Nucleocytoplasmic transport: factors and mechanisms

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Abstract In the past two years, our knowledge concerning the mechanisms of nucleocytoplasmic transport through the nuclear pore complex (NPC) has considerably expanded. The application of in vitro systems that reconstitute nuclear protein import has allowed the identification of cytosolic factors that are required for the import process. Microinjection into *Xenopus* oocytes and yeast genetic systems have provided interesting candidates for RNA export mediators. Functional and structural analysis of nucleoporins has demonstrated the involvement of NPC components in the transport process. Finally, new concepts have emerged such as the integration of the mechanisms of the nuclear protein import and RNA export reactions and the assembly of the transport machinery at specialised domains of the NPC.

Key words: Nucleus transport; Nuclear pore complex; Nuclear localization sequence; Ran/TC4; Nucleoporin

1. Introduction

Nucleocytoplasmic transport takes place through the nuclear pore complexes (NPC), macromolecular assemblies embedded in the double nuclear membrane. The structure, molecular architecture and composition of the NPC have recently been reviewed [1–7]. This mini review will focus on new developments concerning the mechanisms of protein import into and RNA export from the nucleus and the potential functions of nuclear pore components.

2. Protein import into the nucleus

Proteins destined to be imported in the nucleus (karyophilic proteins) contain a nuclear localization sequence (NLS). Two classes of NLS have been defined so far. The first class consists of a short stretch of basic amino acids and has as a prototype the SV40 T antigen NLS, PKKKRKV [8]. The second, originally characterised in nucleoplasmin, is composed of two clusters of basic residues separated by an ill-defined spacer sequence of about ten amino acids in length and is called the bipartite NLS [9]. Longer and thus more complex NLS have also been found in several cases [10–12]. Nuclear import of NLS-containing proteins is an active process and occurs in two steps: first, targeting and binding of the karyophile in a NLS-dependent manner to the cytoplasmic side of the nuclear pores and, second, translocation into the nucleoplasm through the NPCs. While the first step requires no energy and can take place in the cold, the second step is ATP and temperature dependent [13,14]. Both steps require cytosolic factors which, however, are distinct for each step and can be separated biochemically in different cytoplasmic fractions (fractions A and B, respectively) [15].

A variety of techniques such as peptide cross-linking, affinity chromatography, ligand-blotting (reviewed in [16]), anti-idiotypic antibodies [17] or genetic screens [18–20] were used to search for proteins that would bind specifically to NLSs. By these approaches, several proteins were identified as putative NLS-receptors. More recently, the establishment of in vitro nuclear uptake assays employing isolated nuclei or digitonin-permeabilized cells allowed the characterization of cytosolic fractions essential for nuclear protein uptake and subsequently specific nuclear import factors could be purified. Thus, a 9S protein complex that is responsible for the targeting of NLS-containing substrates to the NPC was recently purified from fraction A of *Xenopus* ovary cytosol [21]. This complex was termed karyopherin and was shown to be composed of three subunits of 54, 56 and 97 kDa (karyopherins α1, α2 and β, respectively). An apparently very similar complex was identified at the same time by another group [22]. In the latter case, it was isolated from *Xenopus* egg cytosol and was shown to contain two subunits of 60 and 90 kDa which were named importin 60 and importin 90, respectively. A cytoplasmic protein complex that bound to a NLS-containing protein was also shown to contain components of similar molecular weights in Ehrlich ascites tumour cells [23].

Importin 60 (corresponding to karyopherin α) was initially characterized as an essential nuclear import factor and its cDNA was cloned and sequenced [24]. It belongs to a growing family of proteins that also include the human homologues Rch1 [25], hSRP1 [26,27] and hSRP1α [28], the bovine 54/56 kDa NLS receptor [29] and the essential yeast protein Srp1p [30,31]. Aside from importin 60, the human proteins were found in the two-hybrid system through their interaction with NLS-containing, but otherwise unrelated, polypeptides. Yeast *SRP1* was isolated as a suppressor of RNA polymerase I mutants. Importin 60 is able to physically associate with NLS-containing substrates and promote their docking to the nuclear envelope. hSRP1α, in particular, has affinity for both simple and bipartite NLSs. Importin 60 and the other proteins of the same family contain 'arm' motifs [32] which may constitute a protein–protein interaction domain. Their sequence divergence (40–60% identity even among proteins of the same species) may reflect a functional diversity which is necessary in order to accomodate the large number of different nuclear import substrates and different forms of NLS [33,34].

