The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile

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Abstract We recently showed that a karyophilic protein forms a stable complex, termed nuclear pore-targeting complex (PTAC), with cytoplasmic components prior to nuclear pore-binding. In this study, we cloned a cDNA encoding a 97 kDa of PTAC (PTAC97). Recombinant PTAC97 completely reconstitutes the nuclear binding-step in conjunction with a 58 kDa component of PTAC (PTAC58) in the semi-intact cell-free transport assay. Biochemical analysis reveals that PTAC58 binds to a karyophilic protein, and PTAC97 is associated with PTAC58 in a 1:1 molar ratio. A complex of PTAC97 and PTAC58 targets nuclear pores, depending on the presence of a karyophile. These in vitro results suggest that the first step in nuclear import occurs through the targeting-complex formation of a karyophile with PTAC58 bound to PTAC97.

Key words: Nuclear import; Nuclear location signal; Nuclear pore-targeting complex

1. Introduction

The selective nuclear import of karyophilic proteins is directed by short amino acid sequences termed nuclear location signals (NLSs) [1]. The process of mediated import can be essentially divided into two steps, an ATP-independent binding to the cytoplasmic face of nuclear pores, and the subsequent ATP-dependent translocation through the nuclear pore complex (NPC) [2–4]. Using a semi-intact cell-free transport assay, we recently demonstrated that an SV40 T-antigen NLS containing karyophile forms a stable complex with cytoplasmic components for nuclear pore-targeting [5]. The complex, termed nuclear pore-targeting complex (PTAC), contained two essential proteins having masses of 54 and 90 kDa, respectively, as estimated by electrophoresis. The 54 kDa component, termed PTAC58 (designated based on the calculated molecular mass of 58 kDa deduced from cDNA sequence encoding this protein), was found to be the mouse homologue of a 60 kDa subunit of Xenopus importin [6,7]. In this study, we have cloned, sequenced, and expressed a cDNA encoding the 90 kDa component of PTAC (designated PTAC97). Using the recombinant proteins, we provide in vitro evidence that the first-step in nuclear import occurs through the targeting complex formation of a karyophile with PTAC58 bound to PTAC97.

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2. Materials and methods

2.1. cDNA cloning

Lysyl endopeptidase-digested peptides of the 90 kDa protein excised from an SDS-PAGE gel were used to obtain internal amino acid sequences [8]. Two of the four sequences determined by peptide sequencing were used to obtain partial cDNA fragments of the 90 kDa protein using polymerase chain reaction (PCR) procedures. Four degenerated oligonucleotides were synthesized corresponding to the sense and antisense sequences of the two peptides, NPDWRYRD and TLATWATK. The template for PCR was cDNA synthesized from mRNA prepared from mouse Ehrlich ascites tumor cells using Ready-To-Go cDNA synthesis kit (Pharmacia Biotech). A 1.5 kbp fragment was amplified in one of the two combination of the sense and antisense primers. Sequencing of both ends of the fragment revealed the existence of the amino acid sequences determined by peptide sequencing franking the primers, indicating that the fragment was a partial-length cDNA of the 90 kDa protein. This fragment was used to screen a mouse cDNA library in λgt10 (approx. 1.5×10^6 pfu). 50 clones were isolated and analyzed by PCR and restriction mapping. Two overlapping clones #4 and #33 covered a full-length 90 kDa protein cDNA. 1.8 kbp and 2.1 kbp *Eco*RI fragments composing the full-length were cut out from DNA of clones #4 and #33, and subcloned into pUC119 and pGEMZf (−), respectively. The resultant plasmids were designated p4L2 and pN33, respectively. The inserts were sequenced by the dideoxy chain termination method using an automated cDNA sequencer (LI-COR Model 4000L) and Sequitherm (Epicentre Technologies).

The DNA and deduced amino acid sequences were compared to the GSDB, DDBJ, EMBL and NCBI database using the BLAST programs. The cloned cDNA showed 62% amino acid similarity and 38% identity with yeast L8300 15 (ACC U19028), 98% with a 90 kDa subunit of human importin (ACC L38951), and 99% with rat karyopherin beta (ACC L38644).

