 \times 10^5$ cells per assay for 30 min at 30°C. (B) Time course of transport. In vitro transport was carried out for the indicated times with $6 \times 10^5$ cells per assay and 2.5 mg/ml cytosol. (C) Dependence of transport on ATP, temperature and nuclear localization sequence. All reactions were carried out for 30 min with 2.5 mg/ml cytosol and $4 \times 10^5$ cells. (Standard) 30°C, B-BSA-NLS as transport ligand. (-ATP) addition of 0.1 U hexokinase/5 mM glucose before start of the standard reaction. (0°C) 30 min at 0°C with standard ligand. (Reverse) B-BSA coupled to “reverse” NLS peptides as transport ligand under standard conditions. (B-BSA) Biotinylated BSA as transport ligand under standard conditions.

Figure 2. Nonhydrolyzable analogues of GTP inhibit nuclear protein import. Transport was carried out in the presence of varying amounts of GTP analogues for 30 min with $3 \times 10^5$ cells in the presence of an ATP regenerating system (see Materials and Methods). (A) Inhibition with GTPγS was measured in the presence or absence of 1 mM GTP in the transport mixture. The nucleotides were added to the complete transport mixture on ice immediately before the reaction was started. The final concentrations of GTPγS in the reaction are indicated. (B) The effect of varying amounts of the GTP analogues GMP-PNP and GMP-PCP in the assay were tested. The inhibitors were added to the complete transport mixture on ice immediately before the reaction was started. The difference in the total amount of protein transported in A vs. B is due to different ligand preparations used in the two assays.

in transport inhibition (Fig. 2). This inhibition was specific for guanine nucleotides, as it occurred in the presence of excess ATP (1 mM) but was diminished with an excess of GTP. As shown in Fig. 2 A, half-maximal inhibition was achieved with approximately 10 μM GTPγS in the absence of GTP or with 80 μM GTPγS in the presence of 1 mM GTP. With the other inhibitors, half maximal inhibition was achieved with somewhat higher concentrations, corresponding to ap-
proximately 100 μM GMP-PNP or 100 μM GMP-PCP (Fig. 2 B). The observation that all three analogues inhibited
transport argues against the possibility that thiophosphorylation of ATP led to transport inhibition. Instead these data
suggest that hydrolysis of GTP is involved in nuclear protein import.

The inhibitory effect of GTPγS on nuclear import was also
evident when a fluorescent ligand (allophycocyanin coupled
with synthetic NLS peptides; Adam et al., 1990) was used
to monitor transport. Addition of 200 μM GTPγS to the as-
say greatly reduced nuclear ligand accumulation compared
to a control sample (Fig 3 A). Interestingly, in the presence
of nonhydrolyzable GTP analogues we never observed ac-
cumulation of fluorescent ligand in a rimlike pattern at the
nuclear periphery, which is indicative of ligands docked at
the NPC (Newmeyer and Forbes, 1988; Richardson et al.,
1988). If GTP hydrolysis were required only at a transport
step distal to docking, one would expect accumulation of
ligand in a docked configuration in the presence of GTPγS.

In other experiments, we investigated the effects of GTPγS
on docking by carrying out nuclear import assays at 30°C un-
der conditions of ATP depletion (Newmeyer and Forbes,
1988; Richardson et al., 1988), wherein docked ligands ac-
cumulate at the cytoplasmic surface of the NPC (Newmeyer
and Forbes, 1988; Richardson et al., 1988). In this case, we
found that GTPγS strongly inhibited the rate of accumula-
tion of ligands at the nuclear periphery (data not shown),
supporting the notion that GTP hydrolysis is required at a
transport step prior to or involving ligand docking at the
NPC.

To test whether the effects of GTPγS are trivially due to
disruption of nuclear integrity or wholesale occlusion of
NPCs, we carried out transport in the presence of GTPγS
and one of two different fluorescent dextrans (Figs. 3, B and
C). An ~10-kD dextran, which is small enough to readily
diffuse into the nucleus in vivo, was used to measure
diffusional permeability of the NPC, and an ~150-kD dex-
tran, which is too large to diffuse into an intact nucleus in