The second subunit of the heterodimeric importin complex, importin 90 (corresponding to karyopherin β), only weakly binds to NLS peptides and alone is not sufficient for the targeting of NLS-conjugates to the nuclear envelope [22]. Despite this, when added in the in vitro nuclear uptake assay, it cooperatively enhances the function of importin 60. Importin 90

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may therefore constitute the docking subunit of the complex while importin 60 functions predominantly as the NLS binding subunit. The *Xenopus* importin 90, for which only partial peptide sequences are known, and the human and rat homologues, for which full-length cDNAs are available, are highly conserved. A bovine 97 kDa protein that, in conjunction with the 54/56 kDa NLS-receptor, reconstitutes the first step in nuclear protein import [35], is probably related to importin 90.

Biochemical fractionation of the *Xenopus* fraction B that mediates the energy-requiring second step in nuclear protein import, i.e. translocation through the nuclear pore channel and accumulation in the nucleoplasm, has led to the identification of Ran/TC4 as an essential nuclear import factor [36,37]. Ran (Ras-related nuclear protein) is small GTPase of 27 kDa and an abundant nuclear protein [38]. Activation of Ran requires the exchange of bound GDP for GTP, which is stimulated by its specific nucleotide exchange factor RCC1 (regulator of chromosome condensation), a 45 kDa chromatin-binding protein [39]. Ran and RCC1 are highly conserved proteins both structurally and functionally. They are thought to be basic components of a nuclear regulatory pathway involved in the cell cycle, maintenance of nuclear structure and RNA processing and export into the cytoplasm [40]. In fraction B, Ran forms a 60 kDa complex with a dimer of a 15 kDa protein termed Ranip (Ran-interacting protein) [41]. Ran and Ranip are sufficient to catalyze translocation of NPC-docked karyophilic substrates into the nucleus in a reaction that requires GTP hydrolysis. The human homologue of the *Xenopus* Ranip has also been recently purified and cloned and it was named NTF2 (nuclear transport factor 2) [42]. NTF2 appears to interact directly with the nuclear pore protein p62 and thus it may function in targeting Ran to the nuclear pore.

The identification of Ran as an essential factor for nuclear protein uptake provided the first clue as to how nuclear import and export reactions could be functionally linked. It is, however, not clear whether a common Ran/RCC1-cycle is required for both nuclear import and export events or whether, via a variety of effectors, Ran participates in different cellular pathways. In search of such effectors, RanBP1 (ran binding protein 1), RanBPX and RanGAP1 (RanGTPase-activating protein) have recently been discovered [43–47]. RanBP1 binds to Ran-GTP and affects GTP exchange and hydrolysis [45] while RanBPX appears to be a component of the nuclear pore (see also below). The yeast RanGAP1 is the product of the *RNA1* gene [47], mutations in which result in defects in tRNA and rRNA processing and mRNA export [48,49]. Surprisingly enough, Rna1p appears to be localized in the cytoplasm [50]. The fact that the two antagonistic Ran regulators, RCC1 and RanGAP1, are physically separated by the nuclear envelope suggests that Ran may shuttle between the nuclear and cytoplasmic compartments. One of the observations that substantiated the involvement of Ran in the translocation step of nuclear protein import was the requirement for GTP hydrolysis [36,51]. This implies, in spite of some controversy [52], that the active form of Ran for protein import is the GTP-bound form. Conversion of cytoplasmic Ran-GTP to Ran-GDP by RanGAP1 may trigger the translocation of karyophiles through the nuclear pore. Ran-GDP has then to enter the nucleus in order to be re-activated by nuclear RCC1 that catalyses the exchange of GDP for GTP. The GTP-bound form of Ran could finally be re-exported from the nucleus or fulfill its nuclear functions including RNA export. A recent report has demonstrated that overexpression of a mutant form of the yeast homologue of Ran that is able to bind but not hydrolyse GTP, leads to a block in both protein nuclear import and poly(A)$^+$ RNA export [53]. On the other hand, a Ran mutant lacking a carboxy-terminal domain, is still active in the nuclear protein import assay, but loses its ability to associate with RanBP1 and to affect cell cycle progression [54] suggesting that different domains of Ran may be responsible for the diverse functions of Ran.

