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Cytosolic Factors in Nuclear Transport: What's Importin?

Minireview

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To enter the nucleus, a protein must transit through one of the largest and most complex gateways in the eukaryotic cell, the nuclear pore. Consisting of perhaps 1000 proteins (60–100 different ones), the 120 million Dalton pore recognizes and imports proteins involved in all walks of nuclear life (Forbes, 1992; Rout and Wente, 1994). The proteins imported include replication enzymes, transcription factors, steroid receptors, and histones, as well as small nuclear ribonucleoproteins (snRNPs). At the same time, messenger RNAs, tRNAs, ribosomal precursors, and shuttling proteins are exported. This minireview will focus on nuclear import.

Small proteins can enter the nucleus by diffusion through the ever-open central channel of the pore (90 Å), but even small nuclear proteins take advantage of a more specific path, one that relies on possession of a nuclear localization signal (NLS). Nuclear proteins contain within their amino acid sequence one or more NLS sequences, which cause the central channel of the pore to expand transiently up to 260 Å in diameter to allow nuclear protein entry. NLS sequences fit no consensus but fall in general into two classes, short basic sequences of four to seven amino acids and longer bipartite sequences consisting of two stretches of basic amino acids separated by ten less-conserved amino acids. The majority of nuclear proteins analyzed contain one (or several) of the simple NLS signals above, although more complex nuclear signal sequences and domains also exist. As long as a macromolecule bears an NLS, the nuclear pore will open. The pore's liberal view of what constitutes an import substrate was made most obvious when it was discovered that 20–260 Å gold particles coated with NLS-containing proteins are readily transported through the nuclear pore (Dworetzky and Feldherr, 1988). Such assays, which visually follow nuclear transport as it occurs, allowed the identification of an energy requirement and the definition of two distinct steps in nuclear transport. First, an NLS-bearing protein binds to the pore in an ATP-independent manner. Next, the NLS-bearing protein translocates through the pore, a process that is energy dependent (Newmeyer and Forbes, 1988; Richardson et al., 1988; Akey and Goldfarb, 1989).

Initial Thoughts

A decade ago it was thought that the nuclear pore must surely contain among its 60–100 different proteins an NLS receptor. Such an NLS receptor would initiate the binding step in import. Since only a single pore protein had been identified at the time, there were certainly no data to refute this supposition. Indeed, there appeared to be no mechanistic reason to invoke cytosolic factors at all in nuclear import. Nuclear proteins are not thought to be unfolded during import, as are mitochondrial proteins, which require the cytosolic factor hsc70 for their import. Nuclear transport also differs substantially in concept from import into the endoplasmic reticulum (ER), which requires the cytosolic RNP complex SRP. In that instance, the SRP complex binds to the ER signal sequence as soon as the nascent peptide emerges from the ribosome, arresting translation until docking and cotranslational ER import can occur.

Despite this assumption, evidence that we will review below indicates that not one but a number of cytosolic factors are involved in nuclear import. The identification of these factors in different systems and by different techniques, however, has led to confusion as to which are authentic, as well as to which of the many are equivalent in function. The recent description and cloning of one such factor, importin (Görlich et al., 1994), now allows the proposal of a somewhat unifying picture of the cytoplasmic factors involved in nuclear import. As often happens, clarification on the one hand leads to new questions on the other, discussed at the end of the minireview.

Cytosolic Factors and NLS Receptors

In initiating a search for the NLS receptor, perhaps fully expecting it to be a pore protein, Adam et al. (1989) used a radiolabeled NLS peptide derived from the SV40 T antigen NLS and by chemical cross-linking looked for proteins that would specifically bind to the NLS. Two putative NLS receptors were identified. Somewhat surprisingly, both were present not only in the nuclear envelope, as expected, but also in the cytosol and nucleoplasm of rat liver cells. Were they the real NLS receptor(s)? Had they fallen off the pore during cell fractionation? Other researchers, using related experimental methods, also identified putative NLS receptors in organisms as disparate as yeast and human (for review see Yamasaki and Lanford, 1992). One protein, NBP70, seemed to be present both in the nucleus and cytoplasm of yeast (Stochaj and Silver, 1992), although others were nucleolar (Xue and Melese, 1994). To make matters more complex, some of the putative NLS receptors proved to be binding to the NLS for nonspecific reasons. For example, one putative NLS receptor proved upon sequencing to be protein disulfide isomerase (PDI) that had stuck to the cysteine residue added to the synthetic NLS for cross-linking (Yamasaki and Lanford, 1992). Thus, whether any of these proteins were the true NLS receptor(s) was a contentious issue, much less whether they were cytosolic proteins.

