The molecular mechanism of translocation through the nuclear pore complex is highly conserved

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Summary

In this report we investigated the activity of vertebrate nuclear transport factors in a primitive organism, *Amoeba proteus*, to better understand evolutionary changes in the transport mechanisms of organisms expected to have different requirements for nucleocytoplasmic exchange. It was initially determined that FxFG-containing nucleoporins and Ran, both of which are essential for nuclear import in vertebrates, as well as yeast, are also present and functional in amoebae. This suggests that there are fundamental similarities in the transport process; however, there are also significant differences. Transport substrates containing either the hnRNP A1 M9 shuttling signal (a GST/GFP/M9 fusion protein) or the classical bipartite NLS (colloidal gold coated with BSA-bipartite NLS conjugates), both of which are effectively transported in vertebrate cells, are excluded from the nucleus when microinjected into amoebae. However, when these substrates are injected along with transportin or importin α/β, respectively, the vertebrate receptors for these signals, they readily accumulate in the nucleoplasm. These results indicate that although the molecular recognition of substrates is not well conserved between vertebrates and amoebae, vertebrate transport receptors are functional in *A. proteus*, showing that the translocation machinery is highly conserved. Since selected nuclear import pathways can be investigated in the absence of competing endogenous transport, *A. proteus* might provide a useful in vivo system for investigating specific molecular interactions involved in trafficking.

Key words: Nuclear transport, Nuclear pore complex, Amoebae

Introduction

Signal-mediated exchange of proteins through the nuclear pores is a multistep process initiated by import (NLSs) or export signals (NESs) that bind to an appropriate transport receptor or a receptor-adapter complex. In the case of import, the receptor, along with its bound cargo, then docks to filaments that extend from the cytoplasmic surface of the pores and is subsequently translocated into the nucleus through a central channel in the pore complex. Ran, a 24 kDa GTPase, helps to provide directionality to the exchange process. It is present predominantly in its GDP form in the cytoplasm and in its GTP form in the nucleoplasm. This intracellular gradient facilitates the release of the cargo in the nucleus, since the cargo-receptor complex dissociates in the presence of RanGTP but not RanGDP (for reviews, see Nakielny and Dreyfuss, 1999; Bayliss et al., 2000a; Wente, 2000; Rout and Aitchison, 2001).

There are considerable data demonstrating that the basic transport process outlined above functions in organisms as diverse as yeast and vertebrates. However, specific components of the transport machinery exhibit different levels of conservation. The transport receptors that function in the import and export of proteins through the nuclear pores are all members of the importin-β (or karyopherin-β) superfamily (Wozniak et al., 1998; Gorlich and Kutay, 1999; Jans et al., 2000). The characteristic features of these transport receptors are an N-terminal RanGTP-binding domain, a relatively large mass (approximately 90-140 kDa), and an acidic isoelectric point. In addition, the receptors contain binding sites for specific NLSs, NESs or adaptors, and are able to interact with nucleoporins, mainly by binding to FG motifs (see below). Fourteen and 21 importin-β family members have been identified in yeast and humans, respectively. Of the yeast members, five are involved in export, and the remainder function in protein import. Kutay et al. suggested that the greater number of receptors in vertebrate cells could (1) increase redundancy in the pathways available for specific signals or classes of signals or (2) provide pathways for transport substrates that are not present in yeast (Kutay et al., 2000).

Significant differences have also been detected in the organization and composition of the nuclear pore complexes. In yeast, the pore complex has a mass of about 66 MDa (Yang et al., 1998) and contains multiple copies of approximately 30 different nucleoporins (Rout et al., 2000). The pore complex in vertebrate cells has a mass estimated to be 125 MDa, and it is thought to be made up of over 50 different proteins. A detailed comparison of the nucleoporins in the two organisms has recently been published by Vasu and Forbes (Vasu and Forbes, 2001). Six of the known vertebrate nucleoporins show strong homology with yeast nucleoporins; however, the majority are only weakly homologous or distantly related. Despite this diversity, one feature that is common to both yeast and vertebrate nucleoporins are FG-repeat motifs. This general classification includes FG, FxFG or GLFG repeats.

There are data showing that FG-containing nucleoporins are present throughout the pore complex (e.g. Grote et al., 1995; Stoffler et al., 1999; Rout et al., 2000; Stewart et al., 2001); some are distributed symmetrically, whereas others are localized specifically on the nuclear or cytoplasmic face of the complex. Consistent with this distribution, there is evidence that the FG repeats are required for translocation through the pores. For example, binding studies, reviewed by Ryan and Wente, have revealed that all of the transport receptors that have been studied interact with one or more FG-containing nucleoporins (Ryan and Wente, 2000). Although a number of receptors bound to the same nucleoporins, differences in the overall binding patterns have been detected. Microinjection or overexpression of peptides containing FG-repeat domains interferes with nuclear transport, providing further evidence that FG motifs are involved in the translocation (Bastos et al., 1996; Iovine et al., 1996; Stutz et al., 1996).

