The Nup358-RanGAP Complex Is Required for Efficient Importin α/β -dependent Nuclear Import

Saskia Hutten, Annette Flotho, Frauke Melchior, and Ralph H. Kehlenbach

Department of Biochemistry I, Faculty of Medicine, Georg-August University of Göttingen, 37073, Göttingen, Germany

Submitted December 26, 2007; Revised February 1, 2008; Accepted February 15, 2008
Monitoring Editor: Karsten Weis

In vertebrate cells, the nucleoporin Nup358/RanBP2 is a major component of the filaments that emanate from the nuclear pore complex into the cytoplasm. Nup358 forms a complex with SUMOylated RanGAP1, the GTPase activating protein for Ran. RanGAP1 plays a pivotal role in the establishment of a RanGTP gradient across the nuclear envelope and, hence, in the majority of nucleocytoplasmic transport pathways. Here, we investigate the roles of the Nup358-RanGAP1 complex and of soluble RanGAP1 in nuclear protein transport, combining *in vivo* and *in vitro* approaches. Depletion of Nup358 by RNA interference led to a clear reduction of importin α/β -dependent nuclear import of various reporter proteins. *In vitro*, transport could be partially restored by the addition of importin β , RanBP1, and/or RanGAP1 to the transport reaction. In intact Nup358-depleted cells, overexpression of importin β strongly stimulated nuclear import, demonstrating that the transport receptor is the most rate-limiting factor at reduced Nup358-concentrations. As an alternative approach, we used antibody-inhibition experiments. Antibodies against RanGAP1 inhibited the enzymatic activity of soluble and nuclear pore-associated RanGAP1, as well as nuclear import and export. Although export could be fully restored by soluble RanGAP, import was only partially rescued. Together, these data suggest a dual function of the Nup358-RanGAP1 complex as a coordinator of importin β recycling and reformation of novel import complexes.

INTRODUCTION

The giant nucleoporin Nup358/RanBP2 (Wu *et al.*, 1995; Yokoyama *et al.*, 1995) is a major component of the filaments that emanate from the cytoplasmic ring of the nuclear pore complex (NPC) into the cytoplasm (Walther *et al.*, 2002). As many other nucleoporins, Nup358 interacts via phenylalanine-glycine repeats (FG repeats) with karyopherins, transport receptors that mediate import and export across the NPC (for review see Tran and Wenthe, 2006). In recent nuclear transport models, a hydrophobic milieu that is established by FG repeats derived from several nucleoporins is suggested to allow selective translocation of karyopherins, with or without cargo molecules, across the NPC (for review see Weis, 2007). Individual nucleoporins do not play a major role in these models. Nevertheless, several nucleoporins have been shown to affect specific transport pathways. Nup153, for example, interacts with various import receptors, regulating late steps in nuclear import (Shah and Forbes, 1998). Very recently, specific FG nucleoporins were shown to be required for mRNA export (Terry and Wenthe, 2007). In this study, we investigate the role of Nup358 in nuclear protein transport in detail.

Ran is a small GTP-binding protein that binds to karyopherins and plays an essential role in the majority of nucleocytoplasmic transport pathways. RanGTP is generated in the nucleus, resulting from the activity of the chromatin-bound guanosine nucleotide exchange factor RCC1. In the cytoplasm, vertebrate RanGAP1 (RanGAP for short), together with the RanGTP-binding protein RanBP1 or the RanBP1-like domains of Nup358 strongly promotes GTP-hydrolysis on Ran. RanGDP is then reimported into the nucleus by a dedicated nuclear import factor, NTF2. Localized nucleotide exchange and hydrolysis on Ran are key to the assembly and disassembly of import and export complexes, respectively (for review see Fried and Kutay, 2003). In CRM1-mediated nuclear protein export (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997), RanGTP is an integral component of the transport complex. In the cytoplasm, RanGAP-promoted GTP-hydrolysis on Ran leads to the dissociation of the export cargo. In nuclear import, by contrast, binding of RanGTP to import receptors leads to the disassembly of import complexes in the nucleus (Rexach and Blobel, 1995). The best-studied import pathway involves the importin α/β heterodimer, where importin α serves as an adapter protein that binds to import cargos with a “classic” nuclear localization signal (NLS), whereas importin β functions as the RanGTP-binding karyopherin (for review see Fried and Kutay, 2003).

In vertebrate cells, a substantial portion of RanGAP is modified by SUMO1 and targeted to Nup358 (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). Plant cells also localize RanGAP to the nuclear envelope, yet by a mechanism that does not depend on SUMO modification (Rose and Meier, 2001). Together, these observations led to the hypothesis that NPC-associated RanGAP is required for efficient nuclear protein import (Mahajan *et al.*, 1997) and export (Kehlenbach *et al.*, 1999). Yeast cells, on the other hand, do not express a

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-12-1279>) on February 27, 2008.

Address correspondence to: Ralph H. Kehlenbach (rkehlen@gwdg.de).

Abbreviations used: FG repeats, phenylalanine-glycine repeats; GFP, green fluorescent protein; NES, nuclear export signal; NFAT, nuclear factor of activated T-cells; NLS, nuclear localization signal; NPC, nuclear pore complex; Nup, nucleoporin; WGA, wheat germ agglutinin.

Nup358 homolog, and yeast RanGAP (rnlp) does not associate with the nuclear envelope. Hence, localization of RanGAP to the nuclear pore is not a prerequisite for transport per se. Consistent with this, Nup358 is not required for nuclear import of some substrates *in vivo*, like the glucocorticoid receptor (Salina *et al.*, 2003) or the transcription factor NFAT (nuclear factor of activated T-cells; Hutten and Kehlenbach, 2006). Furthermore, in *Xenopus* oocytes, Nup358 was reported to be dispensable for nuclear import of BSA-NLS *in vitro* (Walther *et al.*, 2002). In *Drosophila* cells, however, a reduced import rate was reported for the PYM protein upon depletion of Nup358 (Forler *et al.*, 2004). In addition, Nup358 was identified as one of three nucleoporins whose depletion led to reduced nuclear import in *Drosophila* cells (Sabri *et al.*, 2007).

Here, we address these opposing findings and investigate the role of the Nup358-RanGAP complex and of soluble RanGAP in importin α/β -dependent import and CRM1-dependent export. Our results point to a function of the Nup358-RanGAP complex as a coordinator of importin β recycling and reformation of importin α -containing import complexes.

MATERIALS AND METHODS

Cell Culture and Transfections

HeLa-P4 cells (Charneau *et al.*, 1994) were grown in DMEM (GIBCO, Rockville, MD) containing 4500 mg/l glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The stable cell line expressing green fluorescent protein (GFP)-NFAT has been described previously (Kehlenbach *et al.*, 1998). For expression of GR₂/nuclear export signal (NES)-GFP₂-NLS constructs, HA-importin β , HA-transportin, and HA-Nup358 (aa 2595-2881), HeLa-P4 cells were transiently transfected using Polyfect (Qiagen, Chatsworth, CA), according to the instructions of the manufacturer.

RNA Interference

Cells were either mock treated or transfected with small interfering RNA (siRNA) against Nup358 (CACAGACAAAGCCGUUGAA, corresponding to nucleotides 351-369; accession no. NM_006267) as described (Hutten and Kehlenbach, 2006). Where indicated, mock-treated and nucleoporin-depleted cells were mixed 1 d before the analysis at a ratio of 1:3-1:4 to allow for a direct comparison of protein levels and transport efficiencies.

Plasmids

The K526R mutation was introduced into mouse RanGAP in pET11d (Mahajan *et al.*, 1997) by site-directed mutagenesis. For generation of the NES-GFP₂-NLS shuttling constructs, the coding sequence of GFP was PCR amplified and inserted into the BglII and HindIII sites of pEGFP-C1 (Clontech, Palo Alto, CA). To introduce an NLS, oligonucleotides coding for the SV40 cNLS (5'-ATTCAGGCCCAAGAAAAAGAGGAAAGTTGGGTGAG and 5'-TC-GACTACCCCACTTCTCTTTTCTTTGGGCCTG) were annealed and inserted into the EcoRI and SalI sites of this construct. Finally, fragments of the human immunodeficiency virus 1 (HIV-1) Rev protein containing the NES (amino acids 48-116 or 68-90) were PCR amplified and inserted into XhoI and SpeI sites of a newly generated multiple cloning site 5' of the GFP₂-NLS fusions, generating Rev₄₈₋₁₁₆-GFP₂-cNLS and introducing amino acids ERQ after the initiating methionine, and Rev₆₈₋₉₀-GFP₂-cNLS, respectively. Oligonucleotides coding for the NES of the NS2 protein (amino acids 76-97) of the minute virus of mice (Askjaer *et al.*, 1999) were annealed and inserted accordingly, generating the NS2P₇₆₋₉₇-GFP₂-cNLS construct. The GR₂-GFP₂-cNLS construct was generated by insertion of two copies of the hormone responsive domain (aa 511-795) of the rat glucocorticoid receptor, 5' to the GFP₂-cNLS fusion. The coding sequence of human importin β and transportin were PCR-amplified and inserted into the NcoI and XbaI sites of the elongation factor-hemagglutinin (pEF-HA) plink vector (Gasteier *et al.*, 2003). The sequence coding for amino acids 2595-2881 of human Nup358 was PCR amplified and inserted into the NcoI and EcoRI sites of the EF-HA plink vector. All constructs were verified by DNA sequencing.

