Ran-dependent Signal-mediated Nuclear Import
Does Not Require GTP Hydrolysis by Ran*

(Received for publication, September 16, 1998)

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Nuclear import of classical nuclear localization sequence-containing proteins involves the assembly of an import complex at the cytoplasmic face of the nuclear pore complex (NPC) followed by movement of this complex through the NPC and release of the import substrate into the nuclear interior. This process has historically been thought to require nucleotide hydrolysis as a source of energy. We found, using hydrolysis-resistant GTP analogs and a mutant Ran unable to hydrolyze GTP, that transport of classical nuclear localization sequence-containing substrate through the NPC and release of the substrate into the nucleus did not require hydrolysis of GTP by Ran. In fact, for movement of this type of import substrate into the nuclear interior we did not observe a requirement for hydrolysis of any nucleotide triphosphate. We did, however, find that a pool of free GTP (or its structural equivalent) must be added, probably because the GDP Ran that is added must be converted to GTP Ran during the import process. We found that a requirement for GTP hydrolysis can be restored to an import mixture consisting of recombinant import factors by the addition of RCC1, the Ran guanine nucleotide exchange factor.

Many nuclear proteins contain a nuclear localization sequence (NLS), the classical type of which consists of either a single or bipartite stretch of primarily basic amino acids. The presence of an NLS on a reporter results in its rapid transport from the cytoplasm through the nuclear pore complex (NPC) and into the nuclear interior. This process can be reconstituted in vitro using cells that have been treated with a low concentration of digitonin, which permeabilizes the plasma membrane to the passage of macromolecules but leaves the nuclear membrane intact (1). Addition of cytosol as a source of import factors, a nucleoside triphosphate (NTP) as a source of energy, and import substrate to these permeabilized cells results in import of the substrate into the nucleus. From this cytosol, four import factors have been identified that together efficiently support nuclear import of a basic NLS-containing substrate in permeabilized cells in the absence of cytosol. The receptor for the classical or basic NLS (karyopherin/importin α), together with karyopherin/importin β1, targets the import substrate to the NPC (2–8). Thus the addition of these two import factors alone to an in vitro import assay results in binding of import substrate at the nuclear envelope in a characteristic rim pattern. Subsequent passage of import substrate from the NPC into the nuclear interior requires the addition of two other import factors: p10/NTF2 (9, 10) and the small GTPase Ran (11, 12). The exact role of the nuclear import factor p10, which preferentially binds GDP-Ran rather than GTP-Ran, has yet to be defined. Ran, however, has been shown to be a key player not only for the nuclear import of a variety of substrates bearing different types of NLSs, but also for the nuclear export of most RNAs and a number of proteins containing different types of nuclear export signals (13, 14).

Two additional proteins, which are among those retained in large amounts in permeabilized cells, are considered vital to regulation of nuclear import. RCC1, the Ran guanine nucleotide exchange factor (GEF), contains a basic NLS and is localized primarily inside the nucleus bound to chromatin (15, 16). In contrast, the majority of RanGAP1, the Ran GTPase-activating protein (GAP), is found both free in the cytoplasm and bound on the cytoplasmic filaments of the NPC (17–19). The differential localization of these two proteins is predicted to play a role in controlling the direction of nuclear transport by controlling the assembly and disassembly of transport complexes in response to the relative predicted concentrations of GDP-Ran (predominantly cytoplasmic) and GTP-Ran (predominantly nuclear) (20). Significantly, the addition of GTP-Ran (instead of GDP-Ran) to the cytoplasmic side of the nuclear envelope is known to inhibit nuclear import, probably due to the ability of GTP-Ran (but not GDP-Ran) to disrupt the karyopherin α-β1 complex and to release karyopherin β1 from docking sites on the NPC (21, 22). Other proteins, for example RanBP1 and components of the NPC, are thought to play a critical role in nuclear import, but do not have to be added to permeabilized cells to achieve efficient import (see below).