Currently there are three general models for translocation through the pores, all of which are centered around interactions between FG repeats and transport receptors. Rout et al. have proposed that pore-associated FG-containing filamentous proteins, assisted by Brownian movement, exclude substances that lack FG-binding domains, but, at the same time, bind to receptor-cargo complexes for subsequent diffusion through the pores (Rout et al., 2000). Ribbeck and Gorlich suggested that the transport channel located in the center of the pore complex is occupied by a meshwork of FG repeat nucleoporins that functions as a semi-liquid phase into which receptor-cargo complexes, containing FG-binding sites, could partition and thereby translocate through the pores (Ribbeck and Gorlich, 2001). A third model (reviewed by Stewart et al., 2001) proposed that receptor-cargo complexes initially concentrate at the cytoplasmic face of the pores by binding to FG repeats. Transport across the envelope would then involve sequential steps of adsorption and desorption to FG motifs that line the central channel of the pores. It is also possible that directional migration across the envelope is enhanced by an ‘affinity gradient’ for different nucleoporins within the pore complexes (Ben-Efraim and Gerace, 2001). Distinguishing among these models remains a major challenge in the field.

It is likely that the major evolutionary changes in nuclear transport capacity needed to accommodate the additional regulatory requirements of higher organisms are caused by increases in the number of transport receptors and the complexity of the pore complex. The translocation process itself appears to be more highly conserved. Thus, transport in both yeast and vertebrates is typically Ran dependent, consistent with the conserved Ran-binding domain being located in the N-terminus of the receptors. FG-containing nucleoporins, which are also required for translocation, are abundant in yeast and vertebrate cells. These similarities raise the possibility that there is an underlying, universal pathway for translocation through the pores.

This study is a continuation of an earlier investigation on nuclear transport in *Amoeba proteus*. The basic premise of these experiments is that evolutionary changes in the properties of the transport machinery are related to the changing regulatory requirements of cells as complexity increases. Thus, an understanding of the nucleocytopasmic exchange in primitive systems should help to distinguish between the basic, essential elements of the transport process and regulatory elements that evolved to satisfy the requirements of more complex organisms. Previously (Feldherr and Akin, 1999), it was determined that the nuclear transport apparatus in amoebae is different from vertebrate cells. First, the functional diameter of the central transport channel is smaller in amoebae. This was established by analyzing the import of different size gold particles that were coated with BSA conjugated to peptides containing the classical large T NLS. In amoebae, particles larger than 140 Å in diameter were essentially excluded from the nucleoplasm, whereas in vertebrates the exclusion limit is approximately 230 Å. Presumably, this reflects differences in the nature and organization of the nucleoporins. It was suggested (Feldherr and Akin, 1999) that variations in functional pore size could be related to differences in the dimensions of the ribosomal subunits that exit the nucleus. Second, although large T NLSs facilitated the transport of gold through the pores in amoebae, particles coated with conjugates containing bipartite NLSs were largely excluded from the nucleus. In vertebrate cells both signals utilize the same receptor (the importin α/β heterodimer) and are equally effective in initiating transport. Data were also obtained suggesting that the large T NLS mediates nuclear export as well as import. Thus, there also seem to be differences in the specificity and activity of the transport receptors.

The present report compares the molecular mechanism of protein import in *A. proteus* with that of vertebrate cells. It was initially found that FG repeats are present in amoebae nucleoporins and that these repeats, along with Ran, are necessary for signal-mediated nuclear import. Although proteins containing the classical large T NLS were efficiently transported into the nucleoplasm in amoebae, substrates bearing other common vertebrate import signals, specifically the M9 shuttling sequence and the bipartite NLS, were retained in the cytoplasm unless the corresponding vertebrate transport receptors were also present. These results demonstrate that the nuclear protein import machinery in *A. proteus* is able to recognize and utilize vertebrate receptors, which argues in favor of a highly conserved translocation process.

Materials and Methods

Amoebae cultures

Amoeba proteus were purchased from Carolina Biological Supply (Burlington, NC) and cultured at room temperature (21°C) in amoebae medium, which contained 0.5 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.16 mM K_2HPO_4, and 0.12 mM KH_2PO_4 (pH 6.9-7.0). The cells were fed *Tetrahymena pyriformis* as described previously (Prescott and James, 1955).

Western blot analysis of Ran

100 μl of packed amoebae were collected by low speed centrifugation in amoebae medium to which was added 0.1 mg/ml each of leupeptin, aprotinin and pepstatin. An equal volume of 2× sample buffer [125 mM Tris (pH 6.8), 20% glycerol, 10% BME, 4.6% SDS, and 0.02 mg/ml bromophenol blue] was added to the pellet, and the cells were homogenized and boiled for 5 minutes. Samples were then run on a 10% SDS-polyacrylamide gel (Laemmli, 1970). A431 cell lysate (Transduction Labs, Lexington, KY) served as a positive control for the Ran antibody.

The gel was blotted onto nitrocellulose (MSI, Inc., Westborough, MA) for 1 hour at 12 amps. The blot was incubated in blocking buffer (1% BSA, 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20)

for 12 hours at 4°C. This was followed by incubation for 1 hour at room temperature in 1:5000 dilution of monoclonal antibody against human Ran (Transduction Labs) in blocking buffer. The blot was then rinsed for 1 hour and stained using alkaline-phosphatase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO), according to Blake et al. (Blake et al., 1984).

Transport factors

Previously described methods were used for the bacterial expression and purification of human importins α and β (Bayliss et al., 2000b), His-tagged transportin (Pollard et al., 1996), canine Ran (Stewart et al., 1998a), rat NTF2 and its W7A mutant (Bayliss et al., 1999), the 18 FxFG repeat construct of yeast nucleoporin Nsp1p (Clarkson et al., 1997) and the 11 FxFG repeat region of rat nucleoporin p62 (Buss and Stewart, 1995). All proteins were over 95% pure by SDS-PAGE stained with Coomassie Brilliant Blue. The Ran mutant Q69L was kindly supplied by Mary S. Moore, and the FxFG repeat region of Nup153 (amino acids 618-828 of the *Xenopus* Nup153 sequence) was provided by Douglass Forbes. The monoclonal antibody, MAb414, against FG-containing nucleoporins, was purchased from Berkeley Antibody Company (Richmond, CA).