Protein Purification

Importin α (Hu *et al.*, 1996), importin β (Chi and Adam, 1997), transportin (Baake *et al.*, 2001), CRM1 (Kehlenbach and Gerace, 2002), RanBP1 (Kehlenbach *et al.*, 1999), RanGAP, RanGAP-K526R (Mahajan *et al.*, 1997), and Ran (Melchior *et al.*, 1995) were expressed as described. All proteins were dialyzed against transport buffer (TPB; 20 mM HEPES-KOH, pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)₂, 1

mM EGTA, 2 mM DTT, and 1 μ g/ml each of aprotinin, leupeptin, and pepstatin), frozen in liquid nitrogen and stored at -80°C .

Import Ligands

FITC-BSA-NLS, Cy3-BSA-NLS, and Cy5-BSA-NLS were prepared as described (Paschal and Gerace, 1995; Kehlenbach and Gerace, 2002).

RanGAP Assays

Labeling of Ran with [γ -³²P]GTP and RanGAP assays were essentially performed as described (Askjaer *et al.*, 1999; Kehlenbach *et al.*, 2001). For antibody inhibition experiments, 25 ng of RanGAP was preincubated with increasing concentrations of anti-RanGAP antibodies for 15 min at 20°C. After the addition of [γ -³²P]RanGTP to a final volume of 25 μ l and incubation for 10 min at 20°C, the reaction was stopped with stop solution (7% charcoal, 10% ethanol, 0.1 M HCl, 10 mM NaH₂PO₄). After centrifugation, the released [³²P]phosphate in the supernatant was measured by scintillation counting. Background counts from a reaction without RanGAP were subtracted and GTP-hydrolysis was expressed as the percentage of the maximal value of recovered radioactivity. In reactions with HeLa cells as a source of RanGAP activity (Yaseen and Blobel, 1999), cells were permeabilized with digitonin, washed twice with transport buffer, and incubated with the anti-RanGAP antibody or an unspecific IgG in the presence of 4 mg/ml bovine serum albumin (BSA) as a blocking reagent. [γ -³²P]RanGTP was added as above to the antibody-containing suspension or after washing the cells and resuspension in TPB. ~20,000 cells were used per reaction.

Nuclear Transport Assays

To induce the import of the GR₂-GFP₂-NLS fusion protein, cells grown on poly-L-lysine-coated coverslips were treated with 5 μ M dexamethasone (Sigma, St. Louis, MO) for 15 min at 37°C, fixed, and subjected to indirect immunofluorescence. For analysis of nuclear import *in vitro*, adherent cells were grown on poly-L-lysine-coated coverslips, permeabilized with 0.008–0.015% digitonin in transport buffer, and incubated for 30 min at 30°C with an ATP-regenerating system (1 mM ATP, 2.8 mM creatine phosphate, 0.4 U creatine phosphokinase, Sigma), ~25 μ g/ml fluorescein isothiocyanate (FITC)-BSA-nuclear localization signal (NLS) or ~3.5 μ g/ml Cy3-BSA-NLS, 500–750 nM importin α , 100–250 nM importin β , 1–6 μ M Ran, 25 nM RanBP1, and 20–200 nM RanGAP, as indicated. As a specificity control, cells were preincubated with 200 μ g/ml wheat germ agglutinin (WGA; Sigma) for 10 min at 4°C. After import reactions, cells were washed, fixed, and either analyzed directly by fluorescence microscopy or after immunofluorescence staining. For quantification of nuclear import, the nuclear FITC fluorescence of 50–100 control cells and cells with a strongly reduced Nup358-staining was measured using the Zeiss AutMessPlus-Software (Jena, Germany).

For the analysis of nuclear export, a stable cell line expressing GFP-nuclear factor of activated T-cells (NFAT) was used (Kehlenbach *et al.*, 1998). Briefly, cells were treated with trichostatin A overnight to induce the expression of GFP-NFAT and with ionomycin for 30 min to induce its import into the nucleus. After permeabilization with digitonin and washing with transport buffer, cells were preincubated with anti-RanGAP antibodies or unspecific IgG for 40 min at 20°C, washed, and subjected to export reactions. After 30 min at 30°C, cells on coverslips were washed, fixed with 3.7% formaldehyde, and analyzed by fluorescence microscopy. For a quantitative analysis, cells were trypsinized before digitonin-permeabilization, and export reactions with ~40,000 cells in suspension were performed in a final volume of 20 μ l. In some reactions, ~2.5 μ g/ml Cy5-BSA-NLS was included for simultaneous analysis of nuclear import. After the reaction, cells were washed with transport buffer. The average nuclear fluorescence emitted from GFP-NFAT or Cy5-BSA-NLS in ~5000 cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Reactions were standardized by arbitrarily assigning a value of 100 to the nuclear fluorescence of GFP-NFAT in a 4°C control reaction and to the maximal nuclear fluorescence of Cy5-BSA-NLS after incubation at 30°C.

Antibodies

Antibodies against full-length mouse RanGAP were raised in goats (Pichler *et al.*, 2002) and used for inhibition experiments, Western blotting, and immunofluorescence (see Figure 7) or were raised in rabbits and used for immunofluorescence (see Figure 6). Antibodies were affinity-purified using RanGAP and immobilized to cyanogenbromide-activated Sepharose. After binding, beads were washed with PBS containing 500 mM NaCl, and antibodies were eluted with elution buffer (PBS, pH 2.3, containing 200 mM acetic acid and 500 mM NaCl) and dialyzed against PBS or TPB. The goat-anti Nup358 antibody was raised against a recombinant fragment (aa 2595-2881) and purified as described for the anti-RanGAP antibody. The rabbit anti-importin β antibody was kindly provided by Larry Gerace (The Scripps Research Institute, La Jolla, CA). The goat anti-GST antibody was obtained from Amersham Biosciences (Piscataway, NJ). The monoclonal mouse anti α -tubulin and mouse anti importin β -antibodies were obtained from Sigma and Affinity BioReagents (Golden, CO), respectively. For the detection of HA-epitope-tagged proteins, a monoclonal mouse anti-HA antibody (clone

12CA5) was used. For immunofluorescence, donkey anti-goat Alexa 594, donkey anti-rabbit Alexa 594, donkey anti-mouse Alexa 488, and donkey anti-goat Alexa 633 (1:2000; Molecular Probes, Eugene, OR) were used as secondary antibodies. For immunoblotting, HRP-coupled donkey anti-goat, donkey anti-mouse, or donkey anti-rabbit IgG (Dianova, Rodeo, CA) were used as secondary antibodies.

Cell Fractionation

For nucleocytoplasmic cell fractionation, 2×10^6 cells were trypsinized and either lysed directly in protein sample buffer or permeabilized with 0.015% digitonin in transport buffer on ice. After centrifugation at $230 \times g$ for 5 min, the supernatant was mixed with an equal volume of $2 \times$ SDS sample buffer. The pelleted nuclei were washed once, resuspended in transport buffer, and incubated with $1 \mu\text{M}$ Ran for 30 min at 30°C in the presence of an ATP-regenerating system. Nuclei were collected by centrifugation, washed once with transport buffer, and lysed in SDS sample buffer. The supernatant was mixed with sample buffer as above. Equivalent volumes of the individual fractions were analyzed by Western blotting.

SDS-PAGE and Western Blotting

Proteins were analyzed by SDS-PAGE and Western blotting using standard methods. The ECL system (Pierce, Rockford, IL) and a luminescent image analyzer (LAS-3000; Fuji, Tokyo, Japan) were used for visualization of proteins.