Figure 3. GTPγS does not affect nuclear integrity. Transport reactions were carried out in the presence or absence of 200 μM GTPγS
for 30 min using 2 × 10⁴ cells per assay and 2.5 mg/ml cytosol. The cells were then mounted onto coverslips without washing and photographed directly using epifluorescence (left micrograph of each pair) or phase contrast (right micrograph of each pair) optics. (A) Allophycocyanin coupled to NLS-containing peptides was used as the transport ligand. (B) After 15 min of transport (using B-BSA-NLS as the transport ligand) 0.5 mg/ml fluorescein-dextran with a molecular weights of 9,400 D was added to the reaction. (C) After 15 min of transport (using B-BSA-NLS as the transport ligand) 0.5 mg/ml fluorescein-dextran with a molecular weight of 149,000 D was added to the reaction.
Transport Inhibition Is Dependent on Concentrations of Cells and Cytosol

The inhibitory effects of GTPγS on nuclear import were strongly influenced by both cell and cytosol concentrations in the assay (Fig. 4). While low cell concentrations yielded almost complete transport inhibition with GTPγS, higher cell concentrations resulted in markedly reduced or no transport inhibition. As shown in Fig. 4 A, when 1.5 × 10⁶ cells per 40-μl reaction was analyzed, GTPγS yielded virtually complete transport inhibition over a broad range of cytosol concentrations. By contrast, essentially no inhibition was obtained with 6 × 10⁵ cells per 40-μl reaction up to a cytosol concentration of 5 mg/ml. However, further increasing the cytosol concentration in this sample again resulted in transport inhibition. An intermediate concentration of cells in the assay (3 × 10⁵ cell per 40-μl reaction) yielded a level of inhibition intermediate between that obtained with 1.5 × 10⁶ cells and 6 × 10⁵ cells (data not shown; see also Fig. 5). Hence, depending on the concentration of cytosol and cells in the assay, the inhibition can vary from 0 to 95%. This phenomenon was observed with all cytosol and permeabilized cell preparations tested, although it should be emphasized that the concentration of cells required to obtain specific degrees of transport inhibition varied with different cytosols as well as with different permeabilized cell preparations. We presently have no clear understanding of this effect, which could involve complex interactions between nuclear and cytoplasmic pools of GTP-binding proteins and

Figure 4. Inhibition by GTPγS is dependent on concentrations of permeabilized cells and cytosol. The effect of 200 μM GTPγS on in vitro transport was measured as a function of cytosol concentration for 1.5 × 10⁵ cells (A) or for 6 × 10⁵ cells (B) per 40-μl transport reaction. Transport and determination of the amount of ligand transported per assay were otherwise carried out as described in Materials and Methods. As the degree of inhibition obtained by GTPγS varied with the batch of cytosol and also with the preparation of digitonin permeabilized cells, the experiments in this figure were done in parallel using the same materials for A and B.

Figure 5. GTPγS affects the rate of nuclear protein import. Accumulation of ligand over time was measured in the presence or absence of 200 μM GTPγS. 2.5 mg/ml cytosol and 6 × 10⁵ cells were used per assay. The transport was stopped at the indicated times and processed as described in Materials and Methods. As shown in the experiment shown, 6 × 10⁵ cells led to 70% inhibition, while in a similar experiment conducted with a different batch of cytosol the same degree of inhibition and the same shape of the curve was achieved with 3 × 10⁵ cells (data not shown).
A low molecular weight fraction is required for nuclear protein import and enhances the inhibitory effects of GTPγS. (A) A Superose 12 column was used to fractionate cytosol into 13 fractions. Each fraction was then concentrated fourfold (with respect to the initial cytosol concentration) and tested for its effects when added to a standard transport assay in the presence or absence of 200 μM GTPγS. The standard transport assay contained 2.5 mg/ml complete cytosol and 3 × 10⁶ cells, and the amount of each added fraction was equivalent to that derived from 40 μl complete cytosol. (Cytosol) Standard assay without additions; (+13) transport with the standard mixture plus each of the individual concentrated cytosol fractions. The small arrowheads indicate the position of molecular weight standards in the Superose column profile. (B) An independent experiment with a different batch of cytosol was carried out as described above in (A). cytosol or cytosol enriched with fraction 9 (equivalent to 40 μl complete cytosol) was tested in the absence or presence of 200 μM GTPγS. (C) An experiment was carried out with assay samples containing permeabilized cells without complete cytosol but containing the combined Superose fractions 1-12 (+fr.9) or combined fractions 1–8 + 10–12 (−) only. (D) Transport in the absence of GTPγS; (■) transport in the presence of 200 μM GTPγS.