2.2. Recombinant expression and purification

An epitope-tagged PTAC97 was constructed in a glutathione-S-transferase (GST) gene fusion vector pGEX-2T. A tag encoding an influenza virus hemagglutinin (HA) epitope with *Bam*HI and *Sac*I sites at the ends was generated by annealing of two synthetic oligonucleotides with the sequences 5′-GATCCTACCCATACGACGTCCCA-GATTACGCTAGCGGTATGGAGCT-3′ and 3′-GATGGGTATG-CTGCAGGGTCTAATGCGATCGCCATACC-5′. This tag, the *Sac*I–*Eco*RI fragment corresponding to residues 2–642 from pN33 and *Eco*RI–*Kpn*I fragment containing a partial-length cDNA corresponding to residues 643–876 from p4L2, were subcloned into pBluescript SK(−) and the resulting plasmid was sequenced to confirm the fidelity of the oligonucleotide insert. A *Bam*HI–*Kpn*I fragment assembled from the three fragments was excised and inserted into the *Bam*HI and *Kpn*I sites of a modified pGEX-2T in which *Kpn*I site was introduced at the *Eco*RI site by linker ligation. The construct was called pGEX HA-PTAC97 and was used for the expression of the GST fusion protein in *E. coli*. The plasmid expressing a full-length PTAC97 fused to GST without HA tag was constructed from pGEX HA-PTAC97 by cutting out the *Bam*HI–*Sac*I fragment corresponding to the HA epitope followed by blunting and ligation. The resultant plasmid pGEX PTAC97 was sequenced to confirm in-frame ligation of the fused region. The expression of the recombinant PTAC97 fusion proteins was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14 h at 20°C.

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E. coli cells expressing the fusion proteins were disrupted using a french press, and the fusion proteins were purified from the lysates using glutathione-Sepharose (Pharmacia) affinity chromatography. Recombinant PTAC97, obtained after cleavage of the GST portion with thrombin, co-migrated with the authentic 90 kDa component of the nuclear pore-targeting complex. The activity of recombinant PTAC97 was not affected by the presence of the GST portion, nor modification by epitope tagging when examined in the digitonin-permeabilized cell free transport assay.

Recombinant PTAC58 was expressed in *E. coli* strain BL21 as GST-fusion protein by induction of 1mM IPTG for 14 h at 20°C. The fusion protein was purified from the *E. coli* lysate by glutathione affinity chromatography.

2.3. Cell-free import assay

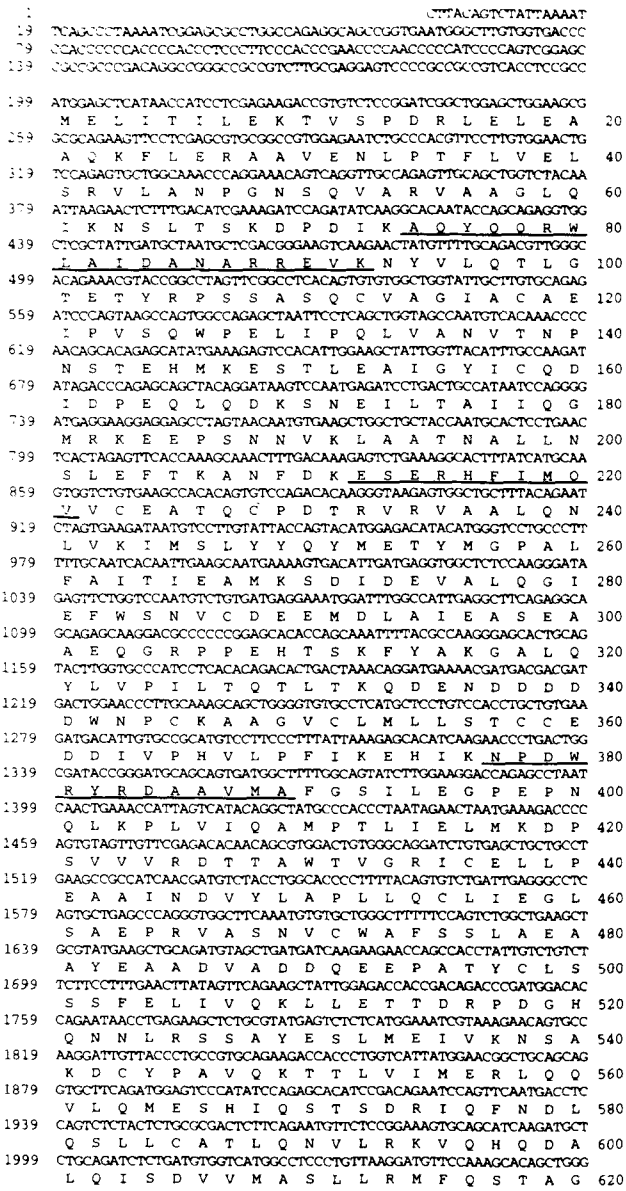
Digitonin permeabilized Ptk2 cells were prepared as described previously [5]. Ten μl testing solutions constitutively contained 6 pmol of T-bBSA (unless indicated), 60 pmol of mutantT-BSA, 10% BSA and transport buffer (20 mM HEPES pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM ethylene glycol bis (β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM DTT and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Transport assays were performed in the presence of either or both recombinant PTAC58 and PTAC97 proteins at amounts indicated in the respective figure legends, with or without competitor proteins. After incubation, cells were fixed, and stained with fluorescein isothiocyanate (FITC)-labeled Avidin (Pierce) to visualize the localization of T-bBSA as described previously [5].