The identification of the importin (or karyopherin) complex, Ran and Ran-associated proteins has by no means exhausted the list of candidate nuclear import factors. The heat-shock protein hsc70 has been proposed to play a role in nuclear transport on the basis of several observations and can shuttle in and out of the nucleus [55,56]. Interestingly, hsc70 has also been found to physically interact with components of the RCC1/Ran system [57]. Several other proteins that were initially characterized by their affinity for NLS-containing peptides, were finally shown to reside mainly in the nucleolus (reviewed in [58]). The NLS-binding protein Nopp140 in particular shuttles between the nucleolus and the cytoplasm, and it may do so on intranuclear tracks that extend from the nucleolus to the nuclear periphery and are sometimes directly connected to the nuclear pores [59]. It has been suggested that these nucleolar NLS-binding proteins may have a specific function in the biogenesis and transport of pre-ribosomal particles [58]. Finally, the import of snRNPs into the nucleus depends on signals both on their snRNA as well as their associated proteins and accordingly their import pathway is distinct from that of classical NLS-containing proteins ([60] and references therein). The factors involved in snRNP import have not been found so far.

3. RNA export

As for nuclear protein import, nuclear export of RNA seems to be a carrier-mediated and energy-dependent process, taking place in at least two steps. Ribonucleoprotein particles (RNPs) have to move from their site of transcription and RNP assembly to the nuclear envelope before translocation into the cytoplasm through the NPC [61–69]. A general targeting signal (export signal) analogous to that of a NLS has not yet been found for RNA. It appears that different classes of RNA depend for their export on specific signals which could either lie in the RNA moiety or be provided by the protein components of the RNPs. Experiments involving microinjections into *Xenopus* oocytes have demonstrated that the export of different classes of RNA (tRNA, 5S rRNA, U snRNA, mRNA) is mediated by different saturable factors, as the export of a given class of RNA, e.g. snRNA, can be saturated by itself or other snRNAs, but not by mRNA, rRNA and tRNA [70]. On the other hand, homopolymeric RNA inhibits the export of more than one class of RNA, suggesting also the existence of common factors required for efficient export [70]. Another, apparently general determinant for export is the dissociation of RNAs from common intranuclear retention sites that may represent a rate-limiting step for the export reaction [71].

Analysis of mutations which affect the export capability of a specific RNA molecule has led to the identification of features that constitute *cis*-acting signals important for export. These include the 5′ cap structure of U snRNAs and mRNAs [72], the 3′ ends of histone mRNAs [73] and sequence-specific determi-