Immunofluorescence

Immunofluorescence staining was essentially performed as described (Hutten and Kehlenbach, 2006). For analysis of endogenous Nup358, importin β , and RanGAP, cells were grown on poly-L-lysine-coated coverslips and either fixed directly in 3.7% formaldehyde or after permeabilization with 0.015% digitonin in transport buffer and, when indicated, a subsequent incubation with $1 \mu\text{M}$ Ran in the presence of an ATP-regenerating system for 30 min at 30°C . Cells were analyzed by fluorescence microscopy using a Zeiss Axioskop2 microscope and AxioVision software. Pictures were processed using Adobe Photoshop (San Jose, CA).

RESULTS

Depletion of Nup358 Inhibits Importin α/β -dependent Import in Living Cells

To analyze the role of Nup358 in nuclear protein import, we took advantage of our RNA interference (RNAi) protocol for this nucleoporin (Hutten and Kehlenbach, 2006), which allows a strong reduction of the Nup358 level (Figure 1 and see Figures 5–7). A quantitative RT-PCR analysis revealed that the Nup358-RNA level in cells that had been treated with specific siRNAs was strongly reduced as well (Supplementary Figure S1), suggesting that the observed depletion

of the nucleoporin resulted from this reduced RNA level. We first used shuttling reporter proteins with pathway-specific NESs and NLSs and analyzed their subcellular localization in control cells and in Nup358-depleted cells. In steady state, GFP-reporter proteins containing a classic importin α/β -dependent NLS (cNLS) and either the CRM1-dependent NES of the HIV-1-Rev protein (Rev₄₈₋₁₁₆-GFP₂-cNLS; Fischer *et al.*, 1995) or of the NS2P protein of minute virus of mice (NS2P₇₆₋₉₇-GFP₂-cNLS; Askjaer *et al.*, 1999) localized predominantly to the nucleus in control cells, suggesting that the NLS is dominant over the NES. Strikingly, these proteins were largely excluded from the nuclear volume upon depletion of Nup358 (Figure 1A). This effect was also observed with other siRNAs targeting different regions of the Nup358-mRNA (Supplementary Figure S2). Formally, the relocation of the NES-GFP₂-cNLS (Rev₄₈₋₁₁₆-GFP₂-cNLS; NS2P₇₆₋₉₇-GFP₂-cNLS) constructs to the cytoplasm of Nup358-depleted cells could result from inhibited nuclear import or stimulated nuclear export. On the basis of previous findings that Nup358-depletion slightly inhibits CRM1-mediated export (Bernad *et al.*, 2004; Hutten and Kehlenbach, 2006), we consider an inhibition of nuclear import the more likely explanation. To confirm our results for another importin α/β -dependent substrate, we used an inducible nuclear import system (Love *et al.*, 1998). The reporter protein, a fusion of a portion of the glucocorticoid receptor and GFP linked to a classic NLS (GR₂-GFP₂-cNLS) was found predominantly in the cytoplasm in control cells as well as in Nup358-depleted cells (Figure 1B). On induction with the receptor ligand dexamethasone, we observed rapid nuclear import in control cells, but strongly reduced transport in depleted cells, corroborating our results with the shuttling reporter proteins. A quantification of this effect is shown in Figure 1C. Inducible import of GR₂-GFP₂-cNLS depended on the cNLS, as a protein lacking this import signal did not accumulate in the nucleus upon dexamethasone induction (data not shown). Together, using steady-state analysis of shuttling reporter proteins as well as analysis of nuclear import of an inducible substrate, our data show that Nup358 (or the Nup358-RanGAP complex) is required for efficient importin α/β -dependent import *in vivo*.

Figure 1. Nup358 promotes importin α/β -dependent nuclear import. (A) Mock-treated or Nup358-depleted cells were transiently transfected with constructs coding for Rev (aa 48-116)-GFP₂-cNLS or NS2P (aa 76-97)-GFP₂-cNLS, as indicated. Reporter proteins with shorter NES-fragments of the HIV-1-Rev protein showed the same effects (cf. Figure 7, B and C). (B and C) Mock-treated or Nup358-depleted HeLa cells were transfected with a plasmid coding for GR₂-GFP₂-cNLS and either fixed before (– dexamethasone) or after induction of import by the addition of dexamethasone for 15 min (+ dexamethasone). (A and B) Cells were stained for Nup358 and analyzed by fluorescence microscopy. Bar, 10 μm . (C) The mean distribution of GR₂-GFP₂-cNLS fluorescence 15 min after induction of import with dexamethasone is shown for mock-treated or Nup358-depleted cells in the three categories: N > C, N = C, and N < C. Bars, SD from the mean of three independent experiments.

Inhibition of NPC-associated RanGAP with Specific Antibodies

Nup358 is a giant nucleoporin with multiple functional domains (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). Furthermore, it stably associates with SUMOylated RanGAP (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). Hence, RanGAP is expected to be codepleted from the NPC upon depletion of Nup358 (see below). We therefore set out to systematically analyze the contribution of RanGAP and of the nucleoporin part of the Nup358-RanGAP complex in nuclear transport in vitro, using an antibody inhibition approach as well as Nup358-depleted cells.

Permeabilization of cells leads to a release of the soluble pool of RanGAP, leaving SUMOylated, Nup358-bound RanGAP behind (Matunis *et al.*, 1996; Mahajan *et al.*, 1997). Such cells do support nuclear import and export upon addition of Ran and appropriate transport receptors, suggesting that Nup358-associated RanGAP is capable and sufficient for efficient nuclear transport. Previously, rabbit anti-RanGAP antibodies have been described that strongly inhibited the catalytic activity of the enzyme. These antibodies also inhibited nuclear import in permeabilized cells, and NPC-associated RanGAP was suggested to be required for efficient transport (Mahajan *et al.*, 1997). Nuclear export was not analyzed in these experiments. We now reinvestigated these issues, using our established in vitro nuclear import and export systems and goat anti-RanGAP antibodies (Pichler *et al.*, 2002). We first tested the ability of affinity-purified goat anti-RanGAP antibodies to inhibit the activity of NPC-associated RanGAP, using digitonin-permeabilized cells as a source of RanGAP. The permeabilized cells did indeed promote the GTPase activity of Ran. The anti-RanGAP antibodies (Figure 2A), but not unspecific IgGs (data not shown), inhibited this activity. Half-maximal inhibition was observed between 16 and 32 $\mu\text{g/ml}$ antibody. The antibodies also efficiently inhibited the activity of soluble, recombinant RanGAP, as described previously for rabbit antibodies (Mahajan *et al.*, 1997; data not shown). We next compared the levels of GTPase inhibition in cells that were or were not washed after a preincubation with anti-RanGAP antibodies. The antibodies led to a strong inhibition of the GTPase activity, independent of the washing step (Supplementary Figure S3). We also tested the stability of the RanGAP-anti-

RanGAP interaction in the presence of soluble RanGAP (i.e., under conditions that resemble those in some transport reactions; compare Figure 3C). Adherent cells were permeabilized, preincubated with the anti-RanGAP antibody, and subjected to a second incubation in the absence or presence of recombinant RanGAP. Cells were then fixed and treated with a fluorescent secondary antibody. No significant differences in signal intensities at the nuclear rim could be detected (Supplementary Figure S4). These results suggest that our anti-RanGAP antibodies do not dissociate from their antigen during a subsequent incubation, at least not to a readily detectable extent. Thus, they appear suitable for the analysis of RanGAP requirements in nuclear transport in permeabilized cells.