2.4. Transport substrates

Bovine serum albumin (BSA) (Sigma), biotinylated BSA [5], or allophycocyanin (APC)(Calbiochem) was chemically conjugated to synthetic peptides containing wild-type (T-peptide: CYGGPKKKRKVEDP), point-mutated (mutant T-peptide: CYGGPKTKRKVEDP), or reverse (reverse T-peptide: CYGGPDEVKRKKKP) sequence of SV40 T-antigen NLS as described previously [9]. All conjugates contained 10–15 peptides per carrier molecule (analyzed by SDS-PAGE).

2.5. Antibodies

Anti-HA monoclonal antibody 12CA5 [10] was purchased from



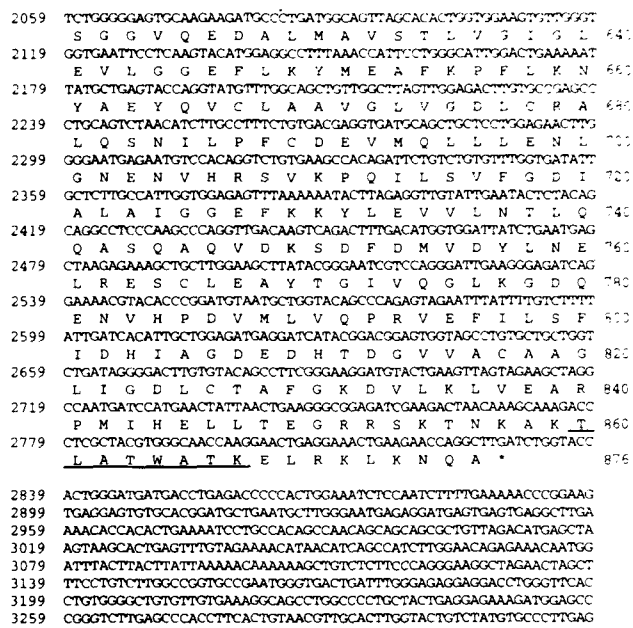


Fig. 1. Nucleotide and deduced amino acid sequence of a 90 kDa component of PTAC (DDBJ accession number D45836). A cDNA library was screened with partial cDNA fragments obtained from PCR using degenerate oligonucleotides corresponding to determined amino acid sequences. Sequences obtained by peptide sequencing of the 90 kDa component are underlined.

Boehringer Mannheim. Anti-PTAC58 antibodies were affinity purified on recombinant PTAC58-Sepharose column from antisera of rabbits immunized with GST-PTAC58 fusion protein.

3. Results and discussion

In order to characterize the 90 kDa component of the nuclear PTAC, we first cloned the cDNA encoding this protein. Fig. 1 shows the DNA sequence and its predicted amino acid sequence (DNA Data Bank of Japan (DDBJ), accession number D45836). The open reading frame encodes a polypeptide of 876 amino acids with a calculated molecular mass of 97.2 kDa. The recombinant 90 kDa component was then expressed in *E. coli*, and its activity examined using a digitonin permeabilized cell-free transport assay [11]. Biotinylated bovine serum albumin, chemically coupled to a synthetic peptide containing the SV40 T-antigen NLS (T-bBSA) was used as a transport substrate. As shown in Fig. 2, neither PTAC58 alone nor the recombinant 90 kDa protein alone mediated efficient nuclear rim-binding of T-bBSA. However, in the presence of both proteins, T-bBSA accumulated efficiently at the nuclear rim. The rim-binding of T-bBSA was competitively inhibited in the presence of excess non-biotinylated T-BSA while the same amount of transport incompetent reverse sequence of SV40 T-antigen NLS [12] conjugated BSA had no effect. The binding was not affected by incubation temperature, or, by either the absence or presence of ATP. Under the same assay conditions, the nuclear entry of the substrate (homogeneous nuclear staining) was not observed even in the presence of ATP (data not shown). These results confirm that the recombinant 90 kDa protein, in conjunction with PTAC58, reconstitutes the NLS-dependent, and ATP-independent initial binding step of the transport. The maximal rim-binding of T-bBSA was achieved when the two components were present in equimolar concentration. Based on the activity of the recombinant protein and the molecular mass calculated from the predicted amino acid sequence, we designate this protein PTAC97 (97 kDa component of PTAC)