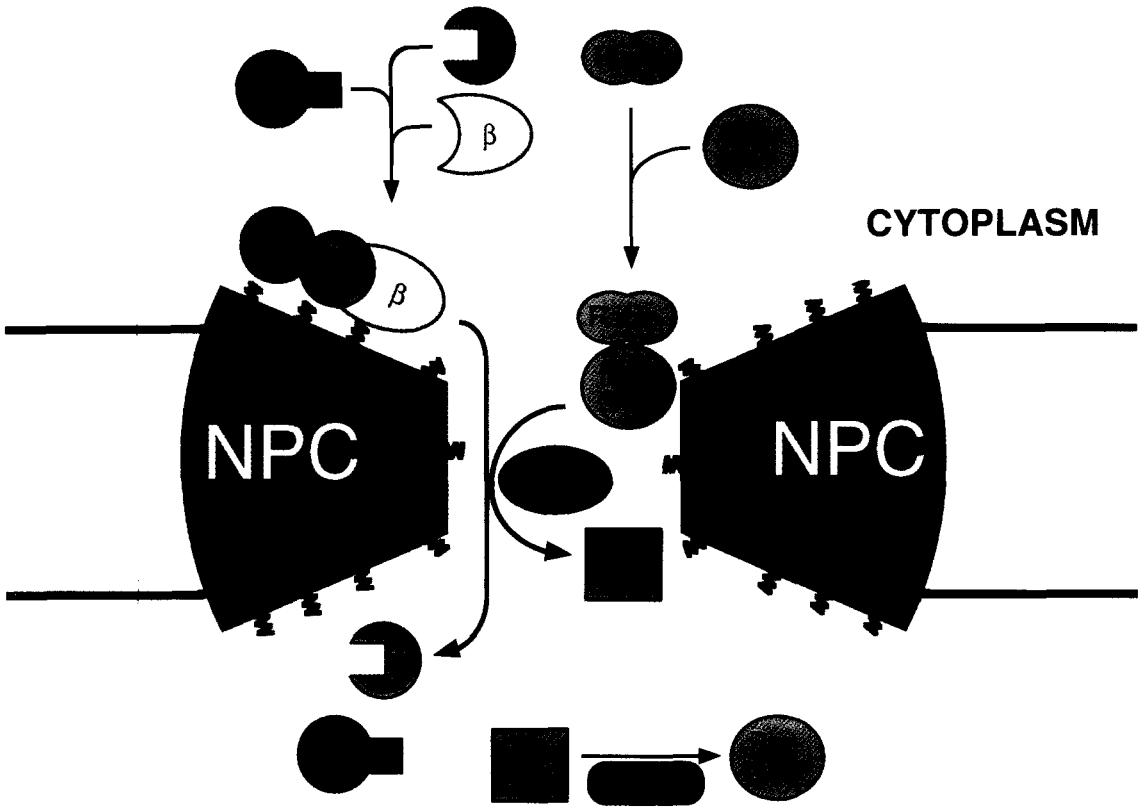

Fig. 1. Schematic representation of the nuclear protein import mechanism. The two subunits of the NLS-receptor α (karyopherin α, importin 60 or SRP1) and β (karyopherin β or importin 90) are responsible for binding a NLS-containing protein in the cytosol and for docking it to the cytoplasmic side of the NPC. Docking is mediated through interactions with the repeat domains of nucleoporins (represented by the zigzagged heavy lines). Ran-GTP and Ranip are also recruited to the NPC by Ran-binding nucleoporins and p62 respectively. Hydrolysis of GTP by Ran assisted by RanGAP is required for the translocation of the karyophile/NLS receptor complex into the nucleoplasm, probably through a series of docking and undocking steps [21,133]. It is not known whether the α or the β or both of the NLS receptor subunits follow the karyophile into the nucleus. Ran-GDP can be reactivated in the nucleus by nucleotide exchange catalysed by RCC1. Ran-GTP has then to return to the cytoplasm to ressume its function in protein import. Other relevant references are mentioned in the text.

nants within tRNA and 5S rRNA transcripts [74–76]. Proteins that specifically recognize these features would be candidates for nuclear export factors. Indeed, a protein complex consisting of the two cap binding proteins, CBP80 and CBP20, has been directly shown to be required for U snRNA nuclear export [77]. Other promising candidates for mediators of RNA export include certain hnRNP proteins which shuttle between the nucleus and the cytoplasm [78], the ribosomal protein L5 and TFIIIA for 5S rRNA [76], the tRNA binding proteins glyceraldehyde-3-phosphate dehydrogenase [79,80] and zuotin [81], SRP9/14 for SRP RNA [82] and viral regulatory proteins such as the HIV-1 Rev and the influenza A NS1 (reviewed in [68,83]). It is, however, yet not clear whether these proteins function directly in RNA export or regulate other aspects of RNA metabolism that could indirectly affect the accessibility to the RNA export machinery. Such a concern is realistic in the view of the observation that assembly of pre-mRNAs into functional spliceosomes causes retention inside the nucleus [84].

To identify components involved in mRNA export in yeast, collections of temperature-sensitive mutants were screened for retention of poly(A)$^+$ RNA inside the nucleus at the non-permissive temperature. These genetic screens yielded the mtr (mRNA transport) [85,86] and rat (ribonucleic acid trafficking) mutants [87]. By these and previous screens a number of genes have been identified that could be classified into three groups. The first group contains genes that code for components of the Ran GTPase cycle such as *CNR1/GSP1* (homologues of Ran) [88,89], *MTR1/PRP20/SRM1* (homologues of RCC1) [90,91] and *RNA1* (homologue of RanGAP1) [50]. As already discussed, these discoveries favoured a model that can unify the mechanisms for nuclear protein import and RNA export. Studies on the effects of RCC1 depletion in the Chinese hamster tsBN2 cell line have shown that RCC1 is also required for export of U snRNAs as well as processing and export of rRNAs [92]. Surprisingly, the export of tRNAs under the same conditions was not affected indicating the presence of an additional or alternative export pathway. Moreover, RCC1 depletion inhibited the accumulation of U3 snoRNAs inside the nucleolus suggesting that a functional RCC1 is also required for intranuclear movement of RNPs [92]. These data are in agreement with a recently published hypothesis according to which, the nuclear Ran GTPase cycle is essential in order to promote peripheralization, i.e. to direct macromolecules inside the nucleus towards the nuclear periphery [93].

