

Nucleocytoplasmic Recycling of the Nuclear Localization Signal Receptor α Subunit In Vivo Is Dependent on a Nuclear Export Signal, Energy, and RCC1

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Abstract. Nuclear protein import requires a nuclear localization signal (NLS) receptor and at least three other cytoplasmic factors. The α subunit of the NLS receptor, Rag cohort 1 (Rch1), enters the nucleus, probably in a complex with the β subunit of the receptor, as well as other import factors and the import substrate. To learn more about which factors and/or events end the import reaction and how the import factors return to the cytoplasm, we have studied nucleocytoplasmic shuttling of Rch1 in vivo. Recombinant Rch1 microinjected into Vero or tsBN2 cells was found primarily in the cytoplasm. Rch1 injected into the nucleus was rapidly exported in a temperature-dependent manner. In contrast, a mutant of Rch1 lacking the first 243 residues accumulated in the nuclei of Vero cells after cytoplasmic injection. After nuclear injection, the truncated Rch1 was retained in the nucleus, but either Rch1 resi-

dues 207–217 or a heterologous nuclear export signal, but not a mutant form of residues 207–217, restored nuclear export. Loss of the nuclear transport factor RCC1 (regulator of chromosome condensation) at the nonpermissive temperature in the thermosensitive mutant cell line tsBN2 caused nuclear accumulation of wild-type Rch1 injected into the cytoplasm. However, free Rch1 injected into nuclei of tsBN2 cells at the nonpermissive temperature was exported. These results suggested that RCC1 acts at an earlier step in Rch1 recycling, possibly the disassembly of an import complex that contains Rch1 and the import substrate. Consistent with this possibility, incubation of purified RanGTP and RCC1 with NLS receptor and import substrate prevented assembly of receptor/substrate complexes or stimulated their disassembly.

NUCLEAR protein import in eukaryotic cells occurs through openings in the nuclear envelope that are spanned by nuclear pore complexes (NPC)¹ (Forbes, 1992; Rout and Wentz, 1994) and is characterized by an energy requirement, signal dependence, and receptor involvement. In vitro assays using semipermeabilized cells have allowed the identification of the soluble factors essential for nuclear protein import (Adam et al., 1990; Newmeyer and Forbes, 1990; Moore and Blobel, 1992), and studies of their interactions with each other and with nuclear pore proteins have led to models of the protein import pathway that suggest several distinct steps. In the cytoplasm, the α subunit of the karyopherin heterodimer,

also called importin α , recognizes the nuclear localization signal (NLS) (for review see Dingwall and Laskey, 1991; Garcia-Bustos et al., 1991) of a karyophilic protein in a temperature- and hence energy-independent manner. Subsequently the karyopherin β subunit mediates docking of the complex to the cytoplasmic periphery of the nuclear pore (Adam and Adam, 1994; Görlich et al., 1994, 1995a; Imamoto et al., 1995a,b; Moroianu et al., 1995a,b; Radu et al., 1995; Weis et al., 1995). Ran, a member of the Ras superfamily of guanine nucleotide binding proteins, binds in its GTP form to RanBP2 close to this initial docking site (Melchior et al., 1995). GTP hydrolysis of RanGTP, which has been shown to be necessary for translocation of the import substrate (Melchior et al., 1993; Moore and Blobel, 1993; Schlenstedt et al., 1995), occurs at the same site and probably allows the undocking of the karyopherin/substrate complex. This GTP hydrolysis is stimulated by RanGAP1 at the nuclear envelope (Bischoff et al., 1994, 1995a,b; Corbett et al., 1995; Matunis et al., 1996; Mahajan et al., 1997). Possible links between Ran and the karyopherin/substrate complex are pp15 (Grundmann et al., 1988; Moore and Blobel, 1994; Paschal and Gerace, 1995;

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1. *Abbreviations used in this paper:* aa, amino acid(s); IBB domain, importin- β -binding domain; MBP, maltose binding protein; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; RCC1, regulator of chromosome condensation 1; Rch1, Rag cohort 1.

Nehrbass and Blobel, 1996; Paschal et al., 1996) and RanBP1 (Chi et al., 1996). The import complex is proposed to move to the immediate vicinity of the nuclear pore before translocation into the nucleus via several binding sites for pp15 and karyopherin β (Moroianu et al., 1995b; Paschal and Gerace, 1995; Radu et al., 1995; Nehrbass and Blobel, 1996).

It is likely that all of the soluble transport factors mentioned above enter the nucleus in a complex with the cargo protein, although this has not been demonstrated directly. While karyopherin β remains bound to the nuclear side of the NPC and is very rapidly reexported (Görlich et al., 1995b; Moroianu et al., 1995b), the transport substrate, karyopherin α , RanGDP, and pp15 appear to enter the nucleoplasm. However, it is not known how or when the dissociation of the import complex is induced. Considering that directionality must be maintained during transport, dissociation may occur solely as the termination step of the import reaction in the nucleus (Melchior and Gerace, 1995; Görlich and Mattaj, 1996). Alternatively, reiterated dissociation and association reactions may occur during translocation through the pore (Rexach and Blobel, 1995; Görlich et al., 1996b). Recent data show that RanGTP destabilizes the $\alpha\beta$ -heterodimer of karyopherin (Rexach and Blobel, 1995). Exchange of GDP for GTP on Ran in the nucleus could therefore induce the dissociation of the import complex. The now free import substrate would be retained in the nucleus by association with, for example, DNA or chromatin, while the import factors could be recycled. The GDP/GTP exchange factor for Ran in the nucleus is RCC1 (regulator of chromosome condensation) (Bischoff and Ponstingl, 1991a,b; for review see Dasso, 1993), first identified as the mutant gene in tsBN2 cells, a thermosensitive mutant line derived from BHK21 cells (Nishimoto and Basilico, 1978; Nishimoto et al., 1978).

The mechanism by which the NLS receptor subunits are returned to the cytoplasm after completion of the import reaction is still unknown. Export of karyopherin β is faster and therefore apparently distinct from that of karyopherin α (Görlich et al., 1995b; Moroianu et al., 1995b). Both subunits are too large to return to the cytoplasm by simple diffusion and must migrate against a concentration gradient since the NLS receptor resides mostly in the cytoplasm. These observations suggest that they probably require active reexport. It is also clear that the mechanism of nuclear exit of karyopherin α must be different from its entry, as it returns to the cytoplasm without the NLS-bearing protein. Consistent with this, the importin β -binding domain (IBB domain) of karyopherin α is responsible for nuclear entry of the NLS receptor α subunit but does not mediate its export (Görlich et al., 1996a; Weis et al., 1996).

To study the requirements for nucleocytoplasmic shuttling of the NLS receptor α subunit in vivo, we used two forms of this protein, Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉. Both were originally identified through their interaction with the RAG-1 recombination-activating protein (Cuomo et al., 1994) and have been shown to interact directly and specifically with NLSs (Cuomo et al., 1994; Gallay et al., 1996). Rch1₃₃₋₅₂₉ was reported to substitute for karyopherin α 1 in an in vitro nuclear import assay (Moroianu et al., 1995b), whereas Rch1₂₄₄₋₅₂₉ inhibited nuclear transport of HIV-1 matrix protein when coexpressed in human 293 cells with

HIV-1 matrix protein cDNA (Gallay et al., 1996) and also inhibited RAG-1-mediated recombination in vivo (Cuomo et al., 1994). The mechanism of this inhibition is not known.

We show here that recycling of Rch1 to the cytoplasm is dependent on a nuclear export signal within residues 207 and 217 and on temperature. Loss of RCC1 activity leads to accumulation of the receptor in the nuclei of microinjected tsBN2 cells, although RCC1 is not necessary for the export reaction itself. We propose that the RCC1-catalyzed exchange of GDP for GTP on Ran is necessary to destabilize or disassemble the import complex and is therefore required for recycling of karyopherin α to the cytoplasm.

Materials and Methods

Plasmids and Proteins

Bacterial expression constructs for human Rch1 were derived by subcloning the Rch1 cDNAs coding for amino acids (aa) 33–529 or aa 244–529 (Cuomo et al., 1994) from a pET21a vector into pET19a using NdeI and XhoI sites from the pET19a multiple cloning site. The resulting fusion constructs contained NH₂-terminal T7- and His-tags. The Rch1 cDNA encoding for aa 185–529 (Cuomo et al., 1994) was cloned from a yeast expression vector (Cuomo et al., 1994) into the pET21a vector using EcoRI and XhoI restriction sites and subsequently into the pET19a vector using NdeI and XhoI restriction, resulting in a construct analogous to that for aa 33–529 or aa 244–529. For nuclear export signal (NES)-Rch1 constructs, the following oligonucleotides were used: 5'-TAT GCT CCC TCC TCT AGA ACG ACT CAC GCT ACA and 5'-TAT GTA GCG TGA GCT GTT CTA GAG GAG GGA GCA for *HIVNES-Rch1*₂₄₄₋₅₂₉; 5'-TAT GGC AGT GGA TCC ACT GTT GGC TCT CCT TGC AGT TCC TCA and 5'-TAT GAG GAA CTG CAA GGA GAG CCA ACA GTG GAT CCA CTG CCA for *Rch1NES-Rch1*₂₄₄₋₅₂₉; 5'-TAT GGC AGT GGA TCC ACT GGC AGC TGG ACT TGC AGT TCC TCA and 5'-TAT GAG GAA CTG CAA GTC CAG CTG CCA GTG GAT CCA CTG CCA for *Rch1NES-Rch1*₂₄₄₋₅₂₉. They were constructed by annealing the two complementary oligonucleotides and cloning them into the NdeI site of pET19a containing the Rch1₂₄₄₋₅₂₉ cDNA. Correct orientation of the inserted fragment was verified by DNA sequencing. The karyopherin β cDNA in pET30a (Chi et al., 1995) was a generous gift from S. Adam (Northwestern University, Chicago, IL). Ran wt cDNA (Ren et al., 1993) was cloned from pET9c into pET15a using NdeI and BamHI restriction. The RCC1 cDNA (Ohtsubo et al., 1987) was cloned from pET3b (T. Dobner, University of Regensburg, Regensburg, Germany) into the pET15a vector via NdeI and BamHI restriction sites.