The first direct evidence that cytosolic factors were required for nuclear import came from an in vitro nuclear import system. This system, composed of isolated rat liver nuclei, an extract of Xenopus eggs, and a rhodamine-labeled nuclear transport substrate, showed vigorous and specific import of the transport substrate. However, N-ethylmaleimide (NEM) treatment of the cytosol severely depressed import and did so by blocking the binding step of transport (Newmeyer and Forbes, 1990). A 40% ammonium sulfate precipitate of the Xenopus cytosol was able to restore import. This NEM-sensitive cytosolic activity was termed NIF1, for nuclear import factor 1. A second nuclear transport assay, using digitonin-permeablized mammalian

cells and exogenously added cytosol, was developed and confirmed the need for a cytosolic factor in nuclear protein import. As in the Xenopus system, NEM-treated cytosol was completely negative for import (Adam et al., 1990).

Adam and Gerace (1991) continued their search for an NLS receptor, biochemically fractionating bovine erythrocyte cytosol and assaying for proteins that would bind and cross-link to a synthetic NLS. Erythrocyte cytosol appeared at first glance to be an odd choice of starting material since the erythrocytes had no nuclei, but the researchers found one of the same proteins they had previously observed in rat liver nuclei, a 54/56 kDa protein doublet that specifically bound the NLS. The 54/56 kDa protein stimulated transport 2- to 3-fold in a permeablized cell assay containing limiting cytosol, simultaneously suggesting that this was indeed the true NLS receptor and that the NLS receptor was cytosolic. The 54/56 kDa NLS receptor, when NEM treated, continued to bind synthetic NLS peptide, but had lost its ability to stimulate import in permeablized cells.

Ran/TC4

Was there only one cytosolic factor involved in nuclear protein import or multiple factors? Moore and Blobel (1992) set forth to determine this biochemically by combining the permeablized cell assay with fractionation of the Xenopus oocyte extract. Following column chromatography, a fraction of the oocyte extract, fraction A, caused fluorescently labeled nuclear transport substrate to bind to the nuclear envelope in a signal sequence-dependent but ATP-independent manner, mimicking the first step in nuclear import. A second fraction, fraction B, had no binding activity on its own, but when combined with fraction A evoked the same high level of import as unfractionated cytosol. Fraction A was NEM sensitive, like NIF1 and the 54/56 kDa NLS receptor. Fraction B, the translocation factor, was NEM insensitive. One might suspect that fraction A since it stimulated the binding step of transport contained the NLS receptor, but what was fraction B?

To identify the translocation factor in fraction B, Moore and Blobel (1993) further fractionated oocyte cytosol, assaying for an activity able to convert the brightly fluorescent nuclear rim stain seen with fraction A to the fluorescent intranuclear stain indicative of translocation. They identified a single protein of 25 kDa. Peptide sequence analysis led to the surprising conclusion that the major component of the translocation factor was the small GTP-binding protein, Ran/TC4 (Moore and Blobel, 1993). Indeed, nonhydrolyzable GTP analogs blocked nuclear import, and recombinant TC4 could substitute for the purified Xenopus TC4 (Moore and Blobel, 1993; Melchior et al, 1993).

Why was Ran/TC4 a surprising participant in nuclear transport? Previous studies had implicated Ran/TC4 along with its guanine nucleotide exchange factor, RCC1, in various aspects of nuclear function, including chromosome condensation, DNA replication, RNA transcription, and the cell cycle (for review see Dasso, 1993). Although all of these problems could be caused by a defect in nuclear transport, this appears not to be the case. Ran/TC4 GDP-bound mutants arrest the cell cycle in vitro even in the complete absence of nuclei (Kornbluth et al., 1994). The same mutant form of Ran/TC4 causes structural defects in the nucleus, while scarcely altering nuclear protein import (Kornbluth et al., 1994; Dasso et al., 1994). While multiple lines of evidence indicate that this highly abundant Ran/TC4 protein (10^7 copies per cell) plays other roles in the cell, evidence continues to mount that it has a pivotal role in nuclear import. In addition to the data cited above, Schlenstedt et al. (1995) find in yeast that expression of a GTP-bound mutant form of Ran/TC4 results in the failure to import nuclear proteins. This mutant also shows simultaneous nuclear accumulation of poly(A)$^+$ RNA, implying a possible export defect when Ran/TC4 cannot hydrolyze GTP.