The role of nucleotide hydrolysis in nuclear transport is unknown. It has been reported recently that certain types of Ran-dependent nuclear export require the presence of GTP Ran (based on microinjection) but do not appear to require GTP hydrolysis by Ran (23). This is in contrast to studies of nuclear import, in which there have been a number of reports, using both hydrolysis-resistant GTP analogs and Ran mutants incapable of GTP hydrolysis (for example, Q69L Ran) (19), that GTP hydrolysis by Ran is required for import (11, 12, 24, 25). To understand the role of GTP hydrolysis by Ran in nuclear import, we attempted to identify the critical step(s) that required GTP hydrolysis. We verified that hydrolysis-resistant GTP analogs or Q69L Ran inhibited nuclear import in the presence of cytosol as observed previously. However, we found

*This work was supported by National Institutes of Health RO1 GM53678, the Searle Scholar/Chicago Community Trust (to M. S. M.), and National Institutes of Health Fellowship F32 GM10231-01 (to E. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: NLS, nuclear localization signal; NPC, nuclear pore complex; NTP, nucleoside 5'-triphosphate; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; NEM, N-ethylmaleimide; NE, nuclear envelope; BSA, bovine serum albumin; DTT, dithiothreitol; AMP-PNP, adenylyl imidodiphosphate; GMP-PNP, guanylyl imidodiphosphate; GTPγS, guanosine-5'-O-(2-thiodiphosphate); GDPβS, guanosine-5'-O-(2-thiodiphosphate); wt, wild type.
that GTP hydrolysis was not necessary in assays employing purified recombinant factors instead of cytosol. We found that nuclear import could occur in the presence of both poorly hydrolyzable GTP analogs and Q69L Ran. We hypothesize that earlier results were due to the presence of RCC1 outside of the nucleus during import assays, resulting in the inappropriate generation of GTP Ran in the cytoplasm. Accordingly, we confirmed the existence of RCC1 in cytosolic extracts utilized in many earlier studies. Furthermore, we demonstrate that an apparent hydrolysis requirement can be introduced into recombinant import assays by the addition of recombinant RCC1.

**EXPERIMENTAL PROCEDURES**

**Materials**

The hSRP1a expression vector was obtained from A. Lamond and the human karyopherin β expression vector was obtained from D. Görlich. Human Ran and Q69L Ran expression plasmids were obtained from M. Rush and E. Fanning, respectively. The human RCC1 expression vector was from T. Nishimoto. The human p10 was produced from an *Escherichia coli* expression vector obtained from U. Grundmann. When possible, nucleotides were purchased from Boehringer Mannheim. Xanthosine 5′-triphosphate was purchased from Sigma.

**Methods**

**Production of Recombinant Proteins**—All recombinant proteins were snap-frozen in the indicated buffer in single use aliquots and stored at −80 °C.

The human recombinant karyopherin α2 was expressed in bacteria, purified as described, (5) and dialyzed against Buffer A (20 mM Hepes-KOH, pH 7.3, 100 mM potassium acetate, 2 mM DTT).

The human karyopherin βi expression vector was obtained from D. Gärlich and the protein was purified according to his suggested method (26). Transformed BL21(DE3) Rep4 cells were induced at 30 °C for 2 h and centrifuged at 10 000 × g for 90 min. Karyopherin βi was purified by passage of the supernatant over a Q-Sepharose column equilibrated in the same buffer followed by elution with a 200–800 mM NaCl gradient, then fractionated on a Sephacryl S-200 gel filtration column equilibrated in transport buffer (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 10 mM magnesium acetate, 2 mM DTT).

Ran (13) and Q69L Ran (19) were purified identically. Transformed BL21 (DE3) cells were induced with isopropyl-β-d-thiogalactopyranoside for 2 h at 30 °C and then washed, pelleted HeLa cells in SDS-polyacrylamide gel electrophoresis sample buffer. HeLa cytosol was prepared as described in Ref. 30.

**Exchange Assay—**

25 μl of 1 μM Ran was loaded on 2 μl of 5000 Ci/mmol [γ-32P]GTP (3000 Ci/mmol, NEN Life Science Products) was converted to GDP (31) in a reaction containing 280 mM Tris, pH 7.0, 1 mM MgCl2, and 3 μl (6.3 μM) of hexokinase (Sigma catalog no. H-5625) and incubated 4 h at 30 °C. The reaction was terminated by heating to 65 °C for 3 min. Conversion was verified by thin layer chromatography.