Figure 6. A low molecular weight fraction is required for nuclear protein import and enhances the inhibitory effects of GTPγS. (A) A Superose 12 column was used to fractionate cytosol into 13 fractions. Each fraction was then concentrated fourfold (with respect to the initial cytosol concentration) and tested for its effects when added to a standard transport assay in the presence or absence of 200 μM GTPγS. The standard transport assay contained 2.5 mg/ml complete cytosol and 3 × 10⁶ cells, and the amount of each added fraction was equivalent to that derived from 40 μl complete cytosol. (Cytosol) Standard assay without additions; (+13) transport with the standard mixture plus each of the individual concentrated cytosol fractions. The small arrowheads indicate the position of molecular weight standards in the Superose column profile. (B) An independent experiment with a different batch of cytosol was carried out as described above in (A): cytosol or cytosol enriched with fraction 9 (equivalent to 40 μl complete cytosol) was tested in the absence or presence of 200 μM GTPγS. (C) An experiment was carried out with assay samples containing permeabilized cells without complete cytosol but containing the combined Superose fractions 1-12 (+fr.9) or combined fractions 1–8 + 10–12 (−) only. (D) Transport in the absence of GTPγS; (■) transport in the presence of 200 μM GTPγS.

their regulators. Nevertheless, this observation was crucial for identifying the transport factor sensitive to GTPγS (see below).

Fig. 5 shows the kinetics of ligand accumulation in the nucleus in the presence or absence of GTPγS under conditions which lead to 70% inhibition at 30 min. The transport rate was linear throughout the time course both in the presence or absence of GTPγS, but the rate of transport was strongly reduced in the presence of inhibitor. It is important to note that 70% inhibition was already seen during the first 5 min of the reaction, so there is no apparent lag in the effects of the inhibitor. Hence, GTPγS continuously affects the rate of transport throughout the time course of this assay in a cytosol and permeabilized cell concentration–dependent fashion.

**A Low Molecular Weight Cytosolic Fraction Is Responsible for GTPγS-Mediated Inhibition and Is Required for Transport**

As an approach to characterizing the transport factor inhibited by GTPγS, we examined whether inhibition could be stably maintained after a brief exposure of transport components to the GTP analogue. We preincubated either permeabilized cells or cytosol or a combination of both with GTPγS at 30°C, added an excess of GTP to compete with the inhibitor (see Fig. 2 A), and then compared transport in the pretreated fraction with untreated counterparts. However, no irreversible inhibition was achieved under any conditions tested (data not shown), indicating that GTPγS must be present in the assay throughout the transport incubation to exert inhibitory effects. We therefore explored a second approach for characterizing the GTPγS sensitive factor.