Moore and Blobel (1994) found that purified Ran/TC4 could not provide the full import activity of fraction B, implying that an additional stimulatory factor must exist. Very recently, they purified this B-2 activity. Peptide sequence analysis indicates that B-2 is the Xenopus homolog of a previously cloned human protein of unknown function, placental protein p15, which exists as a homodimer (pp15; 15 kDa). There is now strong evidence that the translocation factor present in fraction B is a 60 kDa complex, composed of a monomer of Ran/TC4 and a dimer of B-2. Since Ran/TC4 has a largely nuclear location by immunofluorescence, it is thought that it must shuttle between the nucleus and cytoplasm; whether B-2 accompanies it is unknown. A more interesting question is whether Ran/TC4 acts to carry the NLS-bearing protein in a complex through the nuclear pore. An equally likely scenario, however, is that Ran/TC4's role in import is the release of the nuclear protein from its NLS receptor so that it may move to the next step of import.

A Confusing Array of Other Factors

The experimental progression above is straightforward, outlining the discovery of three cytosolic factors clearly needed for nuclear import. This gives a false impression of clarity in the field from the time of discovery and indeed to the present time. The multiple systems used (Xenopus eggs, HeLa cells, rat fibroblasts) and the multiple factors observed (NIF1; NLS receptor; Ran/TC4; B-2), with little evidence to tie any set of observations together, led to a feeling of confusion. Fraction A might correspond to NIF1 and seemed likely to contain the NLS receptor, but the apparent large size of the active component of fraction A (250 kDa) suggested a complex. Indeed, other factors do exist. Having refined their system to permit the specific assay of nuclear envelope binding, Adam and Adam (1994) found that a 97 kDa cytosolic protein greatly stimulates the 54/56 kDa NLS receptor's ability to promote the binding step of transport.

In addition to the 54/56 kDa NLS receptor, other less-abundant NLS receptors seem almost a certainty. This conclusion stems from the finding that although most nuclear proteins tested compete with one another for import and therefore can use the same NLS receptor, noncompeting import pathways were found for different snRNP particles, implying the existence of additional NLS receptors (for review see Fabre and Hurt, 1994).

Most unexpected was the finding that hsp70 or its cyto-

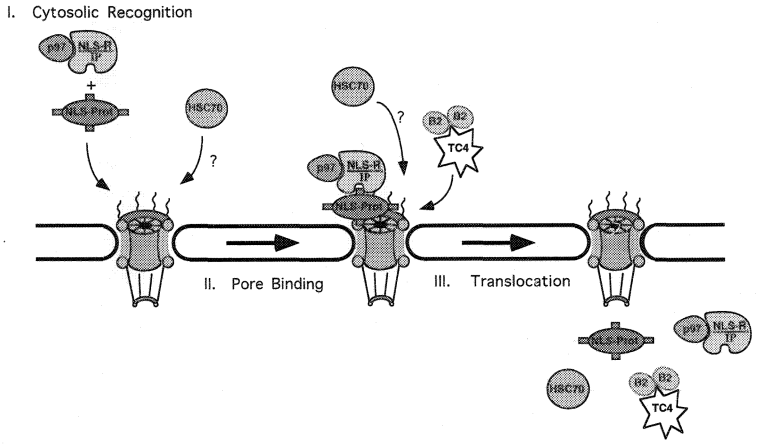

Figure 1. A Model of Cytosolic Factors in Nuclear Import

The NLS receptor (54/56 kDa receptor; importin) is indicated as NLS-R/IP. The receptor recognizes an NLS-bearing protein and directs it to a binding site at the pore. The activity of the receptor is stimulated by its interaction with p97. Once the receptor–NLS-bearing protein complex is bound at the pore, a complex of TC4 and B-2 stimulates translocation. All of these factors or a subset may be imported along with the NLS-bearing protein. The stage at which hsc70 acts in nuclear import is not known, and this is indicated as a potential contribution to binding or translocation. At high concentrations, importin and Ran/TC4 appear sufficient for import; it is likely that at physiological concentrations the 97 kDa and B-2 proteins are necessary.

solic cognate, hsc70, plays a role in nuclear transport (Shi and Thomas, 1992; Imamoto et al., 1992). Cytosol from HeLa cells is defective for import when depleted of hsp70/hsc70 proteins, either by ATP–agarose or antibodies against these proteins. Strikingly, recombinant hsc70 or hsp70 fully restores the import activity; antibodies to hsc70 block import (Shi and Thomas, 1992; Imamoto et al., 1992). Neither protein is sensitive to NEM inactivation. Thus, there appear to be at least five factors required for nuclear import: the NLS receptor, a 97 kDa accessory protein, TC4, B-2, and hsc70. Since nuclear proteins are not thought to be unfolded, the requirement for hsc70 remains unexplained, although a clue may come from the finding that these proteins shuttle back and forth across the nuclear envelope (Mandell and Feldherr, 1990). Interestingly, however, hsp/hsc70 is not required for import of the glucocorticoid receptor (Yang and DeFranco, 1994).