**Exchange Assay—**

25 μl (1 nmol) of Ran was loaded with 3.2 pmol of 3000 Ci/mmol [γ-32P]GTP (3000 Ci/mmol, NEN Life Science Products) and 1 μM GDP. After centrifugation as above, Ran was isolated from the supernatant by a 35–55% ammonium sulfate precipitation. The pellet was resuspended in homogenization buffer containing 250 μM GDP, and Ran was isolated by gel filtration chromatography on a Sephacryl S-200 gel filtration column equilibrated in homogenization buffer. Fractions containing Ran were diluted with an equal volume of 20 mM Hepes-KOH, pH 7.3, 2 mM magnesium acetate, 1 mM DTT and were loaded onto a heparin-Sepharose column equilibrated in the same buffer containing 40 mM potassium acetate and eluted with the same buffer containing 0.5 mM potassium acetate. Ran-containing fractions were dialyzed against Buffer A containing 2 mM magnesium acetate.

The human recombinant p10 was expressed in BL21 (DE3) cells, which were grown to an A590 of 0.6–0.8 and induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 3–4 h at 37 °C. The cells were disrupted by French-pressing in 20 mM Hepes-KOH, pH 7.3, 50 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and centrifuged as above. The supernatant was diluted 1:5 with 20 mM Hepes-KOH, pH 7.3, 2 mM DTT, applied to a Q-Sepharose column equilibrated in the same buffer plus 10 mM NaCl, and eluted in a 0–300 mM NaCl gradient. p10-containing fractions were subjected to 50% ammonium sulfate precipitation of the p10-containing fractions. The pellet was resuspended in Buffer B (20 mM Hepes-KOH, pH 7.3, 100 mM potassium acetate, 1 mM DTT) and loaded on a Sephacryl S-200 gel filtration column equilibrated in the same buffer to further purify the p10.

Recombinant human RCC1 was purified as described (28) and dialyzed against Buffer B + 50 mM sucrose. An anti-RCC1 antibody was produced by immunizing rabbits with recombinant RCC1 followed by affinity purification of the serum against the same protein.

**Inactivation of RCC1—**

RCC1 was incubated with 10 μM N-ethylmaleimide (NEM) for 15 min on ice prior to stopping the reaction by the addition of DTT to 20 mM. For mock-treated samples, the DTT was added to 20 mM final concentration prior to the addition of NEM (10 mM final concentration). The concentration of DTT in the RCC1 samples during NEM treatment was 0.5 mM. NEM stock (0.5% in Me2SO) was prepared fresh daily.

**Nuclear Import Assays—**

The nuclear import assay was performed essentially as described (29) using a reporter import construct consisting of rhodamine-labeled BSA coupled to peptides containing the NLS of the SV40 T antigen. Many of these experiments were also repeated using the red-labeled peptide as an import substrate with identical results. The import assay was performed in HeLa cells permeabilized with 70 μg/ml digitonin. As expected, where noted, the standard import reaction contained 5–10 μg/ml tetramethylrhodamine B isothiocyanate-BSA (74–148 nm), 20 μg/ml (0.33 mM) karyopherin α2, 25 μg/ml (0.25 μM) karyopherin βi, 30–50 μg/ml (1.3–2.0 μM) Ran, 3 μg/ml (0.21 μM) p10, 2 mg/ml BSA, and 1 mM final concentration of the indicated nucleotide in transport buffer. Where included, the following concentrations were used: RCC1 at 0.1 μg/ml (2 nm); GTP, ATP, GMP-PNP, GDP, GDPβS, or AMP-PNP at 1 μM. All reaction mixtures were preincubated at room temperature for 15 min prior to starting the import assay. The import assay was carried out in the permeabilized cells for 20 min at room temperature prior to washing and fixation. The samples were observed with a Zeiss Axiohot microscope equipped with a Hamamatsu charge-coupled device camera (no. C2400) and intensifier (no. C2400–68), and quantitation of the fluorescent images was performed with Optimas 4.1 imaging and quantitation software, including custom macro software written by Matthew M. Batchelor of Meyer Instruments Inc. (Houston, TX). The mean nuclear fluorescence of 30–50 nuclei per sample was determined essentially as described (29). Samples were photographed at identical exposures on T-MAX 400 film and printed identically except where noted.

**Preparation of Cell Extracts—** Xenopus ovariarn cytosol was prepared as described (29). Total HeLa lysate was prepared by resuspending washed, pelleted HeLa cells in SDS-polyacrylamide gel electrophoresis sample buffer. HeLa cytosol was prepared as described in Ref. 30.