Maltose binding protein (MBP)-Rch1 fusion constructs were obtained by PCR amplifying DNA encoding Rch1 residues 197–221 with the primers 5'-TTT GAA TTC GAC TTG GTT ATT AAG TAC GGT G and 5'-TTT TCT AGA TAA AGA TGA CAT ATC AGG AAC TG and residues 218–244 using the primers 5'-TTT GAA TTC ATG TCA TCT TTA GCA TGT GGC and 5'-TTT TCT AGA GGT GCA GGA TTC TTG TTG C. The cDNA encoding Rch1₃₃₋₅₂₉ in pET19 served as the template. The PCR fragments were cloned into the pMal-Vector (New England Biolabs, Beverly, MA) using EcoRI and XbaI restriction sites. The DNA fragments were sequenced and plasmids containing the correct sequence were transformed into BL21 bacteria for expression.

SV40 T-antigen was purified from insect cells as described by Schneider et al. (1994).

Bacterial Expression and Purification of Proteins

To purify nearly full-length (aa 33–529) and truncated (aa 244–529) Rch1 peptides, Ran, RCC1, and karyopherin β , the corresponding pET plasmids were transformed into *Escherichia coli* BL21 (DE3) containing the pLys plasmid. Bacteria were grown in 1000 ml Luria-Bertani broth to an OD₆₀₀ of 0.7 at 30°C (37°C for Ran wt and RCC1) and expression was induced by addition of 2 mM IPTG for 3 h. Cells were harvested by centrifugation and frozen in 0.2 M Tris-HCl, pH 8.0, 0.5 M NaCl, 20 μ g/ml leupeptin, 2 mM PMSF, and 1 mg/ml aprotinin. Cells were thawed at 37°C and disrupted by ultrasonication after a 15-min incubation at 4°C. To the cleared supernatant of the cell lysates, imidazole, pH 7.6, was added to a final concentration of 30 mM and the solution was loaded on 0.5 ml Ni-

NTA-Sepharose (Qiagen, Chatsworth, CA) equilibrated in 0.5 M NaCl and 30 mM imidazole, pH 7.6. After extensive washing with 30 and 60 mM imidazole, pH 7.6, and 0.5 M NaCl, proteins were eluted by step elution with 0.5 M imidazole, pH 7.0, and 0.5 M NaCl, and pooled fractions were dialyzed overnight against 20 mM Hepes/KOH, pH 7.6 (Rch1 derivatives, Ran, karyopherin β), or 20 mM Tris-HCl, pH 7.6 (RCC1), 10% glycerol, and 5 mM mercaptoethanol.

Rch1₁₈₅₋₅₂₉, Rch1NES-Rch1₂₄₄₋₅₂₉, Rch1nes-Rch1₂₄₄₋₅₂₉, and HIVNES-Rch1₂₄₄₋₅₂₉ were purified as insoluble proteins. pET19 plasmids containing the corresponding genes were transformed into BL21 (DE3). Expression of the proteins, harvesting, and disruption of the cells were performed as described above. The cell suspension was centrifuged, and proteins were solubilized from the cell pellet in 8 M urea according to Podust et al. (1995). Solubilized proteins were dialyzed to remove the urea, and the protein-containing solution was applied to Ni-NTA-agarose as described above. The eluate was adjusted to 5% glycerol and then dialyzed against 20 mM Hepes/KOH, pH 7.6, 10% glycerol, and 5 mM mercaptoethanol.

MBP fusion proteins were expressed and purified according to a New England Biolabs protocol (1994). After purification, proteins were dialyzed against 20 mM Hepes/KOH, pH 7.6, 10% glycerol, and 5 mM mercaptoethanol.

Cell Culture

BHK21 (Stoker and MacPherson, 1964), tsBN2 cells, a temperature-sensitive mutant line derived from BHK21 cells (Nishimoto and Basilico, 1978), and Vero cells (Earley and Johnson, 1988) were grown in DME (GIBCO BRL, Gaithersburg, MD) supplemented with antibiotics, 10% FCS (5% for BHK21 cells) (GIBCO BRL), and (for Vero cells) nonessential amino acids (GIBCO BRL) in a humidified incubator at 37°C (Vero) or 33.5°C (tsBN2 and BHK21) under a 10% CO₂ atmosphere.

Microinjection

For microinjection experiments, cells were plated for at least 24 h (Vero) or 36 h (tsBN2 and BHK21) before microinjection on glass coverslips. A microinjector and a manipulator (models 5246 and 5171, respectively; Eppendorf Scientific, Inc., Madison, WI) mounted on an inverted microscope (model IM35; Carl Zeiss, Oberkochen, Germany) were used to deliver samples. All proteins were used at a concentration of 2–4 mg/ml and the solutions were centrifuged for 30 min at 14,000 *g* before injection. Microinjection needles were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) on an automatic pipette puller (Zeitz Instruments, Augsburg, Germany).

Immunofluorescent Staining

Microinjected cells were washed three times with PBS, fixed in 4% ice-cold paraformaldehyde in PBS for 15 min, permeabilized for 15 min in 0.2% Triton X-100, and blocked for 1 h in 10% FCS in PBS. T7-tagged Rch1 proteins were visualized by staining for 2 h at room temperature with a mouse monoclonal anti-T7 antibody (Novagen Corp., Madison, WI) at a 1:500 dilution and a Cy3-linked secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:200 for 1 h at room temperature. MBP fusion proteins were detected with a polyclonal anti-MBP serum (New England Biolabs) at a 1:100 dilution followed by a Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at 1:200 for 1 h at room temperature. The coverslips were mounted in 90% glycerol containing 0.1 mg/ml paraphenylenediamine in PBS (Johnson and Araujo, 1981) and viewed on a fluorescence microscope (model Axiovert 35; Carl Zeiss) using a 63 \times objective.

Quantification

Quantification was done by capturing images of fluorescent cells with a digital camera (CCD camera, model C 4880; Hamamatsu Phototronics, Bridgewater, NJ). The amount of fluorescence in each fixed cell or in the nucleus of the cell was evaluated using the Image-/MetaMorph Imaging System (Universal Imaging Corp., West Chester, PA). Background measurements were obtained by evaluating the fluorescence of noninjected fixed cells or nuclei.

In Vitro Assembly of NLS Receptor–Import Substrate Complexes

Assembly of complexes was performed according to an ELISA protocol

(Goding, 1983). ELISA immunoplates (Nunc, Rochester, NY) were coated with 2 μ g of karyopherin β in 50 μ l of PBS for 2 h at room temperature, followed by saturation of residual nonspecific binding sites with 3% BSA in PBS for 1 h at room temperature. The soluble proteins were added in the following amounts: 1 μ g of Rch1₃₃₋₅₂₉, 1 μ g SV-40 T-antigen, 2 μ g of Ran (RanGDP, RanGTP, or RanGTP- γ S), and 2 μ g of RCC1, as indicated in the figure legend. Additionally, the corresponding nucleotides were present at 2 mM. The binding reaction was performed for 2 h at room temperature in binding buffer (Bischoff and Ponstingl, 1991b) (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, and 5 mM MgCl₂). The plates were washed three times briefly and three times for 10 min each, and either anti-T-antigen antibody Pab101 (Gurney et al., 1980) (1 μ g/well in 50 μ l of PBS) or anti-T7 antibody (0.1 μ l/well in 50 μ l of PBS) was added and incubated overnight, followed by washing of the plates as above and incubation with 1 μ l/well peroxidase-coupled anti-mouse secondary antibody (Jackson ImmunoResearch) in 100 μ l of PBS for 1 h at room temperature. Plates were washed again and the ELISA was developed by adding 100 μ l of peroxidase substrate (50 mM potassium phosphate, pH 5.7, 0.03% H₂O₂, 2 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma Chemical Co., St. Louis, MO) for 5 min. The absorbance of the reaction product was measured at 405 nm using a reference filter at 595 nm in an ELISA reader (model MR5000; DYNEX Technologies, Chantilly, VA).