Transport substrates

The substrates used to assay nuclear import included (1) gold particles coated with BSA conjugated to peptides containing classical NLSs, and (2) a recombinant protein constructed by fusing GST, GFP and the M9 transport signal.

Signal peptides were synthesized by the University of Florida protein core facility. The peptides, CGGGPKKKRKVGG and CGGG-AVKRPAATKKAGQAKKKKLNGG, contain, respectively, the SV40 large T NLS (Kalderon et al., 1984) and the bipartite nucleoplasmin NLS (Robbins et al., 1991). The signal sequences are underlined. Conjugation of the peptides with BSA (Sigma, St Louis, MO) was performed as described previously (Lanford et al., 1986). It was estimated, using SDS-PAGE analysis, that an average of eight peptides were cross-linked to each BSA molecule.

Two GST/GFP fusion proteins containing either the wild-type (wt) or mutant (mt) M9 nuclear transport sequence, designated GST/GFP/M9wt and GST/GFP/M9mt, respectively, were prepared by the University of Florida Molecular Services Core Laboratory. The M9 domain is a bifunctional transport signal that is required for the nuclear import and export of the heterogeneous nuclear ribonucleoprotein A1 [hnRNP A1; (Michael et al., 1995)]. The amino acid sequence of the M9 signal is NNQSSN(FGPM)KGGNFG-GRSSGPYGGGGQYFAKPRNQGGYG (Siomi and Dreyfuss, 1995). In the M9 mutant peptide, the four amino acids in parentheses were replaced with 'AAAA' (Bogerd et al., 1999). The dsDNA fragment encoding the M9 peptide was generated by PCR from overlapping primers. GST fusion vector pGEX-4T-2 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) was used to make the constructs. GFP/M9(wt or mt) was cloned into the vector's *Bam*HI-*Eco*RI site, just downstream of GST. The fusion proteins were overexpressed in F′ *E. coli* and purified on a glutathione-agarose column (Sigma, St Louis, MO). Purity of the proteins was verified by SDS-PAGE.

Colloidal gold

Colloidal gold fractions, containing particles ranging in diameter from 20 to 50 Å (small gold) or 20 to 120 Å (intermediate gold) were prepared as outlined previously (Feldherr, 1965). The gold particles were coated with either BSA-NLS peptide conjugates (for transport assays) or transport factors (for pore localization studies) according to a procedure reported earlier (Dworetzky et al., 1988). It is estimated that the protein coat increases overall particle diameter by about 30 Å. The coated gold preparations were concentrated approximately 200-fold using Ultrafree concentrators (Amicon Inc., Beverly, MA) and dialyzed against amoebae medium.

Microinjection

The amoebae were microinjected using an inverted microscope and a hydraulic micromanipulator (Narishige USA, Inc., Greenvale, NY). The cells were immobilized in an oil chamber and injected with micropipettes that had 1-2 μm tip diameters. The amount injected was approximately 5-10% of the cell volume.

EM and fluorescent analysis

For EM analysis, the cells were fixed for 30 minutes in 4% glutaraldehyde, postfixed for 30 minutes in 2% OsO_4, dehydrated, embedded in Spurr's resin and subsequently examined using a JEOL 100CX electron microscope.

Fluorescent analysis was performed using a Hamamatsu CCD camera and a MetaMorph imaging system (Universal Imaging Corporation, West Chester, PA). Images were collected with a Zeiss Planapo 25× objective lens at the time points indicated. To minimize UV damage to the cells, each exposure was less than 1 second; in addition, a number 16 neutral density filter was used.

The nuclear/cytoplasmic (N/C) gold and fluorescent ratios were obtained by analyzing equal and adjacent areas of nucleoplasm and cytoplasm.

Results

FG repeats and Ran in amoebae

Initially, experiments were performed to determine if FG repeat nucleoporins and Ran, both essential translocation factors in yeast and vertebrates, are present and functional in amoebae.

Small gold particles coated with wild-type NTF2, W7A-NTF2, which contains a mutation in the FG-repeat-binding domain (Bayliss et al., 1999) or antibody MAb414 against FG-containing nucleoporins, were used to assay for FxFG moieties in the pore complex. NTF2 is a 14.4 kDa transport factor (Moore and Blobel, 1994; Paschal and Gerace, 1995) that acts as a receptor for the nuclear import of RanGDP (Smith et al., 1998; Ribbeck et al., 1998). To perform this function, NTF2 has binding sites for both RanGDP and FxFG repeats. It was previously shown in *Xenopus* oocytes that, following microinjection, small gold particles coated with wild-type NTF2 bound extensively to the pore complex (Bayliss et al., 1999). Particles were observed along both the nuclear and cytoplasmic faces of the complex and also within the central transport channel. However, accumulation within the pores was significantly reduced if the particles are coated with W7A-NTF2. In this study, similar experiments were performed on amoebae. The cells were microinjected with the gold preparations, incubated for 30 minutes in culture medium, and then fixed for EM examination. As was observed in oocytes, wild-type NTF2-gold was distributed throughout the pores (Fig. 1A). When the gold was coated with W7A-NTF2 there was a significant decrease in the number of pore-associated particles (Fig. 1B). Correcting for differences in the amount of gold available in the cytoplasm, it was estimated that the decrease in binding was approximately 60%. These results indicate that wild-type NTF2-gold binding to the pores was caused by the presence of FxFG repeats. We also employed the monoclonal antibody MAb414 (Davis and Blobel, 1986) that binds specifically to FxFG nucleoporins. The localization of

MAb414-coated gold particles to the pore complexes following microinjection (Fig. 2) provided additional evidence for the presence of FG-nucleoporins in *A. proteus*.