**Conversion of GTP to GDP—**

125 μCi (42 pmol) of [γ-32P]GTP (3000 Ci/mmol, NEN Life Science Products) and 1 μCi (42 pmol) of [α-35S]GDP (3517 Ci/mmol, NEN Life Science Products) were added to the import reaction (no. C2400–68), and quantitation of the fluorescent images was performed with Optimas 4.1 imaging and quantitation software, including custom macro software written by Matthew M. Batchelor of Meyer Instruments Inc. (Houston, TX). The mean nuclear fluorescence of 30–50 nuclei per sample was determined essentially as described (29).

**Results**

As expected, purified recombinant transport factors (karyopherins α2 and βi, Ran, and p10) supported nuclear import of NLS-BSA in permeabilized cells in the presence of GTP, but not in the presence of GDP, GDPβS, AMP-PNP, or in the absence of nucleotide (Fig. 1, A and B) (29). ATP also supported nuclear import (Fig. 1A), as did all other nucleoside triphosphates tested (CTP, UTP, and xanthine 5′-triphosphate; data not shown). The lack of a specific NTP requirement in this assay is probably due to the presence of nucleoside diphosphokinase-like activity, which is retained in permeabilized cells and capable of generating GTP from an added NTP. Contrary to previous reports, we found that the hydrolysis-resistant GTP analogs GMP-PNP and GTPγS would also stimulate the movement of NLS-BSA into the nucleus, indicating that GTP hydrolysis may, in fact, not be necessary for import of this type of protein.
substrate through the NPC and release into the nucleus (Fig. 1, A and B).

To verify that nuclear import could occur in the absence of GTP hydrolysis by Ran, a mutant Ran that is unable to hydrolyze GTP (Q69L Ran) (19) was substituted for the wt Ran in the in vitro assay. Both the Q69L Ran and the wt Ran were exclusively in the GDP-bound form prior to addition to the assay as determined by fast protein liquid chromatography analysis (data not shown). When added with either GDP, GDP-βS, or AMP-PNP, Q69L Ran (like wt Ran) was unable to support nuclear import (Fig. 1, A and B). However, when added with either GTP, GMP-PNP, or GTP-γS, Q69L Ran was capable of supporting nuclear import (Fig. 1, A and B). These results indicated that free GTP (or its structural equivalent) was required for transport, but that GTP hydrolysis by Ran was not required for passage of import substrate through the NPC and into the nuclear interior. The rates of import supported by Q69L Ran, however, were lower than that supported by wt Ran (Fig. 1C) (see below).

Thus, we observed that import supported by cytosol requires nucleotide hydrolysis (data not shown), while import supported by purified recombinant import factors does not (Fig. 1). Clearly, some component(s) present in cytosol, and not required for import, mediated the observed difference in nucleotide hydrolysis requirements between the two samples. We postulated that RCC1 (the Ran GEF), while predominantly nuclear in vivo, would be present in cytosol (at least in low amounts) and might be responsible for loading Ran with GTP or its structural equivalent (GMP-PNP or GTP-γS), thus inhibiting import. Immunoblotting revealed that RCC1 was, in fact, present in two different cytosol preparations commonly used to support nuclear import in vitro (Fig. 2A, lanes 1 and 3), although, as expected, it was much more abundant in a total cell lysate (Fig. 2A, lane 2) (11, 12.).
We next tested whether the addition of recombinant RCC1 to recombinant import factors could restore the inhibition of nuclear import by hydrolysis-resistant GTP analogs. Since RCC1 itself contains an NLS, it was necessary to inactivate the RCC1 exchange activity such that it would be possible to distinguish inhibition of nuclear import caused by RCC1’s GEF activity on Ran from inhibition caused by competition for import between the two NLS-containing proteins (RCC1 and NLS-BSA). RCC1’s exchange activity was found to be abolished by treatment with the alkylating agent NEM (Fig. 2B). RCC1 was then added to import assays to determine whether it would affect apparent hydrolysis requirements in a manner similar to cytosol. NEM-treated (inactivated) RCC1 was added as a control at the same concentration as mock-treated (active) RCC1 (0.1 mg/ml, 2.2 nM). The addition of hydrolysis-resistant GTP analogs (GMP-PNP or GTPγS) and mock-treated RCC1 to recombinant import factors inhibited nuclear import, while the addition of NEM-treated RCC1 (which lacked exchange activity) had no effect (Fig. 3A). Similarly, the addition of active RCC1 to Q69L Ran and GTP also inhibited nuclear import. Quantitation of these samples (Fig. 3B) clearly demonstrated the inhibition of import mediated by addition of active RCC1. Thus, a requirement for GTP hydrolysis by Ran during this type of nuclear import was not observed unless active RCC1 was added to the cytosolic side of the nuclear envelope.