NPC-associated RanGAP Promotes Nuclear Import and Export

Having established our anti-RanGAP antibodies as tools to inhibit the function of RanGAP, we set out to analyze their effects on nuclear transport. Our assay system allows a parallel analysis of nuclear import and export in the same cells, using a GFP-tagged version of the transcription factor NFAT as an export reporter and BSA-NLS-labeled with the fluorophore Cy5 as an import reporter (Kehlenbach *et al.*, 1998). HeLa cells expressing GFP-NFAT were permeabilized and preincubated in suspension with increasing concentrations of anti-RanGAP antibodies or, as a control, with unspecific IgG. After a washing step, cells were subjected to transport reactions and transport efficiencies were analyzed by flow cytometry. Preincubation with 150 $\mu\text{g/ml}$ antibody led to a complete inhibition of nuclear import (Figure 2B). Half-maximal inhibition was obtained with 40–60 $\mu\text{g/ml}$ anti-RanGAP antibody. The active antibody concentrations in this assay fit very well to those observed in RanGAP assays (Figure 2A), suggesting that reduced nuclear import indeed resulted from inhibited GTP-hydrolysis on Ran. Nuclear export was first visualized by fluorescence microscopy (Figure 2C). In the presence of CRM1 and Ran, nuclear export of GFP-NFAT was strongly inhibited in cells that had been preincubated with the anti-RanGAP antibody, compared with control cells. For a quantitative analysis of this effect, we used flow cytometry (Figure 2D). Maximal inhibition (~50%) of nuclear export was observed at an antibody

Figure 2. Anti-RanGAP antibodies inhibit nuclear import of BSA-NLS and export of GFP-NFAT in vitro. (A) Anti-RanGAP antibodies inhibit RanGAP activity in permeabilized cells. Digitonin-permeabilized cells were used as a source of RanGAP activity. Cells were preincubated with increasing concentrations of anti-RanGAP antibodies. (B–D) Nuclear import of Cy5-BSA-NLS (B) and export of GFP-NFAT (C and D) were analyzed by fluorescence microscopy (C) or flow cytometry (B and D). Permeabilized GFP-NFAT cells were preincubated with increasing concentrations (B and D) or 300 $\mu\text{g/ml}$ (C) of IgG or anti-RanGAP antibodies, followed by a washing step. (B and D) Reactions contained 2.4 μM Ran, 750 nM importin α , 250 nM importin β , and 225 nM CRM1. Note that nuclear export in D was analyzed in parallel in the very same reactions as nuclear import in B. (C) Reactions contained 2 μM Ran and 250 nM CRM1. Reactions were performed at 4 or 30°C, as indicated. Bar, 20 μm .

Figure 3. RanGAP reverses the inhibition of nuclear export by anti-RanGAP antibodies. Nuclear export of GFP-NFAT was analyzed by flow cytometry. Permeabilized cells were preincubated with $\sim 280 \mu\text{g/ml}$ unspecific IgG or anti-RanGAP antibodies. After a washing step, cells were subjected to export reactions. (A) Reactions contained 5 mg/ml cytosol as a source of transport factors. (B and C) Reactions contained 3.6 μM Ran, 700 nM CRM1, and 350 nM RanGAP (B) or increasing concentrations of RanGAP (C), as indicated. (D) Reactions contained 2.4 μM Ran, 750 nM importin α , 250 nM importin β , and 225 nM CRM1. RanGAP or RanGAP-KR at 150 nM was added to the reactions, as indicated. Reactions were performed at 4 or 30°C, and nuclear export of GFP-NFAT was analyzed by flow cytometry.

concentration of 250 $\mu\text{g/ml}$, half-maximal inhibition at $\sim 100 \mu\text{g/ml}$. The observation that significantly lower antibody concentrations were required for inhibition of import compared with export argues for a higher sensitivity of import toward RanGTP that fails to be hydrolyzed by RanGAP. Together, these results show that inhibition of NPC-associated RanGAP reduces both nuclear protein import and export.

Soluble RanGAP Promotes Nuclear Import and Export

We next addressed the question whether soluble RanGAP is able to rescue nuclear import and export in antibody-inhibited cells. First, we measured export kinetics in the presence of cytosol as a source of nuclear transport factors (Figure 3A). Under these conditions, anti-RanGAP-preincubated cells and control cells exhibited identical export kinetics. When cytosol was replaced by recombinant CRM1 and Ran, anti-RanGAP-treated cells showed significantly slower export kinetics compared with control cells (Figure 3B). This suggests that soluble RanGAP and/or other factors that are present in cytosol relieve the inhibition of nuclear export by the anti-RanGAP antibodies. One such cytosolic factor is RanBP1, which led to a significant stimulation of nuclear export in anti-RanGAP-treated cells (data not shown). This is in agreement with our previous observation that RanBP1 stimulated nuclear export in cells that had been preincubated with RanQ69L, a mutant form of Ran that is predominantly in the GTP-bound form (Kehlenbach *et al.*, 1999). This result also suggests that binding of anti-RanGAP antibodies to the NPC does not lead to a steric block of the transport channel, as export can be partially rescued by RanBP1. We next analyzed whether recombinant RanGAP stimulates nuclear export under such conditions as well. Indeed, in a time-course experiment, antibody-pretreated cells showed the same export kinetic as control cells, when soluble RanGAP was added to the reaction, together with Ran and CRM1 (Figure 3B). In intact cells, the cytoplasmic concentration of RanGAP has been estimated to be $\sim 0.7 \mu\text{M}$ (Görllich *et al.*, 2003), a large proportion ($\sim 80\%$; cf. Figure 6C) being SUMOylated. In Figure 3C, we analyzed the concentration range of soluble RanGAP that would stimulate nuclear export in our experimental system. As little as 7 nM soluble RanGAP clearly stimulated nuclear export in cells that had been preincubated with the anti-RanGAP antibody. At RanGAP concentrations of 70 and 140 nM, the nuclear

export efficiency was almost identical in anti-RanGAP-treated cells and control cells. Thus, the RanGAP concentration required for stimulation of export is clearly within the physiological range. These low concentrations also imply that the antibody did not simply inhibit the passage of export and import complexes through the pore by sterically blocking the transport channel. To exclude the possibility that recombinant RanGAP became SUMOylated during the transport reaction and subsequently associated with available binding sites at the NPC, we used a mutant form of RanGAP (RanGAP-KR), where the SUMO-acceptor site (lysine 526) is mutated to arginine. RanGAP-KR, which cannot be SUMOylated, stimulated nuclear export to a similar extent as wild-type RanGAP, indicating that NPC association is not required for activity (Figure 3D). Finally, we used N- and C-terminal fragments of RanGAP that are recognized by our anti-RanGAP antibody but are catalytically inactive. These fragments did not stimulate export in anti-RanGAP-preincubated cells (data not shown), suggesting that a trivial release of the anti-RanGAP antibody from its binding site in the presence of soluble RanGAP (or RanGAP fragments) is not the reason for the observed effects. Taken together, our results demonstrate that either NPC-associated RanGAP or soluble RanGAP can fulfill its function in nuclear export.

In analogy to nuclear export, we also analyzed the ability of soluble RanGAP to rescue nuclear import in anti-RanGAP-inhibited cells. As shown in Figure 4A, preincubation of permeabilized cells with the antibody strongly inhibited nuclear import of the reporter protein in the presence of the recombinant transport factors Ran, importin α , and importin β (cf. Figure 2B). This inhibition could partially be reversed by the addition of soluble RanGAP to the reaction. For a quantitative analysis, we again used the flow-cytometry-based assay. Here, soluble RanGAP clearly stimulated nuclear import in cells that had been preincubated with anti-RanGAP antibodies, but hardly in the control cells (Figure 4B). RanGAP-KR, which cannot be modified by SUMO, was also able to promote nuclear import in anti-RanGAP-preincubated cells. These results show that 1) NPC-associated RanGAP promotes nuclear import in permeabilized cells and that 2) soluble RanGAP can partially substitute for the NPC-bound protein.

Figure 4. Soluble RanGAP promotes nuclear import in vitro. (A) HeLa cells grown on coverslips were permeabilized, preincubated with IgG or anti-RanGAP, and subjected to nuclear import reactions in the presence of buffer or the transport factors importin α (750 nM), importin β (250 nM), Ran (6 μ M), and RanGAP (20 nM), as indicated. Cy3-labeled BSA-NLS was used as import substrate. Bar, 10 μ m. (B) Nuclear import was analyzed by flow cytometry, using GFP-NFAT cells. Permeabilized cells were preincubated with increasing concentrations of IgG or anti-RanGAP antibodies, followed by a washing step. Cy5-labeled BSA-NLS was used as import substrate. All reactions contained 2.4 μ M Ran, 750 nM importin α , 250 nM importin β , and 225 nM CRM1. RanGAP at 500 nM or RanGAP-KR at 150 nM, was added to the reactions, as indicated. Reactions were performed at 4 or 30°C, as indicated. Bars, variation from the mean of three independent experiments.