Several laboratories have reported that nuclear transport is inhibited by peptides that contain FxFG repeats. Thus, peptides containing Nup153 FxFG moieties block importin α/β-mediated protein import (Shah and Forbes, 1998) and poly $(A)^+$ RNA export (Bastos et al., 1996). NTF2-dependent nuclear uptake of RanGDP is inhibited by the FxFG-repeat region of the yeast nucleoporin Nsp1p (Bayliss et al., 1999). Presumably, inhibition of transport is caused by competition between the FxFG peptides and the nucleoporins for receptor binding sites. To determine if FxFG repeat peptides also decrease transport in amoebae, their effect on the nuclear import of intermediate gold coated with BSA-large T NLS conjugates was assayed. In these experiments, FxFG-repeat regions from Nsp1p, p62 or Nup153 were injected along with the gold-large T NLS transport substrate. The concentrations of the injected FxFG peptides were 0.5, 1.25 and 1 mg/ml, respectively. It is apparent from Table 1, which shows the N/C gold ratios in amoebae fixed 30 minutes after injection, that all of the FxFG peptides inhibited import (P<0.0001 in each experiment). In parallel experiments, it was found that all of the above FxFG peptides also inhibited import of the gold tracer in *Xenopus* oocytes (data not shown). It is interesting that the p62 peptide blocked transport, since in vitro assays failed to detect binding of importin β to the N-terminus (FxFG-containing domain) of p62 (Percipalle et al., 1997). This suggests that binding between the p62 repeat peptide and the receptor, although unstable under the stringent in vitro conditions, is sufficient to interfere with transport in vivo. Taken together, the NTF2-gold binding results, and the inhibitory effects of the FxFG peptides, support the view that FxFG moieties are

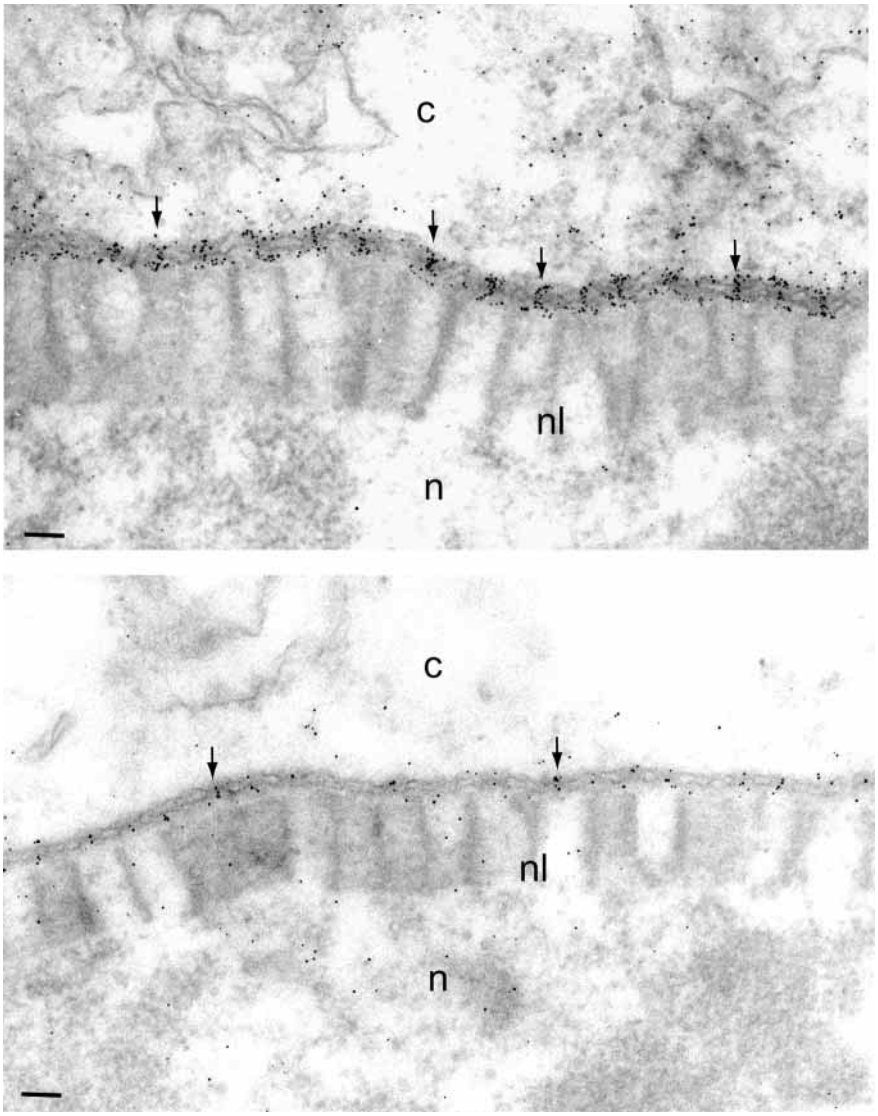

Fig. 1. Electron micrographs of amoebae, fixed 30 minutes after microinjection, showing the distribution of colloidal gold particles coated with either wild-type NTF2 (A) or W7A mutant NTF2 (B). There was significantly less binding of the mutant NTF2-gold to the nuclear pores (arrows). Since the mutation inhibits binding to FxFG repeats, the results suggest that these moieties are present in the pore complexes. The structures (nl) seen extending from the inner surface of the envelope are components of the nuclear lamina. In *A. proteus*, the lamina forms an elaborate honeycomb-like supporting structure. c, cytoplasm; n, nucleus. Bar, 100 nm.