**DISCUSSION**

All previous reports of classical NLS-mediated Ran-dependent nuclear import have either demonstrated a GTP hydrolysis requirement or implied an hydrolysis requirement by including a hydrolyzable NTP in the import assay mixture (32). This is the first report that this type of NLS-mediated import can occur in the absence of NTP hydrolysis in general and GTP hydrolysis by Ran in particular. The decreased level of nuclear import in the presence of hydrolysis-resistant GTP analogs or Q69L Ran as compared with that seen with wt Ran and GTP indicates that GTP hydrolysis by Ran, while not being required for import, may be required for the recycling of some constituent of the transport machinery (see below). We also cannot rule out the possibility that the minor structural differences be-

**Fig. 2.** Identification of RCC1 in cell extracts and inactivation of RCC1 by NEM. A, 15 μg each of HeLa cytosol (lane 1), HeLa total cell extract (lane 2), and Xenopus ovarian cytosol (lane 3) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The immunoblot was probed with antibody against human RCC1. B, RCC1 was either NEM-treated (incubated in NEM followed by DTT) or mock-treated (incubated in DTT followed by NEM). The treated RCC1 was then tested for its ability to catalyze exchange of [α-32P]GDP for unlabeled GTP on wt Ran. Exchange reactions were incubated for 15 min at 30 °C.

**Fig. 3.** RCC1 is capable of mediating inhibition of nuclear import in the presence of hydrolysis-resistant GTP analogs or Q69L Ran. Nuclear import assays were carried out in the presence of 0.1 μg/ml inactivated (NEM-treated) RCC1 or active (mock-treated) RCC1. The effect of RCC1 was also tested on import supported by Q69L Ran and GTP. A, all samples were photographed and printed identically except those denoted by an asterisk, which indicates panels that were printed one f-stop higher because of low signal. B, nuclear import assays were carried out in the presence of wt Ran and either mock- or NEM-treated RCC1 (0.1 μg/ml), followed by quantitation. The means ± S.E. of duplicate samples (n = 30) are shown.
between Q69L Ran and wt Ran, and between GTPγS, GMP-PNP, and GTP may have also been slowing the rate of nuclear import for reasons unrelated to GTP hydrolysis.

Because in the presence of cytosol we observed a requirement for GTP hydrolysis in nuclear import (data not shown), but not when purified transport factors were added (Fig. 1), it was critical to determine which component(s) present in cytosol mediates inhibition of nuclear import by GTP analogs. It has been demonstrated that the addition of GTP Ran to the cytoplasmic side of the NE inhibits nuclear import, probably because GTP Ran disrupts the karyopherin α-β1 complex and releases karyopherin β1 from docking sites on the NPC (21, 22). Either Q69L Ran (in the GTP-bound form) or wt Ran bound to either GMP-PNP or GTPγS are unable to hydrolyze bound nucleotide in response to RanGAP1 and would thus presumably accumulate upon generation. We hypothesized that the RCC1 present in cytosol could generate sufficient quantities of GAP-unresponsive GTPγS Ran or GMP-PNP Ran on the cytoplasmic side of the NE and thus could be responsible for the import inhibition observed in the presence of cytosol containing hydrolysis-resistant GTP analogs. The similarity in hydrolysis requirements between import supported by purified, recombinant factors plus RCC1 and import supported by cytosol strongly reinforces this conclusion. We also find a correlation between the requirement for GTP hydrolysis and the presence of RCC1 in earlier studies (9, 11). Fraction A (29), which contains import factors partially purified from Xenopus cytosol, also contains RCC1.4 Accordingly, like cytosol, hydrolyzable NTP is required for nuclear import in the presence of Fraction A (9, 11).

While the presence of RCC1 would explain the ability of GTP analogs to inhibit import in vitro in the presence of cytosol, in certain previous studies these analogs were also reported to inhibit import in the presence of just recombinant import factors (25). It is possible that differences in techniques may result in different amounts of RCC1 on the cytoplasmic side of the NE. The original protocol (1) for import assays started with cells bound to coverslips that were permeabilized, washed, and tested for import. The modified technique in the cited study utilized cells that were permeabilized and assayed in suspension. Differences in handling between the two techniques, in particular the presumed need to wash by centrifugation and resuspension, could be responsible for lysis and release of RCC1 from nuclei. In addition, although not stated in the cited study, many investigators using this modified technique freeze permeabilized cells for future use (3). This could also be expected to increase the release of RCC1 from the nuclei. As shown in these experiments, increases in the extranuclear concentration of RCC1 have profound effects on apparent nucleotide requirements.