The second group of genes found in genetic screens for mRNA export mutants encode proteins of diverse functions which may be involved in various steps of mRNA processing, prerequisite for export. *RAT1/HKE1/TAP1* is an essential gene

that encodes a nuclear protein with RNase activity in vitro [87,94,95]. *MAS3* encodes a heat-shock transcription factor [86]. Mtr2p is an essential nuclear protein involved in maintaining the integrity of the nucleolus [96]. *NOP3/NPL3/MTS1/MTR13/NAB3* is a nucleolar/nucleoplasmic protein implicated in both protein import and RNA export and contains RNA recognition motifs (RRM) [97–100]. Interestingly, Nop3p exhibits considerable sequence and structure similarities to hnRNP proteins of higher eukaryotes and was shown to shuttle between the nucleus and the cytoplasm in yeast heterokaryons. Finally, the *S. pombe* rae1 gene is also involved in the organisation of the cytoskeleton [101]. The fact that some of the above-mentioned mutations that cause accumulation of poly(A)$^+$ RNA in the nucleus also result in structural changes of the nucleolus or are mapped in genes encoding for nucleolar proteins as well as other observations reviewed in [69] may indicate that the nucleolus is also important for nuclear mRNA maturation and export.

The third and last group of genes involved in mRNA export encode for nucleoporins and will be discussed in the next chapter.

4. The involvement of NPC components in nucleocytoplasmic transport

Besides exploiting in vitro systems and genetics to identify factors involved in nucleocytoplasmic transport, the functional analysis of NPC proteins (nucleoporins) has also provided clues as to how they mediate transport through the nuclear pore channel. Thus, it was demonstrated that monoclonal antibodies raised against a family of NPC components as well as wheat germ agglutinin (WGA) that reacts with O-linked *N*-acetylglucosamine containing nucleoporins blocked nuclear protein uptake both in vitro and in vivo [102–108]. The same reagents were also shown to inhibit nuclear export of various classes of RNA when injected into *Xenopus* oocytes [109–111]. However, these results could also be explained by a steric blockage of the NPC channel or a gross structural perturbation of the NPC rather than indicate a direct role of nucleoporins in active transport. The recent identification of nucleoporins and cytosolic factors by biochemical or genetic means and the generation of specific reagents or mutants provided more insight as to how NPC proteins are involved in this transport process.

In yeast, nucleoporin Nsp1p functionally and physically interacts with nucleoporins Nup49p, Nup57p and Nic96p [112–114]. The first three proteins form a stable heterotrimeric core complex to which Nic96p is less tightly attached. Mutations in Nic96p that lead to its dissociation from the core complex as well as mutations in the individual components of the complex cause cytoplasmic accumulation of NLS-containing reporter proteins, but no apparent defect in RNA export [114–116]. The involvement of Nsp1p and Nup49p in protein import was further confirmed by the use of an in vitro nuclear import assay utilising semi-permeabilized yeast cells [117]. On the other hand, another conditional mutant allele of nup49 caused intranuclear accumulation of poly(A)$^+$ RNA [115]. A fraction of Nsp1p which is not part of the Nsp1p/Nup57p/Nup49p/Nic96 assembly is physically interacting with the essential nucleoporin Nup82p [118]. Depletion of Nup82p causes a defect in poly(A)$^+$ RNA export but does not affect protein import or the nuclear envelope structure. Taken together, these results suggest that Nsp1p through multiple interactions with other nucleoporins forms distinct subcomplexes which have different functions during nucleocytoplasmic transport. By analogy, the mammalian homologue of Nsp1p, p62 which is required for the import of karyophiles into the nucleus [104,119] also exists in two distinct molecular complexes. One consists of p62, p58 and p54 [104,119–121] and the other comprises p62 and p200 [122]. As p62 is localized at both the nucleoplasmic and cytoplasmic sides of the central plug or channel complex of the NPC [7,123] a dual role of this protein in nuclear protein import and RNA export remains possible. Another nucleoporin with potential multiple roles in yeast is Nup1p. In temperature sensitive *nup1* mutants both import of nuclear proteins and export of poly(A)$^+$ RNA are defective [124]. However, only a mRNA export defect was observed when the *NUP1* gene was disrupted in a different yeast laboratory strain [125].