Table 1. The effect of FG peptides on large T NLS-gold import

Experiment		No. of cells analyzed	No. of particles counted	N/C ratio±s.e.	Significance*
Coating agent	FG peptide source				
BSA-Large T NLS	None	8	880	0.589±0.0430	–
BSA-Large T NLS	Nup153	5	421	0.044±0.0076	s
BSA-Large T NLS	p62	6	571	0.004±0.0027	s
BSA-Large T NLS	Nsp1p	5	306	0	**

*Indicates whether the values are (s) or are not (ns) significantly different from those of the controls. Probability values of 0.01 or less were considered significant.

**Significance could not be calculated.

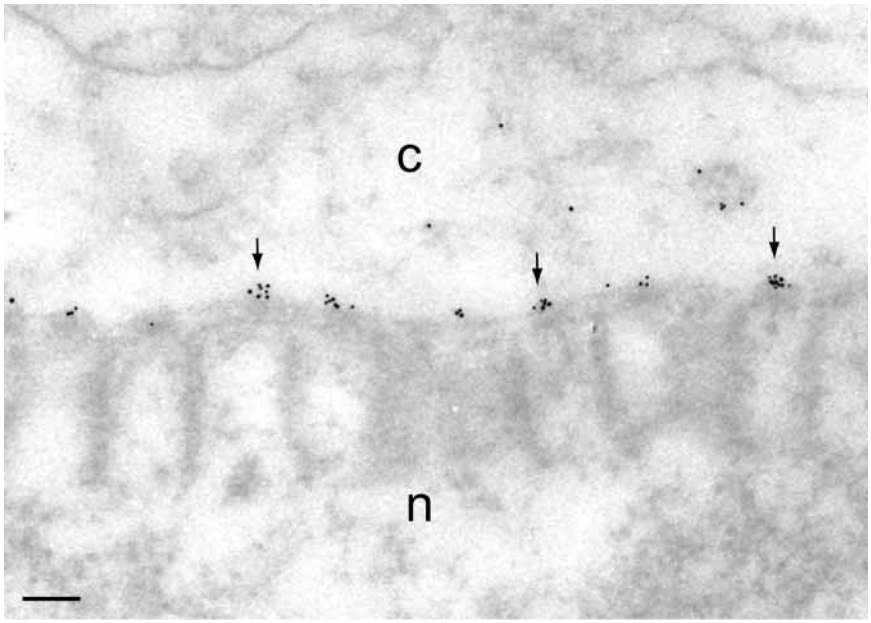

Fig. 2. The distribution of MAb414-coated gold particles in an amoebae fixed for electron microscopy 30 minutes after injection. Since particles coated with antibody are unable to penetrate the pore complexes (arrows), the gold localized to the nucleoporins along the cytoplasmic faces of the pores. c, cytoplasm; n, nucleus. Bar, 100 nm.

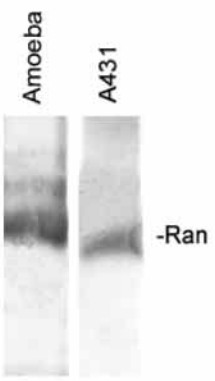

Fig. 3. Western blot analysis for Ran. Blots were probed with monoclonal antibody to human Ran, and similar bands were detected in both the amoebae extract and the A431 cell lysate, which served as a positive control for Ran.

present in amoebae nuclear pore complexes and function in translocation.

Western blotting procedures were employed to determine if Ran is also present in amoebae. Blots of amoebae extracts and A431 lysate, which served as the standard, were treated with a monoclonal antibody against human Ran and subsequently stained. Bands in the 25 kDa range were detected in both cell types; however, the amoebae Ran appeared to have a slightly higher molecular weight. The results are shown in Fig. 3. The Q69L mutant was then used to investigate the activity of Ran in the translocation process in amoebae. References to similar studies in other experimental systems can be found in Stewart et al. (Stewart et al., 1998b). This mutant is not stimulated by RanGAP, and, therefore, is unable to hydrolyze GTP. Since the mutant can undergo nucleotide exchange, it exists in its GTP-bound form when introduced into vertebrate cells, and, thus, inhibits import of proteins containing classical NLSs by preventing the formation of receptor-substrate complexes. In the experiments performed on amoebae, 3 mM wild-type Ran or RanQ69L was microinjected along with transport substrate (intermediate gold coated with BSA conjugated with large T NLS), and the cells were fixed after 1 hour. The results are shown in Table 2. As expected, the N/C gold ratios obtained for substrate alone versus substrate plus wild-type Ran were not significantly different (P=0.11); however, the Q69L mutant caused a highly significant decrease in import (P<0.0001), demonstrating that Ran is involved in the nuclear import in amoebae.

Vertebrate receptors are functional in amoebae

In order to analyze the activity of vertebrate transport receptors in amoebae, it was first necessary to identify vertebrate NLSs that (1) were inactive or marginally active in amoebae and (2) had well characterized receptors. With these objectives in mind, fusion proteins were constructed that contained GST, GFP and either the wild-type or mutant M9 shuttling signal that is present in hnRNP A1. In initial experiments using BALB/c 3T3 cells, it was found that the GST/GFP/M9wt accumulated in the nucleus, as determined by fluorescent analysis, within 10 minutes of microinjection, whereas GST/GFP/M9mt remained cytoplasmic (data not shown). When tested in amoebae, neither of the constructs entered the nucleus. However, when 2 mg/ml transportin was injected simultaneously, the construct containing the wild type, but not the mutant M9 sequence rapidly accumulated in the nucleus.