Importin β Becomes Rate-limiting in Nup358-depleted Cells

Using an antibody-inhibition approach, we showed that the RanGAP-portion of the Nup358-RanGAP complex stimulates both importin α/β -dependent nuclear import and CRM1-mediated nuclear export, at least in vitro. The nucleoporin part of the complex does not appear to have additional

Figure 5. Depletion of Nup358-RanGAP inhibits nuclear import in vitro. (A) Mock-treated and Nup358-depleted cells were mixed, permeabilized, and incubated with FITC-BSA-NLS, 1 μ M Ran, and 500 nM importin α in the presence of 100 nM importin β , 25 nM RanBP1, and 200 nM RanGAP, as indicated by the Roman numerals (I–IX; compare conditions in BI). (B) Quantification of nuclear import. Nuclear fluorescence in the presence of Ran (second row) was subtracted as background and import efficiency (IE) was expressed as the percentage of the obtained value in Nup358-depleted cells compared with control cells (bottom line). Error bars, SD from the mean of three independent experiments (50–100 cells each).

Figure 6. Depletion of Nup358-RanGAP by RNAi. (A) Mock-treated and Nup358-depleted cells were mixed and either fixed before (intact) or after (permeabilized) permeabilization with digitonin. To mimic transport conditions, cells were incubated at 30°C with Ran and an ATP-regenerating system for 30 min. Cells were stained for DNA, Nup358, RanGAP, and importin β , as indicated. Bar, 10 μ m. (B) Mock-treated and Nup358-depleted cells were lysed in SDS-sample buffer (t; lanes 1 and 5) or permeabilized and incubated with Ran and an ATP-regenerating system at 30°C. S₁ (lanes 2 and 6) corresponds to the supernatant after permeabilization, P (lanes 3 and 7) and S₂ (lanes 4 and 8) to the nuclear fraction and the supernatant after the incubation at 30°C, respectively. (C) Mock-treated cells and Nup358-depleted cells were lysed in SDS-sample buffer and analyzed by Western blotting for levels of Nup358 and sumoylated and unsumoylated RanGAP. The equivalents of $\sim 2 \times 10^5$ (100%) or 1×10^5 (50%) cells were loaded. (B and C) α -tubulin served as a loading control.

functions in nuclear export, as this transport pathway was only slightly inhibited in Nup358-depleted cells (Bernad *et al.*, 2004; Hutten and Kehlenbach, 2006). By contrast, our observation of import inhibition in living cells (Figure 1) clearly points to additional functions of the nucleoporin part of the Nup358-RanGAP complex. Nup358 was initially characterized as a RanGTP-binding protein containing four domains with homology to the soluble protein RanBP1 (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). Moreover, importin β has been shown to be targeted to Nup358 by RanGTP (Delphin *et al.*, 1997). Furthermore, transport receptors interact with nucleoporins via FG-repeat regions (Allen *et al.*, 2001), which are also present in Nup358. These interactions may facilitate the nuclear import of transport complexes and/or the recycling of transport receptors. We therefore analyzed the effects of depleting Nup358 on nuclear import in permeabilized cells and possible compensatory functions of the soluble transport factors importin β , RanBP1, and RanGAP. To allow a side-by-side analysis of transport efficiencies, depleted cells and control cells were mixed after the transport reaction, grown on coverslips, and then subjected to transport reactions using BSA-NLS as a substrate. Nuclear import was specific in control cells as well as in Nup358-depleted cells, as it was strongly inhibited by wheat germ agglutinin, a lectin that blocks various nucleocytoplasmic transport pathways (Yoneda *et al.*, 1987; Dargemont and Kuhn, 1992; Figure 5, A and B; cf. conditions I and VI). Importin α together with Ran was able to promote nuclear import of BSA-NLS to a significant level in control cells (condition II). The addition of importin β is not required under these conditions, as the cells retain a certain level of this transport receptor upon permeabilization (cf. Figure 6, A and C). Strikingly, Nup358-depleted cells showed only very limited nuclear import

when no importin β was added to the reaction, even though these cells retained similar levels of the endogenous import receptor, compared with the control cells (cf. Figure 6B). The addition of importin β , RanGAP, or RanBP1 slightly stimulated nuclear import in Nup358-depleted cells and also in control cells (III–V). Stimulation in depleted cells was more pronounced when importin β was added together with either RanGAP (condition VI) or RanBP1 (condition VII). Maximal stimulation of nuclear import in Nup358-depleted cells was observed when importin β , RanGAP, and RanBP1 were all included in the reaction (condition IX). Here, import in depleted cells reached 64% of that observed in control cells. Together, these results lead to the hypothesis that the Nup358-RanGAP complex fulfills additional functions in importin α/β -dependent nuclear import, besides promoting GTP hydrolysis on Ran. First, the Ran-binding domains of Nup358 may have similar functions as soluble RanBP1. Second, importin β is more rate-limiting for nuclear import in Nup358-depleted cells, suggesting that the nucleoporin promotes the utilization of the transport receptor.

A trivial explanation for the observed effect of importin β becoming rate-limiting would be a reduced total concentration of the transport receptor in Nup358-depleted cells. Indeed, this has been reported for *Drosophila* cells (Sabri *et al.*, 2007). We therefore analyzed importin β upon depletion of Nup358 in human cells. In intact control cells as well as in Nup358-depleted cells, similar amounts of importin β localized to the cytoplasm as well as to the nucleus, consistent with previous observations (Salina *et al.*, 2003; Hutten and Kehlenbach, 2006; Figure 6A). On permeabilization with digitonin, association of importin β with the nuclear envelope became apparent. When permeabilized cells were incubated with an ATP-regenerating system and Ran (i.e., under transport conditions), importin β was partially lost from the nuclear envelopes of Nup358-depleted cells. Wild-type cells, by contrast, retained a high level of importin β at the nuclear envelope under these conditions, suggesting that Nup358 is a major binding site for the transport receptor. We also analyzed importin β by Western blotting. As shown in Figure 6B, the total level of importin β did not change significantly upon depletion of Nup358 (cf. lanes 1 and 5). Likewise, very similar amounts of importin β appeared in the soluble fraction after digitonin permeabilization (lanes 2 and 6). The residual importin β that remained associated with the permeabilized cells, however, behaved differently after a subsequent incubation under transport conditions. In control cells, similar amounts of importin β were recovered in the pellet fraction and the soluble fraction (cf. lanes 3 and 4). In Nup358-depleted cells, by contrast, the vast majority of importin β became soluble during the incubation (lanes 7 and 8). Together with the immunofluorescence data, these results suggest that Nup358 serves as a binding site for importin β , keeping the transport receptor in association with the NPC and largely preventing its loss from the permeabilized cells during a transport reaction.

We also investigated the effect of Nup358-depletion on soluble and NPC-associated RanGAP. When visualized by indirect immunofluorescence, RanGAP localizes predominantly to the nuclear envelope, similar to its binding partner, the nucleoporin Nup358 (Figure 6A). As expected, depletion of Nup358 by RNA-interference led to the concomitant loss of RanGAP from the nuclear pore. To investigate, whether total cellular levels of RanGAP were affected by the depletion of Nup358, we performed Western blot analysis. Two forms of RanGAP can easily be distinguished: the unmodified form, migrating at ~70 kDa and the SUMOylated form, migrating at ~90 kDa (Matunis *et al.*, 1996; Mahajan *et al.*,

1997). A large proportion of the 90 kDa-form is known to associate with Nup358. As shown in Figure 6C, SUMO-RanGAP is the predominant form of the protein in a total lysate from control cells. On depletion of Nup358, the level of SUMO-RanGAP decreased, whereas that of free RanGAP significantly increased. Thus, depletion of the binding partner for RanGAP at the nuclear pore does not dramatically affect the total level of RanGAP, but reverses the ratio of the SUMOylated and the unmodified form of the protein. This suggests that the interaction with Nup358 stabilizes the SUMO modification of RanGAP, possibly by protecting it from a de-SUMOylating activity (Zhang *et al.*, 2002). The observation that unmodified RanGAP is as active as SUMOylated RanGAP in activating GTP-hydrolysis on Ran (Swaminathan *et al.*, 2004) together with the notion that soluble RanGAP can stimulate nuclear import (Figure 4) leads to the prediction that cells lacking NPC-associated RanGAP (but retaining Nup358) should not be compromised with respect to nuclear import *in vivo*. To directly test this hypothesis, we devised a strategy to remove RanGAP from the nuclear pore, leaving Nup358 in place. To this end, we transfected a fragment of Nup358 from a region that interacts with RanGAP (Matunis *et al.*, 1998) and that should serve as a competing binding site. Indeed, in cells overexpressing this fragment, the RanGAP concentration at the nuclear envelope was strongly reduced to a level comparable to that observed in Nup358-depleted cells (Figure 7A and cf. Figure 6A). In such cells, the shuttling reporter protein NES-GFP₂-cNLS localized predominantly to the nucleus, as in control cells, suggesting that the RanGAP portion of the Nup358-RanGAP complex is not responsible for the effects observed in Nup358-depleted cells (cf. Figure 1A) and that soluble RanGAP can efficiently promote nuclear import of this reporter *in vivo*. A possible explanation for the observation that maximal import *in vitro* was not achieved in anti-RanGAP-inhibited or in Nup358-depleted cells by the addition of soluble RanGAP (cf. Figures 4 and 5) is that NPC-associated RanGAP helps to optimize nuclear import. Such an effect may be relevant in permeabilized cells, where the reaction volume is very large compared with the nuclear volume and, perhaps, in intact cells with a large ratio of cytoplasm to nucleus, but not in HeLa cells that were used in the *in vivo* experiments in Figure 7A.