Since a high concentration of cytosol can substantially increase the level of transport inhibition by GTPγS in an assay containing a relatively high cell concentration (Fig. 4 B), we reasoned that cytosol contains a factor responsible for mediating or enhancing GTPγS inhibition in a concentration-dependent manner. To identify this factor, we resolved cytosol into 13 fractions by molecular sieving chromatography, concentrated each fraction fourfold (relative to the initial cytosol) and individually added the fractions to transport mixtures containing complete cytosol in the absence or presence of GTPγS (Fig. 6). The concentration of permeabilized cells in the transport incubation was adjusted to yield about 50% transport inhibition in the presence of GTPγS without added cytosolic fractions (Fig. 6 A, leftmost bars). Addition of most cytosol fractions to the transport assay had no significant effect, either on the transport rate without GTPγS or on the degree of inhibition obtained with GTPγS. A few fractions enhanced (e.g., fraction 8) or diminished (e.g., fraction 6) the basal level of transport in the absence of GTPγS, but the relative degree of inhibition in the presence of GTPγS was similar to the control. A strikingly different result was obtained with fraction 9, which corresponds to the peak of 20–30-kD globular proteins (arrow in Fig. 6). Addition of concentrated fraction 9 did not significantly influence the level of transport in the absence of inhibitor, yet dramatically increased the degree of transport inhibition obtained with GTPγS to >90%. The ability of fraction 9 to strongly enhance the inhibitory effect of GTPγS on transport was highly reproducible. For example, Fig. 6 B presents a separate experiment with a different batch of fractionated cytosol. To determine whether this low molecular weight fraction was also required for transport, we pooled fractions 1–8 + 10–12 and tested the transport activity of this
Figure 7. Bacterially expressed Ran/TC4 is the major component of the low molecular weight fraction required for nuclear import and inhibition by GTPγS. Ran/TC4 was expressed in *E. coli* as described in Materials and Methods and was enriched by ion exchange chromatography and molecular sieving on a Superose 12 column. To control for impurities, a mock preparation was done in parallel from *E. coli* containing the expression vector only without the Ran/TC4 insert. (A) Superose fractions of cytosol were obtained as described in Fig. 6. Fractions 1–8 + 10–12 were tested either alone (buffer) or supplemented with 10–µl fraction 9 (+fr.9), 7 µl purified Ran/TC4 (Ran) or 7 µl mock-purified Ran/TC4 (control), using 3 × 10⁵ cells per assay. (B) Unfractionated cytosol was tested alone (buffer) or supplemented with 10–µl fraction 9 (+fr.9) or 10 µl purified Ran/TC4 (Ran) in the presence or absence of 200 µM GTPγS, using 6 × 10⁵ cells per assay. (C) SDS-PAGE of the mock preparation isolated from *E. coli* (control), the Ran/TC4 preparation (Ran), and fraction 9 (fr.9). 5 µl of each sample was applied to the gel, which was stained with Coomassie blue. (D) Immunoblot analysis of cytosol fractions obtained by molecular sieving on Superose 12 using antibodies against Ran/TC4. (Cytosol) Complete cytosol; (1–12) 7 µl of fractions 1–12 respectively (threefold concentrated over cytosol); (Ran) 7 µl of the bacterially expressed Ran/TC4.

We therefore examined whether either of these two proteins was the component of fraction 9 required for nuclear import. Purified ARF from bovine brain, which was active in an ER to Golgi membrane transport assay (Nuoffer and Balch, 1993), had no effect when added to an assay containing a pool of all cytosol fractions except fraction 9 (data not shown). By contrast, striking results were obtained with purified recombinant Ran/TC4. Addition of Ran/TC4 to the transport assay lacking fraction 9 increased the level of nuclear transport in this sample to the degree obtained with concentrated fraction 9 itself (Fig. 7 A; see also Fig. 6 C). This effect was not due to bacterial impurities in the Ran/TC4 preparation, since a control sample purified from control bacterial lysates and containing all of the minor contaminant bands found in the Ran/TC4 sample (Fig. 7 C, compare Ran lane to control lane) was without effect. We estimate that the amount of recombinant Ran/TC4 added to the transport assays in Fig. 7 is no more than severalfold greater than the amount of endogenous cytosolic Ran based on the relative immunoblot signals given by the two samples (Fig. 7 D). Saturating concentrations of Ran/TC4 were added to the samples in Fig. 7 since up to fourfold lower concentrations of added Ran yielded the same effect (data not shown). This suggests a direct involvement of Ran/TC4 in nuclear import. Supporting this conclusion, the chromatographic peak of cytosolic Ran/TC4 coincides with fraction 9, as determined by immunoblotting with antibodies to Ran/TC4 (Fig. 7 D). The residual level of transport in cytosol lacking fraction 9

pool alone or with added fraction 9 (Fig. 6 C). When fraction 9 was omitted, the transport activity was greatly diminished, demonstrating that some factor(s) in this fraction is required for efficient nuclear import.

The Small GTPase Ran/TC4 Is a GTPγS-Sensitive Transport Factor Involved in Nuclear Protein Import

The data above provided evidence for a 20–30-kD cytosolic protein which was required for nuclear protein import and which enhanced inhibitory effects of GTPγS. It seemed plausible that this component itself was a GTPase, since GTPases of widespread importance are often strongly inhibited by GTPγS. Furthermore, many GTPases occur in this molecular weight range, most notably, GTPases of the Ras superfamily (Bourne et al., 1991). Two small GTPases have been previously suggested to have potential roles in nuclear function, the 25-kD protein Ran/TC4 and the 20-kD protein ARF (ADP ribosylation factor). Ran/TC4 appears to be directly or indirectly involved in a large number of nuclear functions including DNA replication, cell cycle feedback control, and RNA synthesis, processing and export (Dasso, 1993). ARF has been shown to be responsible for inhibition of in vitro nuclear reassembly in the presence of GTPγS (Boman et al., 1992), and also has an extensively characterized role in vesicle budding along the secretory pathway (Pfeffer, 1992; Nuoffer and Balch, 1993).