Table 2. The effect of Ran Q69L on large T NLS-gold import

Experiment		No. of cells analyzed	No. of particles counted	N/C ratio±s.e.	Significance*
Coating agent	Ran				
BSA-Large T NLS	None	10	168	1.90±0.2052	–
BSA-Large T NLS	wt Ran	5	681	2.45±0.1812	ns
BSA-Large T NLS	Ran Q69L	5	699	0.08±0.0170	s

*Indicates whether the values are (s) or are not (ns) significantly different from those of the controls. Probability values of 0.01 or less were considered significant.

Fluorescent micrographs taken 60 minutes after injection are shown in Fig. 4A. The N/C fluorescent ratios, at the time points indicated, are plotted in Fig. 4B. The increased nuclear import of GST/GFP/M9wt in the presence of transportin is highly significant (P<0.0001). Also included in Fig. 4B is a curve showing the N/C ratios for fluorescein-labeled BSA. Since this tracer lacks an NLS, it is essentially excluded from the nucleus; thus, the N/C ratios are a measure of background fluorescence from adjacent cytoplasm. The intracellular distribution of transportin-coated small gold particles was also investigated. Consistent with the role of transportin in import, the gold particles accumulated within the pore complexes (Fig. 5) and were able to enter the nucleus (the N/C gold ratio after 30 minutes was 0.41).

Although the large T NLS effectively mediates nuclear import in amoebae, the bipartite nucleoplasmin NLS, a classical import signal that also utilizes the importin α/β pathway, is only marginally functional in these cells (Feldherr and Akin, 1999). Small substrates containing the bipartite NLS (fluorescent conjugates) were able to enter the nucleus, but large substrates (gold coated with BSA-bipartite NLS conjugates) were essentially excluded from the nucleoplasm. To determine if vertebrate receptors could enhance the activity of the bipartite NLS in amoebae, small gold particles coated with BSA-bipartite NLS conjugates were injected either alone or along with importin β plus importin α (1 mg/ml each). As a control, BSA-coated small gold particles that lacked an NLS were simultaneously injected with importin α/β. The cells were fixed 60 minutes after injection. TEMs are shown in Fig. 6, and the N/C gold ratios are listed in Table 3. The addition of importin α/β not only resulted in extensive binding of the transport substrate to the pores but also caused a highly significant (P<0.0001) increase in nuclear uptake. Importin α/β did not facilitate the import of the control, BSA-gold particles. Injection of importin α or importin β alone did not affect nuclear import of the bipartite NLS gold (data not shown).

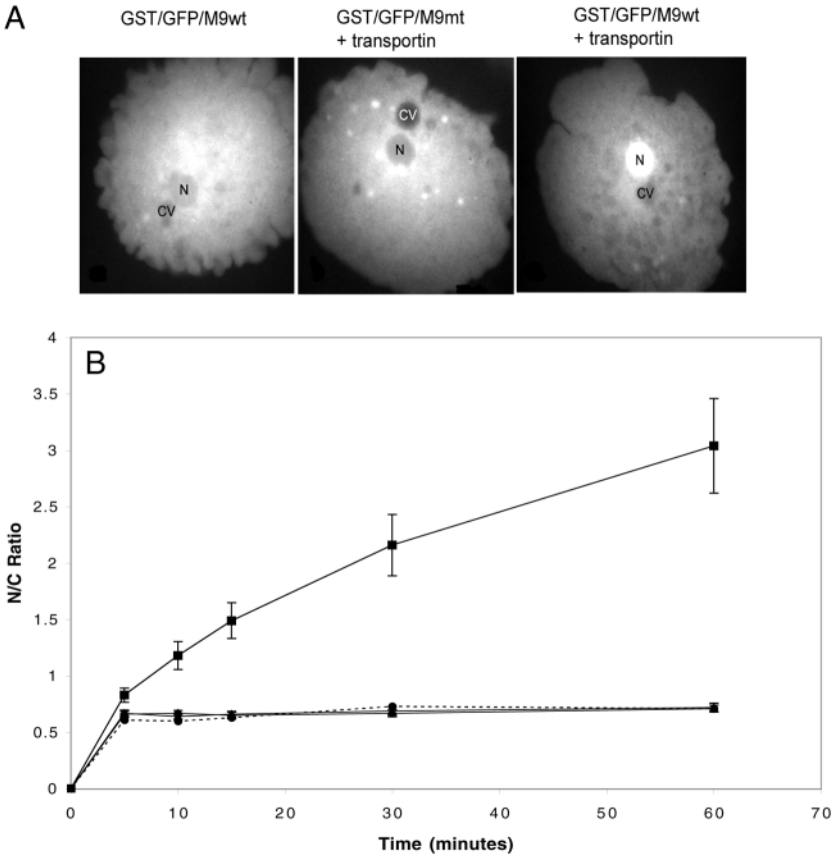

Fig. 4. (A) Fluorescent micrographs illustrating the effect of transportin on the nuclear uptake of GST/GFP/M9 (wt or mt) fusion proteins. The micrographs were taken 60 minutes after the injection of GST/GFP/M9wt alone, GST/GFP/M9mt plus transportin or GST/GFP/M9wt plus transportin. N, nucleus; CV, contractile vacuole. (B) The nuclear uptake kinetics, expressed as N/C fluorescent ratios, are shown for amoebae injected with GST/GFP/M9wt alone (–◆–), GST/GFP/M9mt plus transportin (–▲–), GST/GFP/M9wt plus transportin (–■–), or fluorescein-labeled BSA (…●…), which served as a background control. Respectively, 13, 6, 17 and 7 cells were analyzed.