Results

Subcellular Localization of Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉

To determine the subcellular localization of Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉, each protein was microinjected into the cytosol of Vero cells. The cells were either fixed immediately after injection or after a 2.5-h incubation at 37°C. The exogenous Rch1 was visualized by staining its T7-tag with anti-T7 antibodies. As shown in Fig. 1 *A, a* and *b*, Rch1₃₃₋₅₂₉ was localized predominantly in the cytoplasm at both time points. Although cells injected with Rch1₂₄₄₋₅₂₉ showed cytoplasmic staining immediately after injection (Fig. 1 *A, c*), strong nuclear staining was observed 2.5 h later (Fig. 1 *A, d*). The time course of nuclear uptake of the two proteins was followed by injecting cells with either Rch1₃₃₋₅₂₉ or Rch1₂₄₄₋₅₂₉, fixing them at various time points as indicated (Fig. 1 *B*), and determining the amount of nuclear fluorescence as a percentage of the total cell fluorescence in the injected cells. The graph shows that the fraction of nuclear Rch1₃₃₋₅₂₉ remained at \sim 20% over a period of 2.5 h, consistent with the predominantly cytoplasmic importin α observed in *Xenopus* cells (Görlich et al., 1996a). However, nuclear staining of Rch1₂₄₄₋₅₂₉ increased slowly from 12 to 38% over the same period of time. Since nuclear protein import took place over the entire 2.5-h period, these results suggest that Rch1₃₃₋₅₂₉ was rapidly recycled to the cytoplasm, but Rch1₂₄₄₋₅₂₉ was not.

Export of Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉

To address the question of whether nuclear accumulation of Rch1₂₄₄₋₅₂₉ could be due to a defect in recycling of this protein to the cytoplasm, export of Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉ was examined more closely. The purified proteins were microinjected into the nuclei of Vero cells, and the cells were fixed either immediately after injection or after a 30-min incubation at 37°C. Fig. 2 *A* shows that cells microinjected with Rch1₃₃₋₅₂₉ showed almost exclusively nuclear staining initially (Fig. 2 *A, a*) and strong cytoplasmic staining after a 30-min incubation (Fig. 2 *A, b*). In sharp contrast, the staining pattern of cells injected with Rch1₂₄₄₋₅₂₉ was nuclear at both time points (Fig. 2 *A, c* and *d*). Analysis of

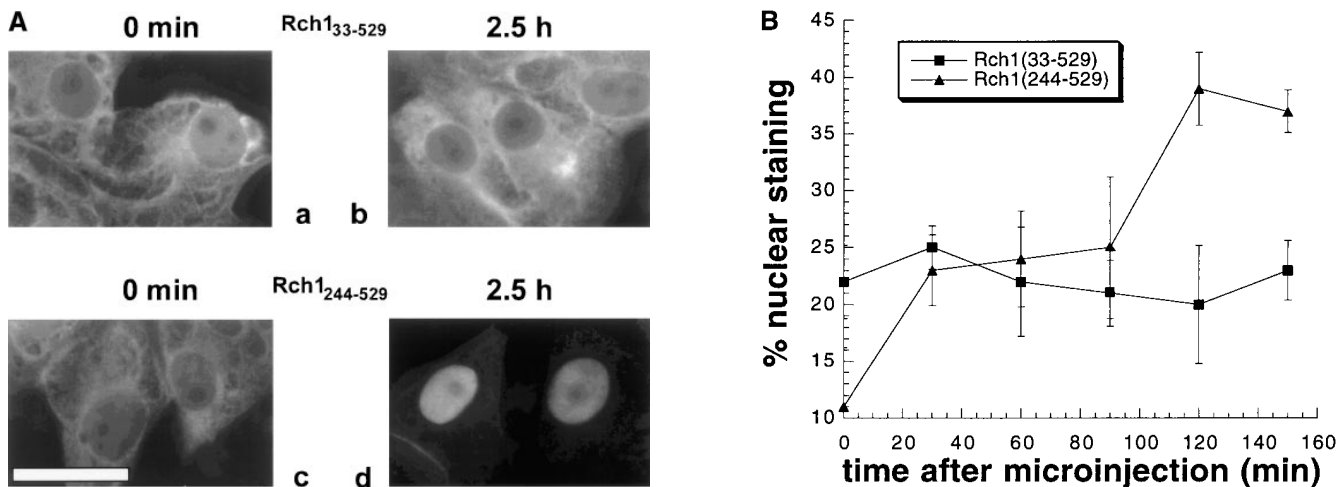


Figure 1. (A) Rch1₂₄₄₋₅₂₉ accumulates in the nuclei of Vero cells in vivo. Vero cells were injected into the cytoplasm with either Rch1₃₃₋₅₂₉ (a and b) or Rch1₂₄₄₋₅₂₉ (c and d) and fixed immediately (a and c) and 2.5 h (b and d) after injection. Immunofluorescence was performed using an antibody against the T7-tag of the extracellular Rch1 proteins and a Cy3-coupled secondary antibody. Micrographs were taken with a digital camera mounted on a fluorescence microscope. (B) Time course of nuclear accumulation of Rch1₂₄₄₋₅₂₉. Vero cells were injected either with Rch1₃₃₋₅₂₉ or Rch1₂₄₄₋₅₂₉ and fixed at various time points as indicated on the x-axis. Cells were immunostained, and the amount of Rch1 fluorescence was measured as described in Materials and Methods. The staining of nuclear Rch1 is expressed as the percentage of total cell fluorescence residing in the nucleus and plotted against time after microinjection. For each time point, the average value obtained from 10–20 cells in two to three independent experiments is shown; the standard deviation of the mean is indicated by error bars. Bar, 25 μ m.

the Rch1 export kinetics (Fig. 2 B) demonstrated a rapid decrease of nuclear fluorescence in cells injected with Rch1₃₃₋₅₂₉. However, the amount of nuclear Rch1₂₄₄₋₅₂₉ fluorescence remained constant over the entire time period (Fig. 2 B). These observations indicate that Rch1₃₃₋₅₂₉ injected into the nuclei of Vero cells was rapidly exported, whereas Rch1₂₄₄₋₅₂₉ was not. The results also suggest that a polypeptide sequence between aa 1 and 244 of Rch1 may be necessary to allow nuclear export of Rch1.

Another Rch1 deletion mutant, Rch1₁₈₅₋₅₂₉, was used to narrow down the region that might contain the information necessary for nuclear export of the NLS receptor subunit. Rch1₁₈₅₋₅₂₉ was injected into the nuclei of Vero cells, and the cells were fixed and stained either immediately af-

ter injection or after a 30-min incubation at 37°C. Fig. 3 A shows that shortly after injection, Rch1₁₈₅₋₅₂₉ was still in the nucleus of injected cells (Fig. 3 A, a), whereas 30 min later, translocation to the cytoplasm had occurred (Fig. 3 A, b). Comparison of the export kinetics of Rch1₁₈₅₋₅₂₉ and Rch1₃₃₋₅₂₉ (Fig. 3 B) confirmed that the rate of export of Rch1₁₈₅₋₅₂₉ was comparable to that of Rch1₃₃₋₅₂₉. These results support the idea that the sequence between aa 185 and 244 of Rch1 is necessary to direct nuclear export of Rch1.

Rch1 Contains a Nuclear Export Signal

To examine the possibility that a nuclear export signal

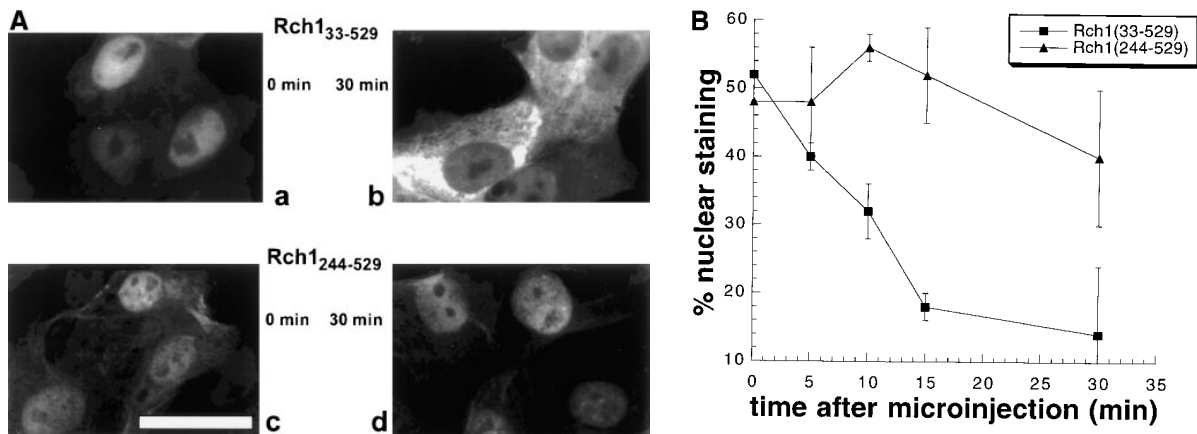


Figure 2. (A) Rch1₂₄₄₋₅₂₉ is defective in nuclear export. Rch1₃₃₋₅₂₉ (a and b) and Rch1₂₄₄₋₅₂₉ (c and d) proteins were injected into nuclei of Vero cells. Fluorescence micrographs show cells fixed and stained 0 min (a and c) or 30 min (b and d) after injection. (B) Export kinetics of Rch1. Vero cells were injected into the nucleus with either Rch1₃₃₋₅₂₉ or Rch1₂₄₄₋₅₂₉ and incubated for the indicated time periods. Quantification was performed as in Fig. 1 B. Bar, 25 μ m.