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is most likely due to the small amount of Ran/TC4 in fractions 8 and 10 which is not resolved by molecular sieving, although we cannot formally exclude the possibility that a low level of nuclear import can occur in the absence of cytosolic Ran/TC4.

In a second experiment, we examined whether Ran/TC4 was responsible for inhibition by GTPγS in this assay (Fig. 7 B). Recombinant Ran/TC4 was added to a transport assay that was adjusted to give a low level of inhibition by GTPγS in the absence of additions. We found that addition of exogenous Ran/TC4 to the system yielded virtually the same degree of transport inhibition as concentrated fraction 9 itself. In conclusion, these data directly demonstrate that Ran/TC4 is a cytosolic factor involved in nuclear import, and that this protein is a major factor involved in inhibition of transport by GTPγS.

Discussion

We have shown that GTPγS and other nonhydrolyzable GTP analogues inhibit the rate of nuclear import in permeabilized cells. We identified the GTPγS-sensitive component as Ran/TC4 and demonstrated that this protein is required for efficient nuclear protein import. It is possible that additional GTPases besides Ran/TC4 are involved in nuclear import, although these are not clearly revealed with our standard assay and inhibition conditions. A requirement for Ran/TC4 in nuclear protein import also was demonstrated with an in vitro system involving Xenopus cytosol, indicating a role in diverse cell types (Moore and Blobel, 1993).

Ran/TC4 was first identified in mammalian cells as a member of the Ras superfamily of GTPases having about 25% identity to Ras (Drivas et al., 1990; Bischoff and Ponstingl, 1991a). It also was genetically defined as a multiplicity suppressor of mutations in yeast RCC1 homologues (see below; Matsumoto and Beach, 1991; Belhumeur et al., 1993; Kadowaki et al., 1993). Ran/TC4 is concentrated in the nucleus of mammalian cells based on immunofluorescence microscopy, but a smaller cytoplasmic pool is also present and is released by permeabilization of cells with digitonin (Ren et al., 1993). The relatively high concentration of Ran/TC4 in the nucleus is presumably due to its interaction with other nuclear components, since monomeric Ran/TC4 is small enough to rapidly diffuse into the cytoplasm. Nevertheless nuclear Ran/TC4 may be in dynamic equilibrium with the cytoplasmic pool, and this equilibrium could be important for its functions. The subcellular distribution of Ran/TC4 could help to explain the variable inhibitory effects of GTPγS related to the concentrations of permeabilized cells and cytosol in the transport assay (Fig. 4) assuming the nuclear and cytoplasmic pools are differentially sensitive to GTPγS. Since there are an estimated 10^7 copies of Ran/TC4 per mammalian cell (Bischoff and Ponstingl, 1991a), recycling of this protein would not be required to drive the levels of transport obtained in our assay conditions (<10^6 molecules per cell), even if Ran/TC4 is a stoichiometric component of the transport apparatus (see below).

Our results on the role of Ran/TC4 in nuclear import are intriguing in light of recent work on Ran/TC4 and the protein RCC1, which is a GNRP for Ran/TC4 (Bischoff and Ponstingl, 1991b). RCC1 was first characterized in the hamster cell line tsBN2 (Nishimoto et al., 1978), which contains a mutant RCC1 protein that is rapidly degraded at the restrictive temperature (Nishitani et al., 1991). TsBN2 cells that are in S phase at the time of the temperature shift enter mitosis without completing DNA replication (Nishimoto et al., 1978), leading to the view that RCC1, which is a chromatin bound nuclear protein, is part of the checkpoint control mechanism for preventing entry into mitosis until completion of DNA replication (reviewed by Dasso, 1993). A homologue of RCC1 with a similar mutant phenotype (piml) was identified in a genetic screen in Schizosaccharomyces pombe (Matsumoto and Beach, 1991). RCC1 has been linked to a large number of additional functions, based on genetic and biochemical studies of RCC1 homologues in S. cerevisiae and vertebrate cells (Aebi et al., 1990; Clark et al., 1991; Dasso et al., 1992; Forrester et al., 1992; Kadowaki et al., 1993; Amberg et al., 1993). These include a role in RNA synthesis, processing, and export as well as in DNA replication. Recent functional studies indicate that mutations in Ran/TC4 can result in a deficiency in DNA replication (Ren et al., 1993), similar to RCC1 mutants. Hence, the Ran/TC4-RCC1 system is directly or indirectly associated with a large number of processes underlying nuclear function.