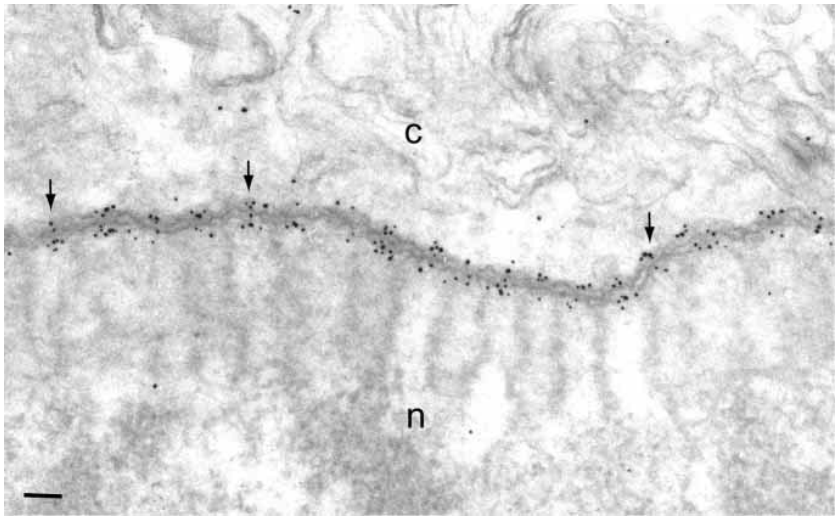

Fig. 5. The distribution of transportin-coated gold in amoebae fixed 30 minutes after injection. Particles bound to the pore complexes (arrows) and were also present within the nucleoplasm. c, cytoplasm; n, nucleus. Bar, 100 nm.

Discussion

Evidence is presented indicating that FxFG motifs and Ran, factors that are essential for translocation in yeast and vertebrate cells, are also present and necessary for nuclear import in amoebae. Furthermore, substrates containing import sequences that were found to be inactive in amoebae (the hnRNP A1 M9 sequence) or have borderline activity (the classical bipartite signal) rapidly accumulated in the nucleoplasm

Table 3. The effect of importin α/β on bipartite NLS-gold import

Experiment		No. of cells analyzed	No. of particles counted	N/C ratio±s.e.	Significance*
Coating agent	Importin α/β				
BSA-bipartite NLS	None	9	1506	0.021±0.0079	–
BSA-bipartite NLS	+	6	2588	3.104±0.2393	s
BSA	+	6	763	0	**

*Indicates whether the values are (s) or are not (ns) significantly different from those of the controls. Probability values of 0.01 or less were considered significant.

**Significance could not be calculated.

when they were microinjected along with the appropriate vertebrate transport receptor (transportin and importin α/β, respectively). Overall, these results suggest that the mechanism for translocation through the nuclear pores is highly conserved.

According to available structural and molecular data, the nuclear pore complex contains a central channel lined by nucleoporins, many of which contain FG-repeat motifs. This channel appears to be a universal conduit used by members of the importin β superfamily to exchange between the nucleus and cytoplasm. Several lines of evidence indicate that many of the FG nucleoporins located within and also adjacent to the central channel serve as common binding sites that function in the translocation of all receptors. For example, in vitro studies have shown that many of the receptors bind to common FG nucleoporins (Ryan and Wente, 2000). Kutay et al. constructed importin β fragments that bound to the nuclear pore complex but were not released by Ran (Kutay et al., 1997). Consistent with the existence of common binding sites, it was found that these fragments had a general inhibitory effect on nuclear transport. They blocked the export of mRNA, U snRNA, and proteins containing the Rev nuclear export signal, as well as the import of an M9 fusion protein. Damelin and Silver used fluorescence resonance energy transfer to investigate the interactions between 13 nucleoporins and two different members of the importin β family, one importin (Kap121) and one exportin (Msn5) (Damelin and Silver, 2000). They found that during translocation, the receptors bound to a subset of seven common nucleoporins, as well as one or two specific nucleoporins. These data suggest that in addition to universal binding sites, different receptor pathways might also require one or more specific nucleoporins. In this regard, Walther et al. obtained evidence that the nucleoporin Nup153 is specifically involved in the translocation of substrates mediated by importin α/β (Walther et al., 2001). Using reconstituted *Xenopus* nuclei, these investigators determined that the assembly of the envelopes in the absence of Nup153 inhibited the transport of substrates that require importin α/β but had no effect on transportin-dependent nuclear import. Shah and Forbes previously identified a Nup153 FG-repeat domain that bound to importin β and blocked the transport of classical NLS substrates but not M9-containing substrates (Shah and Forbes, 1998). They also identified a transportin-binding domain in Nup153, but its general role in transport is controversial (Nakielny et al., 1999).