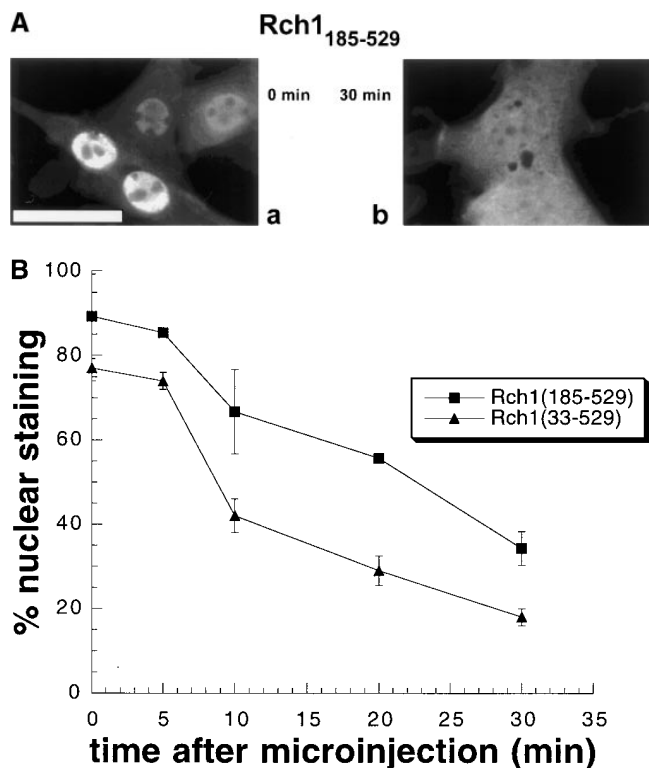


Figure 3. (A) Rch1₁₈₅₋₅₂₉ is efficiently exported from the nucleus. Rch1₁₈₅₋₅₂₉ was injected into the nuclei of Vero cells, which were fixed and immunostained 0 min (a) or 30 min (b) later. (B) Cells were injected into the nucleus with either Rch1₁₈₅₋₅₂₉ or Rch1₃₃₋₅₂₉ to compare export kinetics of the two proteins. After injection, cells were fixed and stained. Nuclear fluorescence was determined and plotted against time after microinjection as in Fig. 1 B. Bar, 25 μ m.

could reside in the region between amino acids 185–244, the cDNA sequences of the mouse, human, *Xenopus*, and yeast NLS receptor α subunit were first compared and screened for conserved leucines in a hydrophobic environment, a motif common to several nuclear export sequences (Gerace, 1995; Nigg, 1997). The region between aa 207 and 217 met these characteristics: **DPL***PLL***LALL**AVPD. The amino acids shown in bold are conserved and amino acids shown in italics are similar among the species mentioned above.

To determine which sequence in the region between aa 185–244 was necessary to direct nuclear export of Rch1, two DNA fragments from this region were chosen, one including the putative NES (coding for aa 197–217) and a second, downstream from the putative NES (coding for aa 218–244). The fragments were obtained by PCR and cloned into the pMal-vector to allow expression of a fusion construct between the MBP, a 42-kD bacterial protein, and the Rch1 cDNA fragment.

The proteins MBP₁₉₇₋₂₁₇ and MBP₂₁₈₋₂₄₄ were then injected into the nuclei of BHK21 cells, and the cells were fixed at different time points after injection to monitor the export reaction. As a control, MBP was also injected. Immunofluorescence was performed using a polyclonal anti-MBP serum and a secondary antibody coupled to Cy3.

The amount of nuclear fluorescence was measured using quantitative fluorescent microscopy.

The fluorescent micrographs (Fig. 4 A) show that both MBP and MBP₂₁₈₋₂₄₄ were nuclear at the 0-min (Fig. 4 A, a and c) and the 10-min (Fig. 4 A, b and d) time points. In contrast, cells injected with MBP₁₉₇₋₂₁₇ showed nuclear staining only at the 0-min time point (Fig. 4 A, e) and cytoplasmic staining after a 10-min incubation (Fig. 4 A, f). The export kinetics shown in Fig. 4 B confirm that neither MBP nor MBP₂₁₈₋₂₄₄ were exported over the time period monitored. However, MBP₁₉₇₋₂₁₇ was very rapidly exported from the nucleus to the cytoplasm. We therefore conclude that the amino acids 197–217, encompassing the putative NES, were sufficient to direct nuclear export of MBP.

In another set of experiments, the putative nuclear export signal was directly investigated. A cDNA fragment coding for amino acids 207–217 was cloned into the Rch1₂₄₄₋₅₂₉ pET19-vector, fusing residues 207–217 to the amino terminus of Rch1₂₄₄₋₅₂₉ and resulting in RchINES-Rch1₂₄₄₋₅₂₉. Similarly, a mutant form of the putative NES with the conserved leucines 110 and 112 exchanged for alanine and glycine (L110A, L112G) was fused to the amino terminus of Rch1₂₄₄₋₅₂₉, resulting in RchInes-Rch1₂₄₄₋₅₂₉. As a positive control for this experiment, sequences encoding the NES of the HIV Rev protein were cloned into the same site of Rch1₂₄₄₋₅₂₉. It was shown earlier that the Rev NES, when coupled to BSA, promoted its export after injection of NES-BSA into *Xenopus* oocytes or mammalian cell nuclei (Fischer et al., 1995).

Nuclear export of RchINES-Rch1₂₄₄₋₅₂₉, RchInes-Rch1₂₄₄₋₅₂₉, and HIVNES-Rch1₂₄₄₋₅₂₉ was then assayed after injection into nuclei of Vero cells. Cells were fixed either at a 0-min time point or 30 min later, followed by immunofluorescent staining of the exogenous protein. The fluorescent micrographs in Fig. 5 A demonstrate that cells injected with either RchINES-Rch1₂₄₄₋₅₂₉ or HIVNES-Rch1₂₄₄₋₅₂₉ showed nuclear staining immediately after injection (Fig. 5 A, a and c) and cytoplasmic staining after a 30-min incubation (Fig. 5 A, b and d), but cells injected with RchInes-Rch1₂₄₄₋₅₂₉ showed nuclear staining at both time points (Fig. 5 A, e and f). To monitor the time course of the export reaction, cells were fixed at different time points after injection, and immunofluorescence was performed using the anti-T7 antibody. The amount of nuclear fluorescence was determined and plotted against time after microinjection. As a control, Rch1₂₄₄₋₅₂₉ was also injected. The export kinetics in Fig. 5 B show that although Rch1₂₄₄₋₅₂₉ and RchInes-Rch1₂₄₄₋₅₂₉ were not exported, RchINES-Rch1₂₄₄₋₅₂₉ was exported at a rate very similar to that of HIVNES-Rch1₂₄₄₋₅₂₉. These results demonstrate that export of the deletion mutant was restored not only by the established nuclear export signal of HIV-Rev (Fischer et al., 1995), but also by amino acids 207–217 of Rch1, indicating that they function as a nuclear export signal. Leucines 110 and 112 of Rch1 appear to be necessary for this function.

Export of Rch1 Is Temperature Dependent

If export of Rch1 from the nucleus depends on the nuclear export signal that is deleted in Rch1₂₄₄₋₅₂₉, one might also expect the export process, like the nuclear import process, to be temperature dependent. To address this question, we

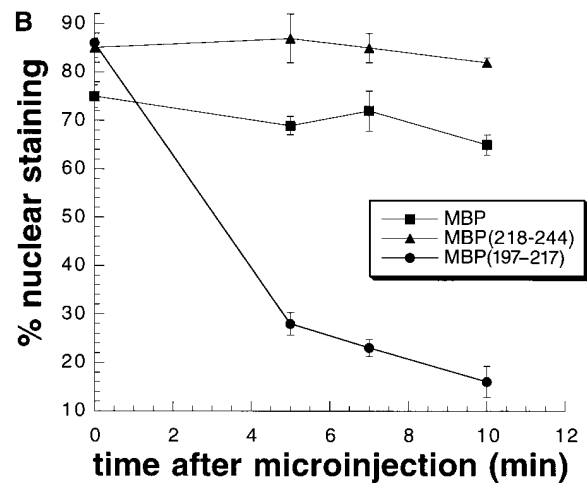
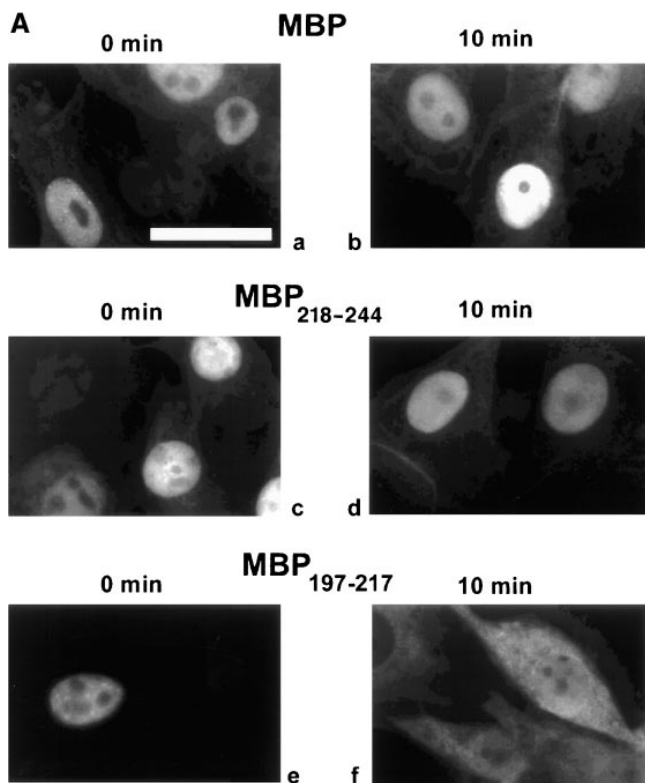