As mentioned above, the genetic screens set up in yeast to identify components involved in mRNA export in several cases yielded genes coding for nucleoporins including Rat3p/Nup133p [126] and Rat7p/Nup159p [127]. Nup133p was independently identified by its genetic interaction with Nup49p [115] and it is not essential for growth but disruption of its gene causes accumulation of poly(A)$^+$ RNA inside the nucleus whereas nuclear protein import is not affected. Rat7p/Nup159p, on the other hand, is essential for growth, genetically interacts with Nup133p and mutations in its gene result in a very rapid and specific cessation of mRNA export [127]. Moreover, in both *nup133* or *nup159* mutants the NPCs cluster at a few sites on the nuclear envelope although this phenotype is not linked to the RNA export defect [115,126,127].

Another group of interacting nucleoporins is that of Nup145p, Nup116p and Nup100p [112,128–131]. These three nucleoporins contain a common motif, the nucleoporin RNA-binding motif (NRM), which allows them to bind to homopolymeric RNA in vitro [131]. Genetic experiments revealed that the three copies of this motif carry out an essential, redundant function. Depletion of Nup145p impairs nuclear export of poly(A)$^+$ RNA and at later stages also blocks nuclear protein import. Not surprisingly, it was shown independently that Nup145p is encoded by the *RAT10* gene (A. Goldstein, T.C. Dockendorff and C.N. Cole, personal communication). All these three nucleoporins contain GLGF repeats (like Nup49p and Nup57p) and mutations in two of them (Nup145p and Nup116p) also result in deformations of the nuclear envelope. It is possible that these RNA-binding nucleoporins constitute a NPC subdomain involved in RNA export or they may provide affinity sites for RNPs during their transport. Alternatively, they may be involved in maintaining the association of the NPC with underlying nucleoskeletal structures.

What is the relationship between nucleoporins and the recently identified cytosolic factors required for nuclear protein import? It has been reported that immobilized WGA-reactive nucleoporins are able to deplete most of the cytosolic import activity suggesting that they provide binding sites for the soluble import factors [132]. More recent data have demonstrated that the *Xenopus* cytosolic fraction A can mediate binding of a NLS-containing import substrate to a discrete set of nucleoporins that include Nup98p, Nup153p and Nup214p [21,133]. The docking subunit of the NLS-receptor complex, karyopherin β, was actually purified by its ability to directly interact with the GLFG repeat domain of nucleoporin Nup98p [21].

Furthermore, immobilized Nup98p can deplete the docking activity of fraction A [133]. Rat Nup98p is localized at the nucleoplasmic side of NPCs and shares sequence homologies with the family of the RNA-binding yeast nucleoporins Nup100p, Nup116p and Nup145p [133]. These data together suggest that nucleoporins that are exposed at either the nucleoplasmic or the cytoplasmic side of the NPC may function as multiple docking sites for the NLS-receptor-karyophile complex and provide trails for bidirectional transport across the NPC [21,133]. Confirmatory evidence for this hypothesis comes also from the yeast field. Srp1p, the yeast homologue of karyopherin α or importin 60 and a potential NLS-receptor, not only genetically interacts with nucleoporin Nup1p but also forms distinct physical complexes with nucleoporins Nup1p and Nup2p, probably through their XFXFG repetitive domains [134]. Taking into account the localization of Srp1p at the nuclear envelope [30], one can assume that the NLS-receptor (Srp1p) in yeast is more stably docked to the NPC via its association with peripherally exposed nucleoporins.

Once a karyophile has been recognized by the NLS-receptor and bound at the NPC, it has to be translocated into the nuclear interior possibly through a series of docking and undocking cycles [21,133]. What triggers or fuels these cycles? The best candidates for this are Ran and Ran-associated proteins and recent data suggest that nucleoporins are also responsible for the recruitment of Ran at the nuclear pores. Nup2p as well as other candidate nucleoporins contain Ran-binding motifs first characterized in RanBP1 [135,136]. The interaction of the Ran-binding domain of Nup2p with the yeast homologue of Ran has also been verified in vivo by the use of the two-hybrid system [136]. Furthermore, RanBPX appears to be a novel Ran-GTP binding nucleoporin [45]. Nup1p genetically interacts with the yeast RanGAP1, Rna1p [124] and p62 may directly associate with NTF2, the human homologue of Ranip [42]. It therefore appears that several nucleoporins serve as assembly sites for the catalytic machinery that couples GTP hydrolysis to the physical movement of nuclear import substrates along the NPC channel.

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