Since the importin α/β heterodimer and transportin are functional in both vertebrate cells and amoebae, and since the

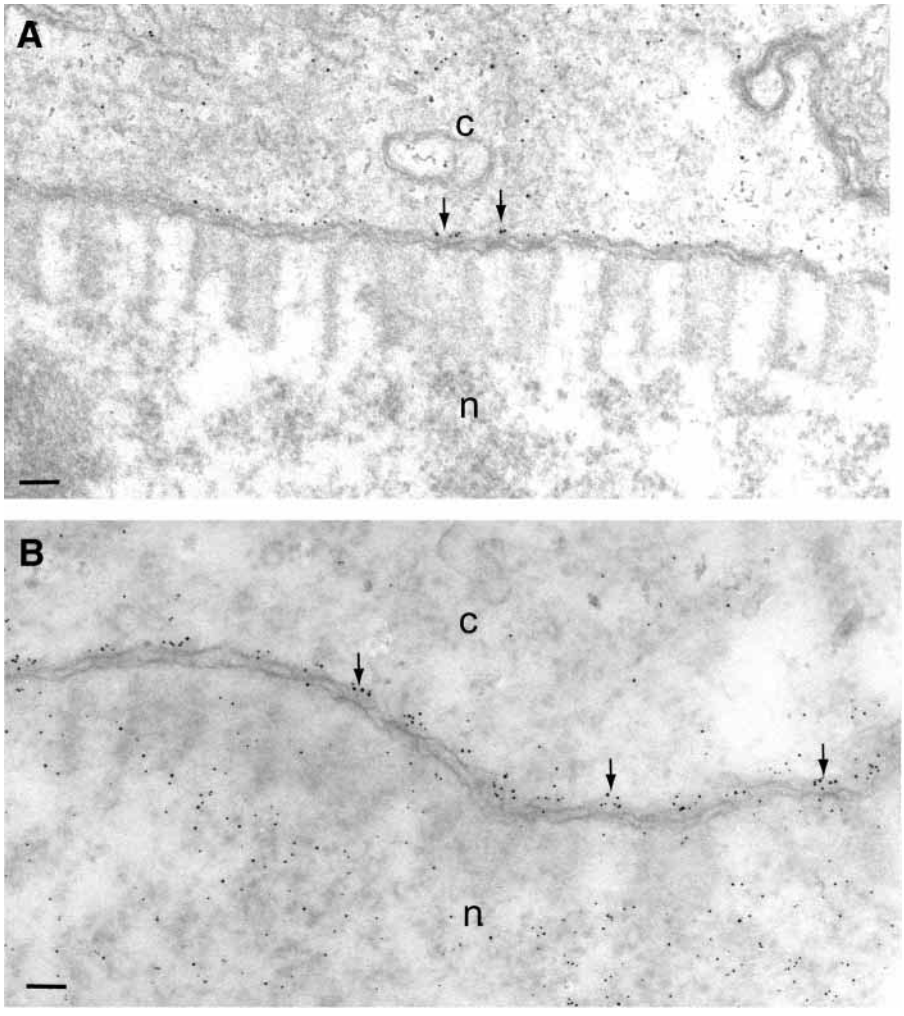

Fig. 6. The nucleocytoplasmic distribution of gold particles coated with BSA-bipartite NLS conjugates, which were injected either alone (A) or along with importin α/β (B). In the presence of the receptor, particles were associated with the pores (arrows) and also accumulated in the nucleus. c, cytoplasm; n, nucleus. Bar, 100 nm.

pore complexes in these organisms are similar in their basic morphology and composition, it is likely that the translocation mechanisms are also comparable. Thus, it is probable that the FG nucleoporins associated with the central transport channels have equivalent functional roles, based on their potential to form multiple weak interactions with the transport factors, thereby facilitating their translocation through the pores. In addition, specific nucleoporins, which are required for different receptor pathways, might also be present. These could include nucleoporins associated with either the initial or terminal phases of the translocation process. Alternatively, the translocation pathway in amoebae, although fundamentally similar in design to that in higher organisms, might be more rudimentary and contain only common FG-repeat-binding sites. Differences could be caused by variations in the proportion of the different FG motifs, as well as their distribution within the pore complexes. According to this model, specific nucleoporins would then function primarily in higher organisms, either as redundant pathways to regulate relative rates of exchange of different receptors or as essential transport factors for particular receptors. Additional experiments, similar to those reported here, but using different transport receptors, should help distinguish between the latter possibilities.

In addition to providing data regarding the evolution of the nuclear transport apparatus, amoebae might also prove to be a useful in vivo experimental system for studying the molecular mechanisms of nuclear trafficking. Nuclear transport assays based on permeabilized cultured vertebrate cells or the yeast *Sacchromyces cerevesiae* have allowed the components of the transport machinery to be identified and the interactions among these components to be explored (e.g. Gorlich and Kutay, 1999; Nakielny and Dreyfuss, 1999; Bayliss et al., 2000b; Wente, 2000; Rout and Aitchison, 2001). However, these experimental approaches have certain limitations. Although the yeast system makes powerful use of molecular genetics and also allows transport to be studied in vivo, kinetic studies, such as those involving microinjection, are not feasible. The permeabilized cell system is useful for investigating the effects of soluble transport factors, but they do not fully replicate in vivo conditions. As we have shown here, in vivo transport studies can readily be carried out in *A. proteus*. Moreover, the fact that the amoebae transport machinery is unable to recognize certain vertebrate NLSs can be exploited to analyze translocation of receptors that do not normally function in these organisms, thereby avoiding 'background' import that would complicate interpretation of the results. This would involve the construction of mutants that would block interactions between the receptor and specific components of the transport machinery and subsequent analysis of the transport of signals specific for the receptor. For example, using this approach it should be possible to establish the importance of different nucleoporin binding sites for transport and also determine if there is a universal requirement for Ran, an issue that is still somewhat controversial.

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