Figure 4. (A) Export of MBP-Rch1 fusion constructs. BHK21 cells were injected into the nucleus with purified proteins MBP (a and b), MBP fused to Rch1 residues 218–244 (c and d), or MBP fused to Rch1 residues 197–217 (e and f), fixed 0 min (a, c, and e) or 10 min (b, d, and f) later, and examined by immunofluorescence. (B) Export kinetics of MBP-Rch1 fusion constructs. After nuclear injection of MBP, MBP_{218–244}, and MBP_{197–217}, the cells were fixed and immunostained using an anti-MBP polyclonal serum. Nuclear fluorescence at different times after injection was determined and plotted against time as in Fig. 1 B. Bar, 25 μ m.

tested export of the NLS receptor α subunit at 0°C. Vero cells were shifted to 0°C immediately before nuclear microinjection of Rch1_{33–529}, and Rch1_{244–529} and then incubated on ice for various time periods. Cells were fixed and the exogenous tagged Rch1 was visualized by immunoflu-

orescence. Cells injected with both forms of Rch1 showed nuclear staining not only immediately after injection (Fig. 6 A, a and c), but also after a 30-min incubation on ice (Fig. 6 A, b and d). Quantitative evaluation of nuclear fluorescence in cells injected with Rch1_{33–529} and Rch1_{244–529}

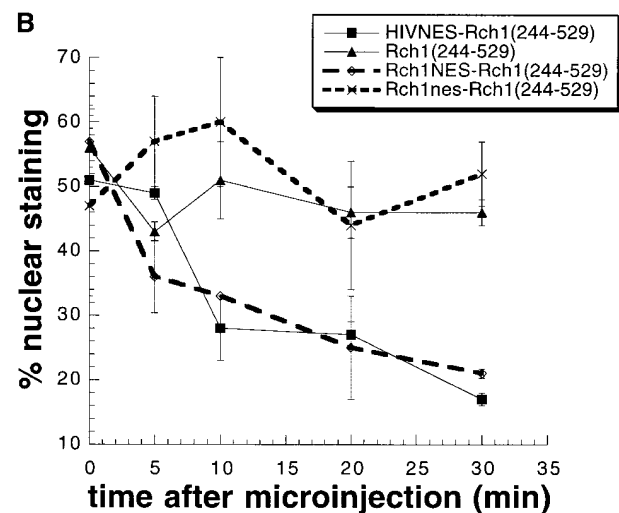
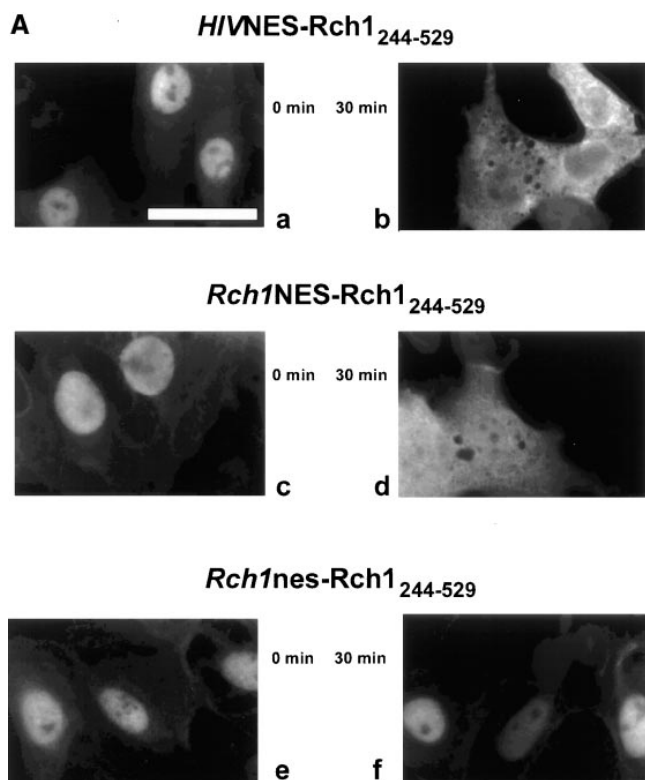


Figure 5. (A) Export of Rch1_{244–529} is restored by a nuclear export signal. HIVNES-Rch1_{244–529} (a and b), Rch1NES-Rch1_{244–529} (c and d), and Rch1nes-Rch1_{244–529} (e and f) were injected into nuclei of Vero cells. Cells were fixed either at a 0-min time point (a, c, and e) or 30 min later (b, d, and f). Immunofluorescence was performed using an anti-T7 antibody. (B) Export kinetics of Rch1_{244–529}, HIVNES-Rch1_{244–529}, Rch1NES-Rch1_{244–529}, and Rch1nes-Rch1_{244–529}. Nuclear fluorescence at different time points after injection was determined as described (Fig. 1 B). Bar, 25 μ m.

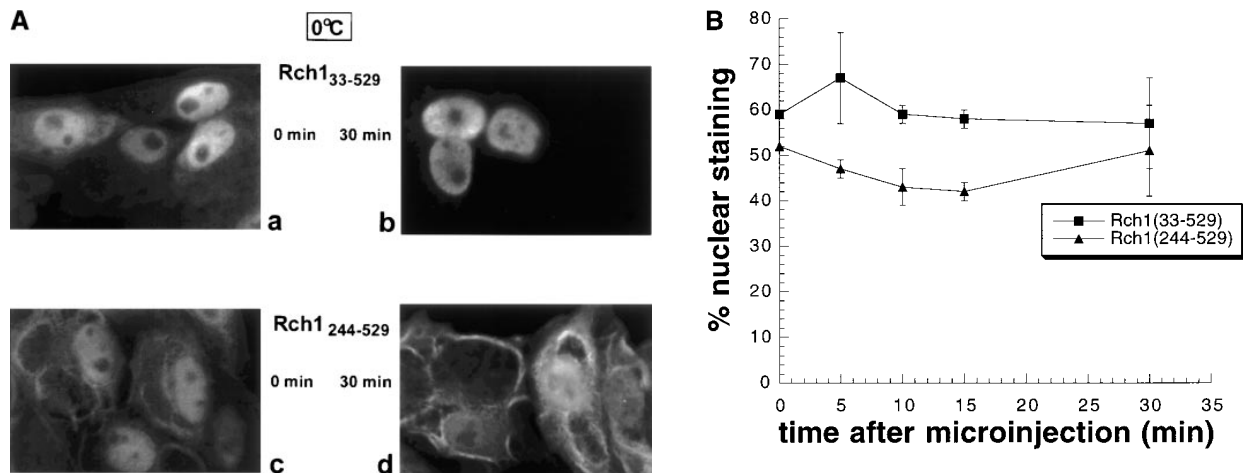


Figure 6. (A) Export of Rch1 is temperature dependent. Rch1₃₃₋₅₂₉ and Rch1₂₄₄₋₅₂₉ were injected into the nuclei of Vero cells at 0°C. The cells were either fixed (*a* and *c*) or incubated on ice for 30 min and then fixed (*b* and *d*). Cells were immunostained and fluorescent micrographs were taken. (B) Export of Rch1 is impaired at 0°C. The graph shows the quantification of the nuclear fluorescence of cells injected into the nucleus with Rch1₃₃₋₅₂₉ or Rch1₂₄₄₋₅₂₉ and incubated on ice as indicated on the x-axis before fixing and immunostaining.

confirmed that the intensity of nuclear fluorescence was constant over the 30-min time period (Fig. 6 B). These results support the idea that recycling of Rch1 is temperature dependent and show that neither Rch1₃₃₋₅₂₉ nor Rch1₂₄₄₋₅₂₉ can exit the nucleus by simple diffusion.