Finally, we tested the hypothesis that importin β is rate-limiting for nuclear import at low Nup358 concentrations in intact cells. As shown before (cf. Figure 1A), depletion of Nup358 strongly reduced nuclear import of the shuttling reporter protein NES-GFP₂-cNLS. Strikingly, overexpression of importin β , but not transportin, in Nup358-depleted cells largely restored the nuclear localization of the reporter protein (Figures 7, B and C), demonstrating that the transport receptor indeed became rate-limiting upon depletion of Nup358.

DISCUSSION

In this study, we show that the Nup358-RanGAP complex plays various roles in importin α/β -dependent nuclear protein import. The RanGAP function of the complex can partially be fulfilled by soluble RanGAP. The nucleoporin part of the complex promotes nuclear import, apparently by facilitated use of the available pool of importin β . In CRM1-mediated export, the RanGAP function can be carried out efficiently by the soluble or the pore-associated form of the protein. Here, the nucleoporin part of the Nup358-RanGAP complex does not appear to have additional functions.

Figure 7. Importin β is the rate-limiting import factor in Nup358-depleted cells. (A) HeLa cells were cotransfected with constructs coding for HIV-1-Rev (aa 48-116)-GFP₂-cNLS and HA-Nup358 (aa 2595-2881) at a ratio of 1:7, stained for RanGAP and HA-Nup358, and analyzed by fluorescence microscopy. (B) Mock- or siRNA-treated cells were cotransfected with Rev (aa 68-90)-GFP₂-cNLS and either empty vector (top panel), a plasmid coding for HA-importin β (middle panel) or HA-transportin (bottom panel) at a ratio of 1:5, stained for Nup358 and HA-tagged protein, and analyzed by fluorescence microscopy. Bars, 10 μ m. A quantification of cells showing a clear nuclear localization ($N > C$) of the reporter protein in the absence or presence of HA-importin β (HA-Imp β) or HA-transportin (HA-Trn) is shown in C. Error bars, the variation from the mean of two independent experiments (>100 cells each).

Soluble and NPC-associated RanGAP Are Able to Promote Nuclear Export and Import

For single round nuclear import and export, RanGTP-hydrolysis is not an essential step (Richards *et al.*, 1997; Schwoebel *et al.*, 1998; Englmeier *et al.*, 1999; Nachury and Weis, 1999). Hence, impaired transport as observed upon inhibition of RanGAP activity with specific antibodies is likely to result from "secondary" effects. In nuclear import, recycling of the transport receptor requires GTP hydrolysis on Ran, allowing the next round of transport. In nuclear export, GTP hydrolysis promotes the release of the export complex from a terminal binding site at the NPC (Kehlenbach *et al.*, 1999), so that the export receptor can recycle back to the nucleus. In cells where soluble RanGAP is lost upon digitonin permeabilization, the NPC-associated form of the protein can efficiently promote nuclear import and export. We now analyzed the contribution of soluble RanGAP to nuclear transport.

Inhibition of NPC-associated RanGAP with specific antibodies led to reduced nuclear export of GFP-NFAT in our *in vitro* assay. The level of maximal inhibition ($\sim 50\%$) is in good agreement with previous observations, where RanQ69L, a Ran mutant that is insensitive to RanGAP, led to a very similar inhibition in the presence of cytosol (Kehlenbach *et al.*, 1998). A possible explanation is that GTP hydrolysis on Ran is not needed for a first round of CRM1-dependent nuclear export. For ongoing transport however, GTP hydrolysis is required, e.g., for the release of CRM1 from the cytoplasmic nucleoporin Nup214 (Kehlenbach *et al.*, 1999). Low levels of soluble RanGAP were able to fully restore nuclear export in anti-RanGAP-treated cells. This observation explains why depletion of Nup358 did not result in strong export defects *in vivo* (Bernad *et al.*, 2004; Forler *et al.*, 2004; Hutten and Kehlenbach, 2006), where soluble RanGAP would be retained in the cytoplasm. Together, both soluble and pore-associated RanGAP can promote CRM1-dependent export. It remains possible that specific transport substrates like certain CRM1-dependent RNAs require very efficient mechanisms involving NPC-associated RanGAP for the dissociation of export complexes.

As for nuclear import, NPC-associated RanGAP is sufficient for nuclear import in permeabilized cells, a notion that

is also supported by our antibody inhibition experiments. Here as well as in Nup358-depletion experiments *in vitro*, free RanGTP will escape from the nucleus and prevent the assembly of functional import complexes in the cytosol. Furthermore, recycling importin β leaves the nucleus in a complex with RanGTP. RanGAP together with RanBP1 (or the Ran-binding domains of Nup358) and importin α are required for disassembly of this complex (Bischoff and Görlich, 1997; Floer *et al.*, 1997), and thus, for the formation of novel import complexes. Under these conditions, nuclear import can partially be rescued by the addition of soluble RanGAP. In intact cells, the inhibition of importin α/β -dependent import did not result from the observed codepletion of RanGAP from the nuclear envelope in Nup358-depleted cells, as a shuttling reporter protein still localized to the nucleus when RanGAP was removed from the pore by overexpression of a Nup358 fragment (Figure 7A). Together, NPC-associated as well as soluble RanGAP are able to promote nuclear import. The question remains, to what extent the two forms contribute to RanGTP hydrolysis under physiological conditions. It has been calculated that only a small percentage of RanGTP would be hydrolyzed in the zone adjacent to the NPC (Görlich *et al.*, 2003). These calculations, however, were made for free RanGTP. In a complex with importins or exportins, it would diffuse much more slowly and could also be retained at the NPC upon interaction of the transport receptor with nucleoporins. Therefore, Nup358-associated RanGAP could have ample time to act on such a complex. Soluble RanGAP may preferentially act on free RanGTP that escapes from the nucleus (see below).

Nup358 Is Required for Efficient Nuclear Import

We and others have recently reported that Nup358 is not strictly required for nuclear protein import (Walther *et al.*, 2002; Salina *et al.*, 2003; Forler *et al.*, 2004; Hutten and Kehlenbach, 2006). By deduction, also NPC-associated RanGAP appears dispensable for nuclear import. How can these published results be interpreted in light of our finding that depletion of Nup358-RanGAP clearly inhibits importin α/β -dependent import *in vitro* and *in vivo*? As importin α/β -dependent import *per se* is clearly possible without Nup358, the subcellular localiza-

tion of a reporter protein will depend on relative import and export rates in depleted cells versus control cells. A key difference between the different experimental systems is that in our study, we used reporter proteins with well-defined import and export signals. NES-GFP₂-cNLS as well as the inducible reporter GR₂-GFP₂-cNLS showed reduced nuclear import in Nup358-depleted cells, similar to the PYM protein that was used previously (Forler *et al.*, 2004). Complex reporter proteins like the glucocorticoid receptor- β -galactosidase fusion protein (Salina *et al.*, 2003) or GFP-NFAT (Hutten and Kehlenbach, 2006) might be imported via alternative import pathways that are not strongly affected by reduced levels of Nup358. Indeed, the glucocorticoid receptor can be imported by importin α/β , importin 7, or importin 13 (Freedman and Yamamoto, 2004; Tao *et al.*, 2006). In our in vitro nuclear import assays, import was inhibited in Nup358-depleted cells as well as in anti-RanGAP-pre-treated cells but was stimulated by RanGAP and other soluble factors like importin β . In *Xenopus* oocytes, nuclear import of BSA-NLS in vitro in the presence of cytosol proceeded with similar rates, in the absence or presence of Nup358 (Walther *et al.*, 2002), probably because the cytosol contained saturating amounts of soluble RanGAP and importin β .