The results on Ran/TC4 described in this study in combination with previous work indicate that the Ran/TC4-RCC1 system can integrate numerous nuclear activities (Fig. 8). The functional state of the cell (related to DNA replication and possibly other processes) is thought to influence the GNRP activity of RCC1 and perhaps other GNRFs (Dasso, 1993), and probably affects the activity of hypothetical Ran/TC4-GAPs as well. This combination of GNRP and GAP activities would determine the relative amounts of Ran/TC4-GTP and Ran/TC4-GDP in the cell, and thereby the activity of the Ran/TC4 system. We assume that in analogy with other GTPases (Bourne et al., 1991) the active form...
of Ran/TC4 is GTP-bound. According to one scenario (pathway 1 in Fig. 8), active Ran/TC4 could interact with only a single target in the cell, which would be associated with the nuclear import machinery. As such, Ran/TC4 could serve as a stoichiometric regulator of nuclear import (similar to the role of EF-Tu in protein synthesis; Bourne et al., 1991). When the Ran/TC4 system is relatively inactive, the rate of nuclear import would decrease. This could indirectly lead to inhibition of DNA replication and defects in cell cycle checkpoint control due to inappropriate concentrations of regulatory molecules in the nucleus compared to the cytoplasm.

In a second scenario (pathway 2 in Fig. 8) active Ran/TC4 could interact with multiple targets in the cell either through a signaling system (e.g. a protein kinase) or through multiple distinct effectors. In this fashion Ran/TC4 could coordinate or regulate the nuclear import machinery and other functional components, such as the DNA replication machinery and the p54nck kinase system.

The observed effects of the Ran/TC4-RCC1 on RNA export could be direct, and Ran/TC4 could affect the nuclear export machinery in a fashion similar to the import machinery. This is compatible with the notion that at least some steps of both nuclear import and export (e.g., channel gating) are likely to be mechanistically similar (Gerace, 1992). Alternatively, the effects of Ran/TC4-RCC1 on RNA export could be a consequence of deficiencies in nuclear import. This possibility is consistent with the observation that numerous RNA binding proteins continuously “shuttle” between nucleus and cytoplasm (Borger et al., 1989; Meier and Blobel, 1992; Pinol-Roma and Dreyfuss, 1992). Trapping these shuttling proteins in the cytoplasm due to deficient nuclear import could have profound effects on nuclear RNA metabolism and export.

By analogy with other GTPases (Bourne et al., 1991; Nuoffer and Balch, 1993), we predict that the active form of Ran/TC4 affecting nuclear import is the GTP-bound form. The inhibitory effects of GTPγS in our nuclear import assay are compatible with either pathway 1 or pathway 2 in Fig. 8, assuming that the functions of Ran/TC4 require hydrolysis of bound GTP. If Ran/TC4 is involved in nuclear import according to pathway 1, GTP hydrolysis most likely would occur at a specific step in transport. Conversely, if Ran/TC4 functions according to pathway 2, then multiple components and steps involved in nuclear import could be affected. We speculate that a transport step prior to or involving ligand docking at the NPC requires GTP hydrolysis, since ligands do not accumulate in a docked configuration at the NE when transport is inhibited by GTPγS (this study) or GMP-PNP (Moore and Blobel, 1993). Furthermore, the rate of accumulation of fluorescent transport ligands at the NE is strongly diminished by GTPγS under standard docking conditions of ATP depletion. Whether the GTPγS sensitive step proximal to docking involves Ran/TC4 or another unidentified GTPase remains to be established.

We are especially grateful to Sandy Schmid, who suggested the design of the ELISA-based assay for quantitating nuclear import and who provided helpful insight throughout the course of this work. We also wish to thank Peter D'Eustachio, who generously provided the cDNA clone for Ran/TC4 and polyclonal antibodies that recognize the protein. Finally, we thank Dave Byrd for help in figure preparation, Mary Keeter for secretarial assistance, Sandy Schmid and Rohit Mahajan for helpful comments on the manuscript, and members of our laboratory for numerous stimulating discussions.

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