Role of RCC1 in Receptor Recycling

Addition of RanGTP to the heterodimeric complex of karyopherin α and β leads to the dissociation of the heterodimer in vitro (Rexach and Blobel, 1995; Görlich et al., 1996b). The fact that the nuclear protein RCC1 is the only known Ran guanine exchange factor, and plays a role in nuclear protein import in vivo (Tachibana et al., 1994; Dickmanns et al., 1996), suggested that RCC1 could be involved in the release of Rch1 from the import complex by providing RanGTP. Since the disassembly of the import complex must be a prerequisite for the recycling of the import factors, RCC1 would then indirectly affect the shuttling of the NLS receptor α subunit. To investigate the possible effect of RCC1 on receptor recycling, we used tsBN2 cells, mutant derivatives of BHK21 cells that express thermolabile RCC1 (for review see Dasso, 1993). To compare shuttling of Rch1 in the presence and absence of RCC1, Rch1₃₃₋₅₂₉ was injected into the cytoplasm of tsBN2 cells. The cells were then fixed immediately or incubated for 4 h at either the restrictive or permissive temperature. The subcellular distribution of Rch1₃₃₋₅₂₉ was then determined by immunofluorescent staining. Fig. 7 A shows that in the presence of RCC1, cells displayed the predominantly cytoplasmic staining typical of injected Rch1₃₃₋₅₂₉ (Fig. 7 A, *a* and *b*). In contrast, Rch1 was predominantly nuclear in the absence of RCC1 (Fig. 6 A, compare *b* [33.5°C] and *d* [39.5°C]).

The import kinetics of Rch1 in the presence of RCC1 demonstrated that Rch1₃₃₋₅₂₉ was mostly cytoplasmic in tsBN2 cells incubated at 33.5°C (Fig. 7 B). The nuclear accumulation of Rch1 in the absence of RCC1 (Fig. 7 B) demonstrates that RCC1 is required directly or indirectly

for recycling of Rch1 to the cytoplasm. In a control experiment using the parental cell line BHK21, Rch1₃₃₋₅₂₉ was mostly cytoplasmic at both temperatures (Fig. 7 B), demonstrating that the altered distribution of Rch1 at the restrictive temperature is caused by the RCC1 mutation rather than by the elevated temperature.

RCC1 Is Not Required for Export of Rch1

Two simple alternatives can be proposed for the role of RCC1 in receptor recycling. In one model, transport of Rch1 back into the cytoplasm might require RanGTP generated by RCC1. Loss of RCC1 would lead to depletion of RanGTP in the nucleus, and subsequently to a defect in receptor recycling. If this idea were correct, export of Rch1 should be inhibited when Rch1 was injected into nuclei of tsBN2 cells that had been incubated at 39.5°C before microinjection and therefore lacked active RCC1. In the other model, RCC1 would act at a step before export of Rch1, for example in the disassembly of the import complex. Rch1 accumulated in tsBN2 nuclei at the restrictive temperature after cytoplasmic injection would then be part of a nondisassembled import complex. If this possibility were correct, free Rch1 injected directly into the nucleus should be exported in a fashion independent of RCC1.

To differentiate between these two possibilities, we injected Rch1₃₃₋₅₂₉ into the nuclei of tsBN2 cells that had been incubated at the permissive and restrictive temperatures. The cells were fixed either immediately after injection (Fig. 8 A, *a* and *c*) or 30 min later (Fig. 8 A, *b* and *d*), and Rch1₃₃₋₅₂₉ was visualized by immunofluorescence. At both temperatures, Rch1₃₃₋₅₂₉ injected into the nuclei was found in the cytoplasm after a 30-min incubation. The time course (Fig. 8 B) shows clearly that nuclear export kinetics of Rch1 were identical in the presence or absence of RCC1. In a control experiment in BHK21 cells, Rch1 export kinetics were also identical at 33.5 and 39.5°C (Fig. 8 B). No export of Rch1 to the cytoplasm was observed at 0°C (data not shown), consistent with the temperature de-

A Import in tsBN2

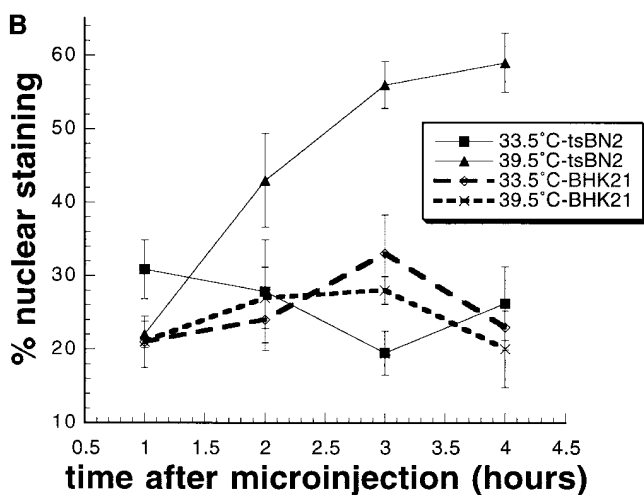
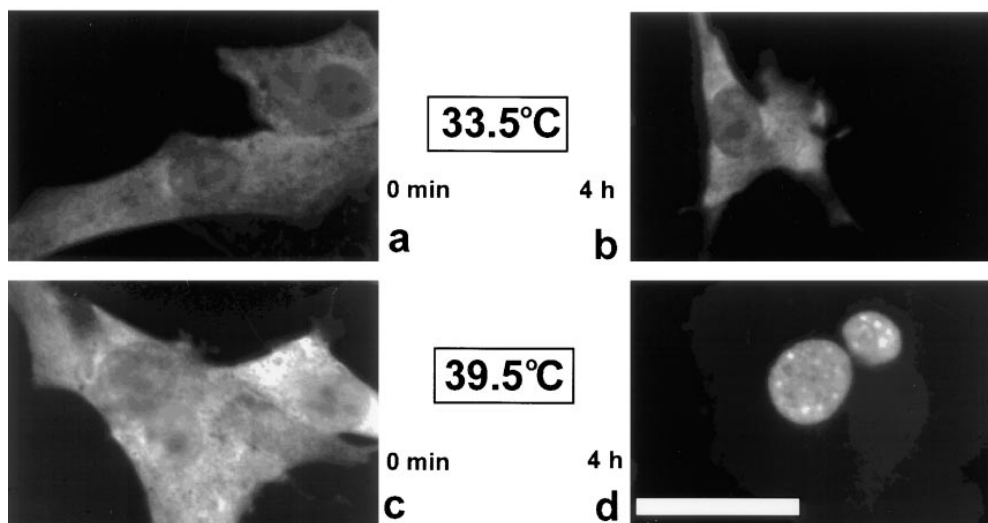


Figure 7. (A) Effect of RCC1 depletion on Rch1 recycling. tsBN2 cells were injected with Rch1₃₃₋₅₂₉ into the cytoplasm followed by incubation at 33.5°C (a and b) or 39.5°C (c and d). Fluorescence micrographs show the localization of Rch1 in cells that were fixed after 0 min (a and c) or 4 h (b and d) of incubation under the conditions indicated. (B) Time course of nuclear accumulation of cytoplasmically injected Rch1₃₃₋₅₂₉ in the absence and presence of RCC1. tsBN2 and BHK21 cells were injected into the cytoplasm with Rch1₃₃₋₅₂₉ and incubated at 39.5°C or, as a control, at 33.5°C for various time periods, fixed and stained. Quantification was done as in Fig. 1 B and the percentage of Rch1 in the nucleus was expressed as a function of time after injection. Bar, 25 μ m.

pendence of the Rch1 export reaction (Fig. 6). These results rule out a requirement for RCC1 in the export reaction itself and support the idea that it is required in an earlier step of receptor recycling.

RCC1 Destabilizes the NLS Receptor/Substrate Complex *In Vitro*

The results presented in Fig. 8 suggested that RCC1 was not required for nuclear export of free Rch1₃₃₋₅₂₉ *in vivo*, but rather for a step before export. One step that must occur before Rch1 recycling is the disassembly of the NLS receptor $\alpha\beta$ complex with the imported cargo protein. RanGTP was reported to disassemble the NLS receptor $\alpha\beta$ complex *in vitro* (Rexach and Blobel, 1995) by binding to karyopherin β at a binding site that overlaps with the karyopherin α binding site (Moroianu et al., 1996b; Görlich et al., 1996b). These results supported the idea that the role of RCC1 might be to destabilize an NLS receptor/substrate complex in the nucleus.

To develop a method to test this possibility, we first assembled NLS receptor/substrate complexes using purified

proteins in a solid phase assay. Karyopherin β and, as a control, BSA were immobilized on ELISA plates and soluble Rch1₃₃₋₅₂₉ was added. After washing, bound Rch1₃₃₋₅₂₉ was detected using the anti-T7 antibody and a peroxidase-coupled secondary antibody. Fig. 9 A shows that Rch1₃₃₋₅₂₉ was bound in the presence of karyopherin β , but not in the presence of BSA, indicating that Rch1₃₃₋₅₂₉ bound specifically to karyopherin β . To test whether the immobilized Rch1₃₃₋₅₂₉/karyopherin β complexes were able to bind T-antigen as an NLS-bearing protein, we immobilized karyopherin β in the ELISA plate and then added either T-antigen alone or T-antigen together with Rch1₃₃₋₅₂₉. Bound T-antigen was detected using a specific monoclonal antibody and a peroxidase-coupled second antibody. Fig. 9 B shows that bound T-antigen was detectable in the presence of karyopherin β and Rch1₃₃₋₅₂₉ but not with karyopherin β alone, indicating the assembly of an $\alpha\beta$ NLS receptor complex with the transport substrate T-antigen.