Notably, when ATP was added instead of GTP, the ability of RCC1 to inhibit import was reduced (Fig. 3B). Under these conditions we believe that there are three reactions occurring (i) production of GTP from added ATP and endogenous GDP by nucleoside diphosphokinase, (ii) loading of Ran by RCC1 with GDP or GTP, and (iii) hydrolysis of GTP by Ran catalyzed by RanGAP1. Inhibition of nuclear import has been reported upon the addition of Ran in the GTP-bound form on the cytoplasmic side of the NE, but the percentage of the total Ran in the GTP-bound form required to cause this inhibition has not been determined (20–22). Presumably, this accumulation of GTP Ran occurs at a slower rate when GTP has to be produced from added ATP as opposed to when 1 mM GTP is added directly to the import mixture, which would mean that more nuclear import can be obtained before the GTP Ran percentage rises to inhibitory levels. Moreover, samples treated with RCC1 and hydrolysis-resistant GTP analogs lacked the typical rim staining pattern seen in samples containing GDP, GDPβS, or AMP-PNP, indicating that the GTP Ran generated under these conditions disrupts the docking of substrate at the cytoplasmic face of the NPC. Our data showing that the addition of RCC1 and hydrolysis-resistant GTP analog (which together would produce GTP Ran) to the cytoplasmic side of the NE abolishes nuclear import is consistent with previous results showing that high levels of GTP Ran on the cytoplasmic side of the NE inhibit nuclear import (20, 21).

Because import requires both GDP Ran and free GTP (or its structural equivalent), GTP Ran must be formed at some point during substrate transport through the NPC or release into the nucleoplasm. This has been predicted to occur when an import complex reaches the nuclear interior, where it encounters RCC1-generated GTP Ran, which releases the import substrate from the NPC. Our data also indicate that this release of the NLS substrate does not require hydrolysis, although this experiment does not demonstrate that the NLS substrate has dissociated from all of the components of the import complex. These data would suggest that the Ran-dependent movement of transport complexes through the NPC itself occurs by facilitated diffusion mechanisms rather than by GTP hydrolysis supplying the energy for movement through the NPC or release into the nucleoplasm.

Since Ran mutants unable to hydrolyze GTP are lethal in vivo, we believe a requirement for GTP hydrolysis by Ran does exist. In vivo, import factors have to be recycled back to their original location and condition before they can support another round of import, and interruption of the GTPase cycle could prevent proper recycling. For example, GTP hydrolysis by Ran may be required for the separation of karyopherin β from Ran. In the presence of hydrolysis-resistant analog, this complex may move back to the cytoplasmic face of the NPC, but be unable to dissociate in response to GTP hydrolysis stimulated by RanGAP1. However, we have been unable to demonstrate changes in the distribution of known import factors in response to different nucleotide analogs by immunofluorescence microscopy in permeabilized cells. A more detailed biochemical examination of these distributions is in progress.

Nuclear import of an NLS-containing substrate requires movement of protein against its concentration gradient. The overall effect of one transport cycle (including recycling of nuclear import factors) is to increase the compartmentalization of the NLS-containing substrate, which must require energy. However, we propose that nucleotide hydrolysis by Ran during the import portion of the cycle is not the source of this energy. Instead, these data indicate that energy may be required either during recycling of import factors or in maintenance of the GDP Ran-GTP Ran gradient. Thus the energy requirements for nuclear transport cannot be fully understood without a more clear understanding of the mechanism of nuclear import.

Acknowledgments—We thank T. Nishimoto, A. Lamond, M. Rush, E. Fanning, D. Görlich, and U. Grundmann for E. coli expression plasmids; B. Brinkley for the HeLa cell line; and P. Casey for much advice on the handling and analysis of nucleotides. We also thank Diane Hoffmaster and Qiongshu Xie for excellent technical assistance and C. Lane for many discussions and a critical reading of the manuscript.

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doi: 10.1074/jbc.273.52.35170

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