Importin β Becomes Rate-limiting in the Absence of Nup358

In previous studies that reported reduced nuclear import in Nup358-depleted cells (Forler *et al.*, 2004; Sabri *et al.*, 2007), the underlying molecular mechanisms were not analyzed. Importin β now appears to be the most rate-limiting factor at reduced concentrations of the nucleoporin, as nuclear localization of our shuttling reporter protein was strongly increased by the overexpression of importin β in Nup358-

depleted cells. In agreement with previous results (Salina *et al.*, 2003), total levels of importin β did not change significantly upon Nup358 depletion, in contrast to what has recently been described for *Drosophila* cells (Sabri *et al.*, 2007). However, our analyses showed significantly reduced amounts of importin β that associated with nuclei of Nup358-depleted and digitonin-permeabilized cells after a transport reaction. Strikingly, control cells showed significant import when only Ran and importin α were added, probably because the cells retained a certain level of importin β after permeabilization and because Nup358 allowed an efficient utilization of this available pool. Nup358-depleted cells, by contrast, showed only very little nuclear import under the same conditions, although the residual level of importin β after digitonin permeabilization did not vary significantly compared with control cells. A combination of soluble Ran, importin α , importin β , RanBP1, and RanGAP strongly stimulated nuclear import in Nup358-depleted cells. These results suggest that Nup358 fulfills multiple functions in nuclear import. First, Nup358 serves as a binding site for RanGAP, which is required for nuclear import, but which also functions as a soluble protein. Second, the Ran-binding domains of Nup358 may stimulate nuclear import, similar to soluble RanBP1 (Chi *et al.*, 1996). This stimulation might result from enhanced RanGAP activity (Bischoff *et al.*, 1995) and/or from facilitated release of RanGTP from recycling importin β (Bischoff and Görlich, 1997; Floer *et al.*, 1997). Third and most importantly, Nup358 decreases the level of importin β that is required for efficient nuclear import. Two scenarios can be envisaged to explain this novel observation: 1) The nucleoporin might function as an assembly platform for incoming import complexes, as suggested before (Yokoyama *et al.*, 1995) and 2) Nup358 might concentrate recycling importin β at the cytoplasmic

Figure 8. The model depicts two alternative pathways for importin α/β -dependent nuclear import. (A) Disassembly of recycling importin β bound to RanGTP and assembly of a new import complex occurs in the cytoplasm, promoted by soluble RanGAP and RanBP1. These soluble proteins may also be used for free RanGTP that escapes from the nucleus in an uncomplexed form. (B) Recycling importin β -RanGTP interacts with the Ran-binding domains of Nup358, which, together with associated RanGAP, coordinates GTP-hydrolysis on Ran and formation of a novel import complex.

side of the NPC. Indeed, RanGTP, which associates with importin β during its export, targets the import receptor to Nup358 (Delphin *et al.*, 1997). GTP hydrolysis at this site has been suggested to be required for reinitiating nuclear import (Yaseen and Blobel, 1999). Recently, import receptors in general have been proposed to be rate-limiting factors in yeast (Timney *et al.*, 2006) and in higher eukaryotes (Yang and Musser, 2006). In the latter study, it was shown that import efficiencies are most sensitive to changes of the importin β concentration around its physiological level. Combining these three functions leads to a model where Nup358 serves as a binding platform that couples nuclear export of RanGTP-importin β with import of an importin β -containing transport complex. To release RanGTP from importin β , RanGAP, RanBP1 (or the RanGTP-binding domains of Nup358) and importin α are required. The dissociation reaction is further stimulated by an NLS-import substrate (Yaseen and Blobel, 1999). Together, Nup358-RanGAP appears ideally situated for coordination of recycling of importin β and reassembly of import complexes. In a model similar to that suggested by Yaseen and Blobel (1999); recycling importin β interacts with the Nup358-RanGAP complex before and after GTP hydrolysis on Ran (Figure 8B) and hence, does not leave the region of the NPC. This would lead to an increase in the active concentration of importin β , in agreement with our observations in Figure 6, A and B. As a result, nuclear import can occur very efficiently. As binding sites for transport factors at Nup358 are limited, GTP hydrolysis on Ran and reformation of an import complex can be also be triggered by soluble RanGAP, together with RanBP1 (Figure 8A). This may also be relevant for free RanGTP that escapes from the nucleus, passing NPC-associated RanGAP unaffected because of its high diffusion rate (Görlich *et al.*, 2003). Yeast cells lack NPC-associated RanGAP and must therefore exclusively use this pathway. It remains to be investigated to what extent the two transport modes actually contribute to nuclear import in vertebrate cells under physiological conditions.

Nup358 can also function as a SUMO-1 E3 ligase (Pichler *et al.*, 2002) and SUMOylation has been suggested to play a role in nucleocytoplasmic transport, at least in yeast (Stade *et al.*, 2002). SUMOylation of our artificial import cargos, however, appears rather unlikely. Furthermore, there is no evidence so far for Nup358-dependent SUMOylation of any component of the transport machinery, e.g., a transport receptor. Nevertheless, we cannot exclude the possibility that defects in SUMOylation are somehow involved in reduced nuclear import in the absence of Nup358.

Although our study shows a clear requirement for Nup358 in efficient importin α/β -dependent transport, similar mechanisms might be at play in other pathways, e.g., in transportin-dependent import. It will be interesting to analyze whether the depletion of Nup358 affects the nucleocytoplasmic distribution of cellular proteins using various nuclear import pathways.

ACKNOWLEDGMENTS

We are grateful to Donna Love (National Institutes of Health, Bethesda, MD), John Hanover (National Institutes of Health), Dirk Görlich (Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany), Ulrike Kutay (ETH, Zürich, Switzerland), Larry Gerace, and Joachim Hauber (Heinrich-Pette-Institut, Hamburg, Germany) for the generous gift of reagents. Christiane Spillner is acknowledged for excellent technical assistance. The project was supported by grants from the Deutsche Forschungsgemeinschaft to R.K. (KE 660/5-1) and F.M. (SFB523, TP18).

REFERENCES

- Allen, N. P., Huang, L., Burlingame, A., and Rexach, M. (2001). Proteomic analysis of nucleoporin interacting proteins. *J. Biol. Chem.* 276, 29268–29274.
- Askjaer, P. *et al.* (1999). RanGTP-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-box helicase. *Mol. Cell. Biol.* 19, 6276–6285.
- Baake, M., Bauerle, M., Doenecke, D., and Albig, W. (2001). Core histones and linker histones are imported into the nucleus by different pathways. *Eur. J. Cell Biol.* 80, 669–677.
- Bernad, R., van der Velde, H., Fornerod, M., and Pickersgill, H. (2004). Nup358/RanBP2 attaches to the nuclear pore complex via association with Nup88 and Nup214/CAN and plays a supporting role in CRM1-mediated nuclear protein export. *Mol. Cell. Biol.* 24, 2373–2384.
- Bischoff, F. R., and Görlich, D. (1997). RanBP1 is crucial for the release of RanGTP from importin beta-related nuclear transport factors. *FEBS Lett.* 419, 249–254.
- Bischoff, F. R., Krebber, H., Smirnova, E., Dong, W., and Ponstingl, H. (1995). Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO J.* 14, 705–715.
- Charneau, P., Mirambeau, G., Roux, P., Paulous, S., Buc, H., and Clavel, F. (1994). HIV-1 reverse transcription. A termination step at the center of the genome. *J. Mol. Biol.* 241, 651–662.
- Chi, N. C., Adam, E. J., Visser, G. D., and Adam, S. A. (1996). RanBP1 stabilizes the interaction of Ran with p97 nuclear protein import. *J. Cell Biol.* 135, 559–569.
- Chi, N. C., and Adam, S. A. (1997). Functional domains in nuclear import factor p97 for binding the nuclear localization sequence receptor and the nuclear pore. *Mol. Biol. Cell* 8, 945–956.
- Dargemont, C., and Kuhn, L. C. (1992). Export of mRNA from microinjected nuclei of *Xenopus laevis* oocytes. *J. Cell Biol.* 118, 1–9.
- Delphin, C., Guan, T., Melchior, F., and Gerace, L. (1997). RanGTP Targets p97 to RanBP2, a filamentous protein localized at the cytoplasmic periphery of the nuclear pore complex. *Mol. Biol. Cell* 8, 2379–2390.
- Englmeier, L., Olivo, J. C., and Mattaj, I. W. (1999). Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. *Curr. Biol.* 9, 30–41.
- Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Lührmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* 82, 475–483.
- Floor, M., Blobel, G., and Rexach, M. (1997). Disassembly of RanGTP-karyopherin beta complex, an intermediate in nuclear protein import. *J. Biol. Chem.* 272, 19538–19546.
- Forler, D., Rabut, G., Ciccarelli, F. D., Herold, A., Kocher, T., Niggeweg, R., Bork, P., Ellenberg, J., and Izaurralde, E. (2004). RanBP2/Nup358 provides a major binding site for NXF1-p15 dimers at the nuclear pore complex and functions in nuclear mRNA export. *Mol. Cell. Biol.* 24, 1155–1167.
- Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* 90, 1051–1060.
- Freedman, N. D., and Yamamoto, K. R. (2004). Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. *Mol. Biol. Cell* 15, 2276–2286.
- Fried, H., and Kutay, U. (2003). Nucleocytoplasmic transport: taking an inventory. *Cell. Mol. Life Sci.* 60, 1659–1688.
- Fukuda, M., Asano, S., Nakamura, T., Adachi, M., Yoshida, M., Yanagida, M., and Nishida, E. (1997). CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 390, 308–311.
- Gasteier, J. E., Madrid, R., Krautkramer, E., Schroder, S., Muranyi, W., Benichou, S., and Fackler, O. T. (2003). Activation of the Rac-binding partner FHOD1 induces actin stress fibers via a ROCK-dependent mechanism. *J. Biol. Chem.* 278, 38902–38912.
- Görlich, D., Seewald, M. J., and Ribbeck, K. (2003). Characterization of Ran-driven cargo transport and the RanGTPase system by kinetic measurements and computer simulation. *EMBO J.* 22, 1088–1100.
- Hu, T., Guan, T., and Gerace, L. (1996). Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J. Cell Biol.* 134, 589–601.
- Hutten, S., and Kehlenbach, R. H. (2006). Nup214 is required for CRM1-dependent nuclear protein export in vivo. *Mol. Cell. Biol.* 26, 6772–6785.
- Kehlenbach, R. H., Assheuer, R., Kehlenbach, A., Becker, J., and Gerace, L. (2001). Stimulation of nuclear export and inhibition of nuclear import by a Ran mutant deficient in binding to Ran-binding protein 1. *J. Biol. Chem.* 276, 14524–14531.