The influence of RanGDP and RanGTP on the assembly or stability of the NLS receptor/substrate complexes was then tested. RanGDP in the presence of GDP and Mg^{2+} did not affect the assembly of NLS receptor/sub-

A Export in tsBN2

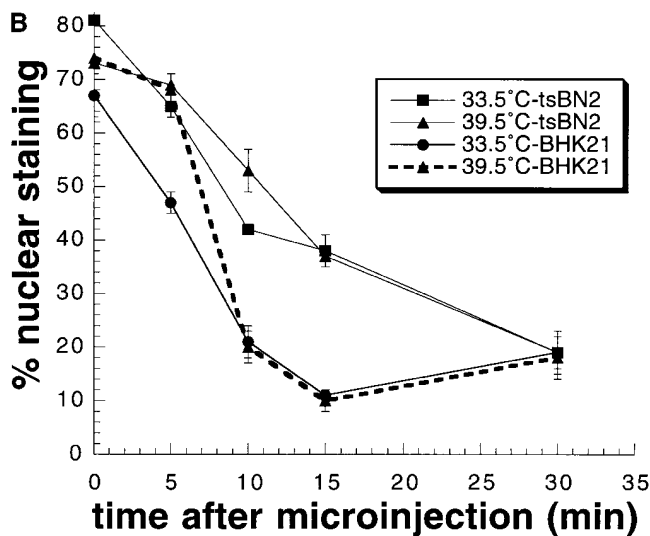
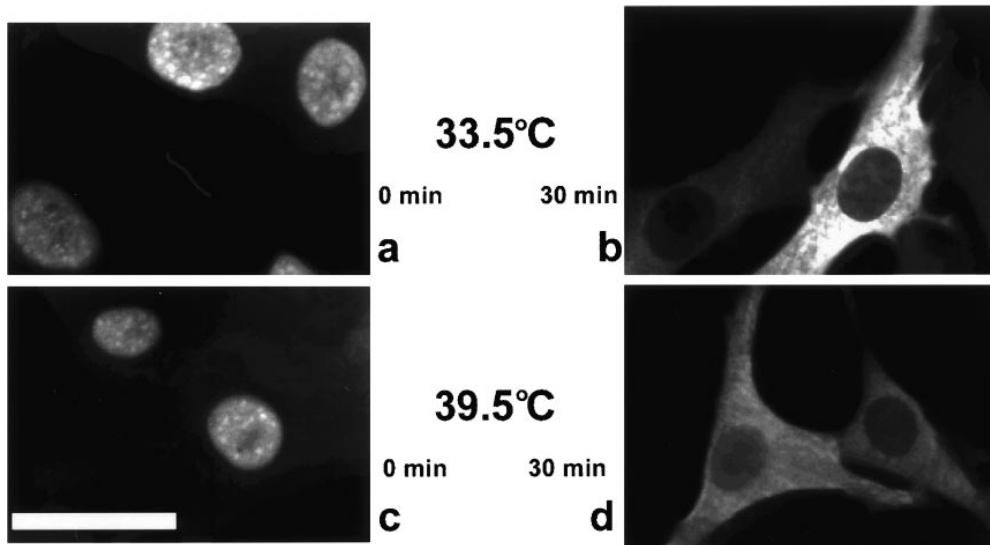


Figure 8. (A) Export of free Rch1 is not dependent on RCC1. Rch1₃₃₋₅₂₉ was injected into the nuclei of tsBN2 cells preincubated for 4 h at 33.5 or 39.5°C. Cells were fixed and stained immediately after injection (a and c) or incubated for 30 min before fixing and immunostaining (b and d). (B) Export of free Rch1 is independent of RCC1. tsBN2 and, as a control, BHK21 cells, were incubated for 4 h at 33.5 or 39.5°C before nuclear injection with Rch1₃₃₋₅₂₉. For injections at 0°C, cells were first incubated at 33.5°C, but shifted to 0°C immediately before injection. After incubation at the indicated temperatures for the indicated time periods, cells were fixed and stained. Quantification was performed as described in Fig. 1 B. Bar, 25 μ m.

strate complexes (Fig. 9 C, compare column 1 with column 3). When RanGTP was added, NLS receptor/T-antigen complex formation was slightly reduced in the presence of Mg²⁺ and GTP (Fig. 9 C, column 5). If Ran was loaded with GTP γ -S, a nonhydrolyzable GTP analogue, complex formation was strongly inhibited in the presence of Mg²⁺ and GTP γ -S (Fig. 9 C, compare columns 5 and 6). However, if RCC1 and GTP were present, NLS receptor complex formation was inhibited by \sim 60% (Fig. 9 C, column 7), while RCC1 in the presence of GDP had no effect (Fig. 9 C, column 4). These results indicate that when RanGTP was added without RCC1, GTP hydrolysis on Ran occurred, but GDP bound to Ran was not exchanged for GTP in the presence of Mg²⁺ (Bischoff and Ponstingl, 1991b), and therefore did not destabilize or impair formation of the NLS receptor/T-antigen complex. On the other hand, when RanGTP, RCC1, and GTP were added, GDP bound to Ran after GTP hydrolysis was exchanged for GTP.

These results confirm that RanGTP prevents formation of the NLS receptor complex or induces its disruption as reported (Rexach and Blobel, 1995; Moroianu et al., 1996b)

and demonstrate that RCC1-catalyzed exchange of GDP for GTP on Ran inhibited formation of the NLS receptor/substrate complex or dissociated the existing complexes.

Discussion

Karyopherin α Sequences Required for Recycling to the Cytoplasm

In this report, we have investigated the requirements *in vivo* for recycling of the α subunit of the NLS receptor (Rch1) from the nucleus to the cytoplasm. Rch1 was found predominantly in the cytoplasm with only \sim 20% of the exogenous α subunit localized to the nucleus in Vero cells and \sim 30% in tsBN2 cells. These results are in accordance with the subcellular distribution of the endogenous α subunit, which was found in higher concentrations in the cytoplasm than in the nucleus in *Xenopus* X1177 cells (Görlich et al., 1996a). This similarity indicates that Rch1₃₃₋₅₂₉ behaves in these assays much like the endogenous α subunit.

The importin β -binding domain of karyopherin α (Gör-

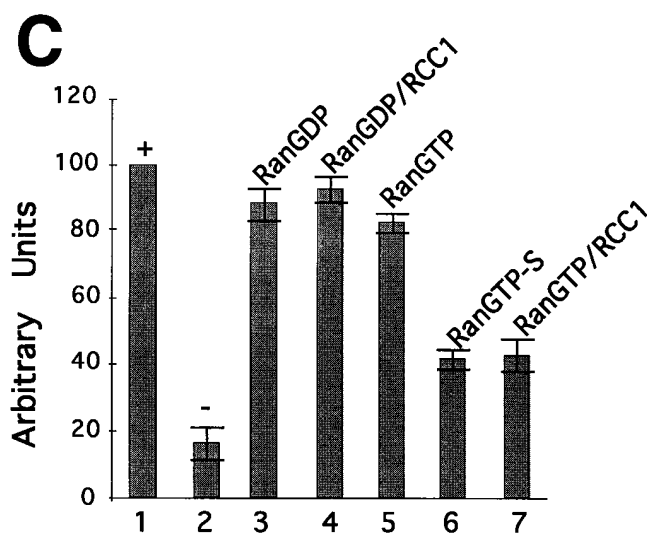
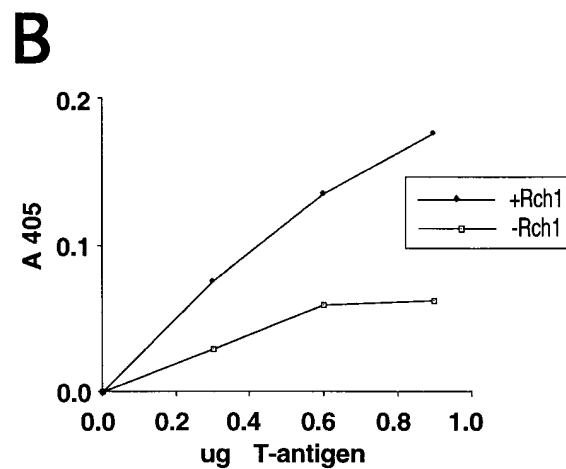
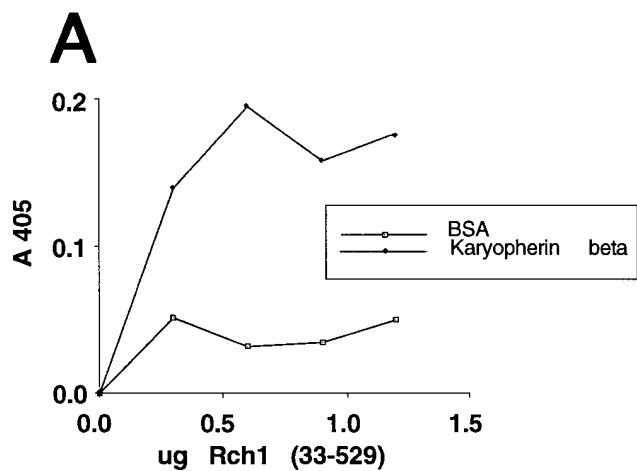