Lastly, in a somewhat different study of cytosolic factors, Sterne-Marr et al. (1992) found that passing cytosol over a column of immobilized pore proteins depletes the cytosol of an NEM-insensitive activity essential for transport. The identity of the depleted protein or proteins remains unknown. They may correspond to the NEM-insensitive TC4 or the B-2 component of fraction B, or the results may indicate the existence of additional cytosolic translocation factors.

Importin

Into the fray comes importin. Görlich et al. (1994) set out to conduct a search for the protein or proteins that are the mediators of the first step in nuclear import, the binding step, by using a functional assay. Essentially, they looked for the active protein component of fraction A. To do this, they fractionated Xenopus egg cytosol and added each fraction to a permeablized cell assay that already contained transport substrate, ATP, and saturating amounts of recombinant TC4, with the assumption that TC4 would carry out the translocation step. They purified from Xenopus eggs a single protein of 60 kDa, which they named importin. When purified importin and recombinant Ran/TC4 were combined (100 μg/ml each), the two proteins together gave high levels of nuclear import. In the absence of TC4, this concentration of importin was sufficient to mediate the pore-binding step.

The authors cloned importin using peptide sequence analysis and polymerase chain reaction. A Xenopus cDNA library yielded six closely related importin clones encoding proteins of 528 amino acids, which differed in 1–22 amino acids. These may represent a family of six related importin genes or multiple alleles of a smaller family. However, when tested in the transport assay, a single recombinant importin protein could act in concert with recombinant Ran/TC4 to allow nuclear transport in the permeablized cell assay. The most interesting result was that when the sequence of importin was analyzed, it showed strong homology with the yeast protein SRP1 (identity 44%) and with the human protein Rch1 (64%).

Why is this interesting? Why is importin important? The answers to these questions are twofold. First, in yeast, SRP1 interacts directly with two nuclear pore proteins, NUP1 and NUP2 (Belanger et al., 1994), and by immunofluorescence associates with the pore (Yano et al., 1992). For the first time a cytosolic factor involved in import has a direct connection to specific pore proteins. The *SRP1* gene, mutants of which have very pleiotropic effects on the yeast cell, encodes an essential protein of 60.5 kDa (Yano et al., 1992, 1994). (It is no relation to the SRP RNP complex that acts in ER translational arrest.) Interestingly, a large part of both SRP1 and importin consists of eight hydrophobic 42 amino acid repeats, termed arm repeats. The arm repeats have also been found in β-catenin, plakoglobin, armadillo (the Drosophila homolog of β-catenin), as well as in the tumor suppressor protein APC (which binds to β-catenin), and the GDP/GTP exchange factor smgGDS (Peifer et al., 1994). In all cases, it is thought that the arm repeats mediate strong protein–protein interactions.

The second reason that importin is important is that at last a binding factor for nuclear transport has been cloned and sequenced. One would predict from the biochemical assay data that importin and SRP1 must be signal sequence receptors in their respective organisms. However, neither Görlich et al. (1994) nor the SRP1 groups present data that address NLS binding. Fortunately, recent sequence analysis of the rat 54/56 NLS receptor reveals that it is very similar to human SRP1 (S. Adam, personal communication), one of two SRP1-related proteins in hu-

mans, Rch1 and human SRP1 (Cuomo et al., 1994; Cortes et al., 1994). This at last closes the circle of inferences. Importin, a protein with the functional activity expected of an NLS receptor, has sequence homology with the 54/56 kDa NLS receptor, a protein with NLS-binding activity.

A Model

Nuclear transport, at least in the area of cytosolic factors, is beginning to make sense. A model can be proposed that fits the data of many groups (Figure 1). An NLS-containing protein binds in the cytosol to the NLS receptor (54/56 kDa receptor, importin, SRP1) with the aid of the 97 kDa accessory protein. This NLS protein–NLS receptor–p97 complex next binds to the nuclear pore, perhaps via NUP1 or NUP2 in yeast; the mammalian homologs of NUP1 and NUP2 are not known. A complex of Ran/TC4 and B-2 dimer then acts at a step subsequent to the binding step to allow translocation through the pore. It is too early to know whether there is, in fact, one step or many in the translocation process. Ran/TC4–B-2 may act anywhere in this pathway: in releasing the NLS protein from a bound state so that it can move forward, in opening the pore, or in an undiscovered step of translocation. Hsc70 is also involved, but where is not yet known. Since the NLS receptor, Ran/TC4, and hsc70 proteins all have dual localization in the nucleus and the cytoplasm, they may likely enter the nucleus as they carry out their role in transport; equally, they may act to stimulate import on the cytosolic side of the pore and then enter the nucleus for a separate reason, perhaps to play a role in export. As can be seen, the many points of mechanistic uncertainty in this model pose questions for the future.

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