- Kehlenbach, R. H., Dickmanns, A., and Gerace, L. (1998). Nucleocytoplasmic shuttling factors including Ran and CRM1 mediate nuclear export of NFAT in vitro. *J. Cell Biol.* 141, 863–874.
- Kehlenbach, R. H., Dickmanns, A., Kehlenbach, A., Guan, T., and Gerace, L. (1999). A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. *J. Cell Biol.* 145, 645–657.
- Kehlenbach, R. H., and Gerace, L. (2002). Analysis of nuclear protein import and export in vitro using fluorescent cargoes. *Methods Mol. Biol.* 189, 231–245.
- Love, D. C., Sweitzer, T. D., and Hanover, J. A. (1998). Reconstitution of HIV-1 rev nuclear export: independent requirements for nuclear import and export. *Proc. Natl. Acad. Sci. USA* 95, 10608–10613.
- Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88, 97–107.
- Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* 135, 1457–1470.
- Matunis, M. J., Wu, J., and Blobel, G. (1998). SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. *J. Cell Biol.* 140, 499–509.
- Melchior, F., Sweet, D. J., and Gerace, L. (1995). Analysis of Ran/TC4 function in nuclear protein import. *Methods Enzymol.* 257, 279–291.
- Nachury, M. V., and Weis, K. (1999). The direction of transport through the nuclear pore can be inverted. *Proc. Natl. Acad. Sci. USA* 96, 9622–9627.
- Ossareh-Nazari, B., Bachelier, F., and Dargemont, C. (1997). Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* 278, 141–144.
- Paschal, B. M., and Gerace, L. (1995). Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* 129, 925–937.
- Pichler, A., Gast, A., Seeler, J. S., Dejean, A., and Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* 108, 109–120.
- Rexach, M., and Blobel, G. (1995). Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* 83, 683–692.
- Richards, S. A., Carey, K. L., and Macara, I. G. (1997). Requirement of guanosine triphosphate-bound ran for signal-mediated nuclear protein export. *Science* 276, 1842–1844.
- Rose, A., and Meier, I. (2001). A domain unique to plant RanGAP is responsible for its targeting to the plant nuclear rim. *Proc. Natl. Acad. Sci. USA* 98, 15377–15382.
- Sabri, N., Roth, P., Xylourgidis, N., Sadeghifar, F., Adler, J., and Samakovlis, C. (2007). Distinct functions of the *Drosophila* Nup153 and Nup214 FG domains in nuclear protein transport. *J. Cell Biol.* 178, 557–565.
- Salina, D., Enarson, P., Rattner, J. B., and Burke, B. (2003). Nup358 integrates nuclear envelope breakdown with kinetochore assembly. *J. Cell Biol.* 162, 991–1001.
- Schwoebel, E. D., Talcott, B., Cushman, I., and Moore, M. S. (1998). Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. *J. Biol. Chem.* 273, 35170–35175.
- Shah, S., and Forbes, D. J. (1998). Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors. *Curr. Biol.* 8, 1376–1386.
- Stade, K., Ford, C. S., Guthrie, C., and Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* 90, 1041–1050.
- Stade, K., Vogel, F., Schwienhorst, I., Meusser, B., Volkwein, C., Nentwig, B., Dohmen, R. J., and Sommer, T. (2002). A lack of SUMO conjugation affects cNLS-dependent nuclear protein import in yeast. *J. Biol. Chem.* 277, 49554–49561.
- Swaminathan, S., Kiendl, F., Korner, R., Lupetti, R., Hengst, L., and Melchior, F. (2004). RanGAP1*SUMO1 is phosphorylated at the onset of mitosis and remains associated with RanBP2 upon NPC disassembly. *J. Cell Biol.* 164, 965–971.
- Tao, T., Lan, J., Lukacs, G. L., Hache, R. J., and Kaplan, F. (2006). Importin 13 regulates nuclear import of the glucocorticoid receptor in airway epithelial cells. *Am. J. Respir. Cell. Mol. Biol.* 35, 668–680.
- Terry, L. J., and Wente, S. R. (2007). Nuclear mRNA export requires specific FG nucleoporins for translocation through the nuclear pore complex. *J. Cell Biol.* 178, 1121–1132.
- Timney, B. L., Tetenbaum-Novatt, J., Agate, D. S., Williams, R., Zhang, W., Chait, B. T., and Rout, M. P. (2006). Simple kinetic relationships and nonspecific competition govern nuclear import rates in vivo. *J. Cell Biol.* 175, 579–593.
- Tran, E. J., and Wente, S. R. (2006). Dynamic nuclear pore complexes: life on the edge. *Cell* 125, 1041–1053.
- Walther, T. C., Pickersgill, H. S., Cordes, V. C., Goldberg, M. W., Allen, T. D., Mattaj, I. W., and Fornerod, M. (2002). The cytoplasmic filaments of the nuclear pore complex are dispensable for selective nuclear protein import. *J. Cell Biol.* 158, 63–77.
- Weis, K. (2007). The nuclear pore complex: oily spaghetti or gummy bear? *Cell* 130, 405–407.
- Wu, J., Matunis, M. J., Kraemer, D., Blobel, G., and Coutavas, E. (1995). Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.* 270, 14209–14213.
- Yang, W., and Musser, S. M. (2006). Nuclear import time and transport efficiency depend on importin beta concentration. *J. Cell Biol.* 174, 951–961.
- Yaseen, N. R., and Blobel, G. (1999). GTP hydrolysis links initiation and termination of nuclear import on the nucleoporin nup358. *J. Biol. Chem.* 274, 26493–26502.
- Yokoyama, N., Hayashi, N., Seki, T., Pante, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M., and Nishimoto, T. (1995). A giant nucleopore protein that binds Ran/TC4. *Nature* 376, 184–188.
- Yoneda, Y., Imamoto-Sonobe, N., Yamaizumi, M., and Uchida, T. (1987). Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. *Exp. Cell Res.* 173, 586–595.
- Zhang, H., Saitoh, H., and Matunis, M. J. (2002). Enzymes of the SUMO modification pathway localize to filaments of the nuclear pore complex. *Mol. Cell. Biol.* 22, 6498–6508.