Figure 9. RCC1 inhibits formation of the NLS receptor/substrate complex. (A) Karyopherin β was immobilized on an ELISA plate before addition of different amounts of Rch1₃₃₋₅₂₉, as indicated on the x-axis of the graph. Plates were washed and Rch1 bound to the solid phase was detected in a colorimetric reaction. (B) Karyopherin β was immobilized on ELISA plates. T-antigen was added in the amounts indicated on the x-axis of the graph, either alone or together with Rch1₃₃₋₅₂₉. Plates were washed, and the amount of T-antigen bound to the solid phase was determined in a colorimetric reaction. (C) Karyopherin β was immobilized on ELISA immunoplates before addition of either Rch1₃₃₋₅₂₉ and T-antigen (columns 1 and 3–7) or T-antigen alone (column 2). The binding reactions were then supplemented with RanGDP and GDP (column 3); RanGDP, GDP, and RCC1 (column 4); RanGTP and GTP (column 5); RanGTP- γ S and GTP- γ S (column 6); or RanGTP, GTP, and RCC1 (column 7). MgCl₂ was present in all reactions at 5 mM. The T-antigen bound to each solid phase was determined in a colorimetric reaction. The columns depict the mean values from at least two separate experiments; the standard deviation of the mean is indicated by error bars.

lich et al., 1996a; Weis et al., 1996) was mapped to residues 1–65 of importin α and required aa 21–65 or 1–52. In contrast, Rch1₃₃₋₅₂₉ was reported to support nuclear import in vitro as efficiently as full-length karyopherin α 1 (Moroianu et al., 1995b), and even a karyopherin α 1 mutant lacking the first 49 amino acids was reported to bind to karyopherin β in vitro, albeit with reduced efficiency (Moroianu et al., 1996a). Another group observed nuclear entry of Rch1₃₃₋₅₂₉ in vivo (Gallay et al., 1996), consistent with nuclear import activity. The experimental variables that give rise to the discrepancies between these reports have not been resolved. However, our experiments showed nuclear entry and exit of Rch1₃₃₋₅₂₉ in vivo (Figs. 1, 2, and 7), as expected for an α subunit active in nuclear protein import, and our in vitro binding assays confirmed interaction of Rch1₃₃₋₅₂₉ with both karyopherin β and an NLS-bearing cargo protein as expected for an NLS receptor complex (Fig. 9).

The IBB domain is completely absent in the Rch1₂₄₄₋₅₂₉ deletion mutant, precluding its activity in nuclear protein import. Nevertheless, nuclear entry of this protein was still observed. One possible explanation for this apparent contradiction is that Rch1₂₄₄₋₅₂₉ might enter the nucleus piggyback on a protein with more than one NLS. Rch1₂₄₄₋₅₂₉ is capable of binding to NLSs in a transport substrate (Cuomo

et al., 1994), and a substrate with multiple NLSs could still interact with endogenous karyopherin α after binding one molecule of Rch1₂₄₄₋₅₂₉. The endogenous NLS receptor α subunit could thus mediate the transport reaction. However, our data do not rule out the possibility that Rch1₂₄₄₋₅₂₉ could enter the nucleus via diffusion.

In our experiments, Rch1₂₄₄₋₅₂₉ was found to accumulate in the nuclei over a 2.5-h period (Fig. 1), while Gallay et al. (1996) reported that Rch1₂₄₄₋₅₂₉ was exclusively cytoplasmic at \sim 48 h after the corresponding cDNA was transfected into 293S cells. This discrepancy might be explained by the very different times at which localization was assayed. When cells were microinjected with cDNA encoding Rch1₂₄₄₋₅₂₉ and incubated for extended time periods, nuclear accumulation of the protein was initially observed for \sim 4–5 h, but after that nuclear staining was reduced (data not shown). Slow leaking from the nucleus has been observed with other proteins. For example, pyruvate kinase injected into the nuclei of *Xenopus* oocytes was gradually transported out of the nucleus (Schmidt-Zachmann et al., 1993).

Compared with the nuclear export of pyruvate kinase, nuclear export of Rch1₃₃₋₅₂₉ was very rapid (Fig. 2). The high concentration of NLS receptor α subunit in the cytoplasm, which probably depends on the fast export reaction

from the nucleus, could be very important in maintaining directionality in the import reaction. Rch1₂₄₄₋₅₂₉, however, proved to be deficient in this fast nuclear export reaction (Fig. 2). The fast nuclear exit of Rch1 is mediated by a sequence in the region between aa 207 and 217 (Figs. 4 and 5) that probably carries an NES since the HIV Rev NES fused to Rch1₂₄₄₋₅₂₉ restored nuclear export of the deletion mutant to a very similar extent as aa 207–217 (Fig. 5). Typically, NESs contain several Leu-residues and are rich in hydrophobic amino acids (for review see Gerace, 1995; Nigg, 1997). The region 207–217 in Rch1 shows similar characteristics: Rch1, **DPL***PL*LALLAVPD; PKI NES, LALKLAGLDI (Wen et al., 1995); and Rev NES, LPPL-ERLTL (Fischer et al., 1995; Wen et al., 1995). Interestingly, the leucine residues indicated in bold are conserved among human Rch1, mouse importin, *Xenopus* importin 1, and yeast SRP1p α subunits, and residues indicated in italics are similar (Yano et al., 1992; Cuomo et al., 1994; Görlich et al., 1994; Imamoto et al., 1995a), consistent with their probable importance in Rch1 function. We have shown here that the conserved leucines are necessary for the NES activity of amino acids 207–217.

The pathway of karyopherin α recycling must be different from that for its entry into the nucleus to achieve accumulation of nuclear proteins in the nucleus. One mechanistic difference probably lies in the proteins that accompany karyopherin α during the transport through the pore in the import and export reactions. Import of karyopherin α is mediated by karyopherin β , whereas export is not, since the IBB domain of karyopherin α supports only nuclear entry, but not exit of karyopherin α (Görlich et al., 1996a; Weis et al., 1996). Our data indicate that Rch1 export depends on an NES and on energy, suggesting a receptor-mediated export reaction. However, the putative export receptor and other proteins required for recycling remain to be identified. Interestingly, Aitchinson et al. (1995) reported that a yeast mutant that lacked Nup120p and displayed defects in mRNA export also accumulated importin α in the nucleus, pointing to a possible role for NPC proteins in receptor recycling.

A Role for RCC1 in Rch1 Recycling to the Cytoplasm

RCC1 was also found to be a requirement for the recycling of the NLS receptor α subunit. Loss of RCC1 in tsBN2 cells led to nuclear accumulation of cytoplasmically injected Rch1₃₃₋₅₂₉ (Fig. 7). The time course of nuclear accumulation of Rch1₃₃₋₅₂₉ depicted in Fig. 7 B shows that when tsBN2 cells were shifted to the restrictive temperature directly after the injection, the Rch1 distribution was predominantly cytoplasmic for about the first hour. By 2 h after the shift-up, however, the intensity of nuclear staining of Rch1₃₃₋₅₂₉ was markedly increased and continued to increase over the time period monitored. We speculate that during the first hour after shift-up, active RCC1 was still present. This time course is consistent with the observation that inhibition of nuclear protein import in vivo in tsBN2 cells was first detectable after 2 h at the restrictive temperature (Dickmanns et al., 1996). Trapping of Rch1 in the nucleus, as observed here, may well be responsible for the nuclear protein import defect of tsBN2 cells at the restrictive temperature.

On the other hand, export of free receptor from the nucleus to the cytoplasm was not dependent on RCC1 (Fig. 8). Since inactivation of the RCC1 homologue prp20 in yeast also resulted in nuclear accumulation of the yeast Rch1 homologue (Koepp et al., 1996), the authors speculated that RanGTP supplied by prp20 might be necessary for the exit of the yeast NLS receptor α subunit from the nucleus. However, we demonstrate here that at least for tsBN2 cells, the exit event itself was not affected by the loss of RCC1 (Fig. 8). While this manuscript was under revision, Richards et al. (1997) independently demonstrated that RCC1 was not necessary for nuclear export of a NES-bearing protein. Nevertheless, RanGTP did appear to be necessary for export. These findings are consistent with our proposal that RCC1 plays a role in an earlier step of NLS receptor recycling.

RanGTP, but not RanGDP, was reported to destabilize the karyopherin $\alpha\beta$ heterodimer in vitro (Rexach and Blobel, 1995; Moroiianu et al., 1996b). We confirm this result here using a solid phase assay and show that Ran, GTP, and RCC1 release an NLS-bearing protein from an NLS receptor/substrate complex or prevent formation of these complexes (Fig. 9 C). These results, taken together with our in vivo data, suggest that RCC1 could induce the disassembly of the import complex by providing RanGTP, which dissociates karyopherin β from the import complex, thereby lowering the affinity of karyopherin α to NLSs (Rexach and Blobel, 1995). RanGTP will therefore facilitate the release of the imported protein from the NLS receptor α subunit, which in turn would be a prerequisite for the recycling of the α subunit. Interaction of the imported protein with other nuclear proteins or DNA, and/or association of karyopherin α with other proteins involved in the recycling process, could then promote the dissociation of import substrate from the α subunit.

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