Using recombinant proteins, interactions involving T-bBSA, PTAC58, and PTAC97 were examined. For this, mixtures of T-bBSA/PTAC58, T-bBSA/PTAC97, PTAC58/PTAC97, or T-bBSA/PTAC58/PTAC97 were incubated, and complex formation was examined by gel filtration chromatography. As shown in Fig. 3A, T-bBSA associates directly with PTAC58, but not with PTAC97. The association of T-bBSA with PTAC58 was found to be competitively inhibited by the presence of excess non-labeled T-BSA, while transport incompetent reverse or point-mutated SV40 T-antigen NLS conjugates had no effect [7]. PTAC97 Associates directly with PTAC58 in a 1:1 molar ratio, even in the absence of T-bBSA (Fig. 3A and B). This was determined by adding increasing amounts of PTAC97 to constant amounts of PTAC58 and T-bBSA. As shown in Fig. 3B, the amount of PTAC97 incorporated in the targeting complex increased up to that equivalent to PTAC58, but did not lead to additional increases even in the presence of excess PTAC97. These data are consistent with a scenario wherein the targeting complex is formed through interactions of NLS with PTAC58 which, in turn, is bound to equimolar PTAC97. Based on our recent observation that PTAC58 is located mainly in the cytoplasm of mammalian cells [7], PTAC58 and PTAC97, in all probability exist as a complex in the cytoplasm. The PTAC58/PTAC97 complex would be predicted to act as a cargo that carries NLS-containing proteins from cytoplasm to nuclear pores.

In order to determine whether the complex of PTAC58/PTAC97 binds to nuclear pores alone, or it targets nuclear pores only after association with karyophiles, the nuclear binding activity of the PTAC58/PTAC97 complex was examined. In these experiments equimolar mixtures of recombinant PTAC58 and influenza virus hemagglutinin (HA) epitope-tagged PTAC97 proteins were incubated with semi-intact cells, either in the presence or absence of T-peptide conjugate. As shown in Fig. 3A, PTAC58 and PTAC97 form a complex even in the absence of the karyophile under these conditions. The PTAC58/PTAC97 complex did not accumulate at the nuclear rim in the absence of the karyophile (Fig. 4a), but did so efficiently in the presence of the karyophile (Fig. 4b,c). Punctated nuclear rim staining was observed (Fig. 4c), which reflected the staining of nuclear pores as previously reported [13,14]. The data suggest that complex formation of the karyophile with PTAC58 bound to PTAC97 is required for nuclear pore-binding activity of PTAC.

For the nuclear pore-binding of T-bBSA, both PTAC58 and PTAC97 were required. PTAC97 Associates directly with NLS-binding protein, PTAC58, but not with NLS-containing karyophile. To date, bovine NEM-sensitive 54/56 kDa NLS-binding protein [15], bovine p97 [16], importin which consists of a 60

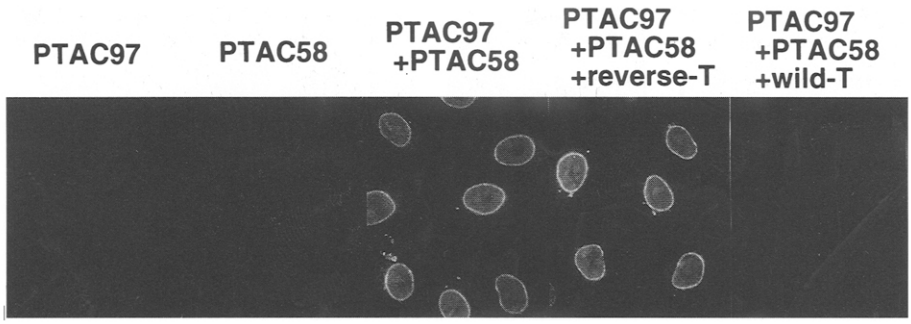


Fig. 2. Effect of recombinant 90 kDa protein (PTAC97) on the nuclear-binding step of transport in digitonin-permeabilized cell-free transport system. Ptk2 Cells permeabilized with digitonin were incubated with 10 μl of test solution for 30 min on ice in the absence of an energy source. Incubation was performed in 10 μl of test solution, which contained 6 pmol of T-bBSA, with or without recombinant PTAC58 or PTAC97 in the presence or absence of competitor proteins. 'PTAC97' indicates the presence of 12 pmol of recombinant PTAC97 protein and 'PTAC58' indicates the presence of 12 pmol of recombinant PTAC58 protein, 'reverse-T' and 'wild-T' indicate the presence of 60 pmol of competitor non-biotinylated BSA conjugates of reverse T-peptide and wild type T-peptide respectively. Localization of T-bBSA was visualized with FITC-Avidin.

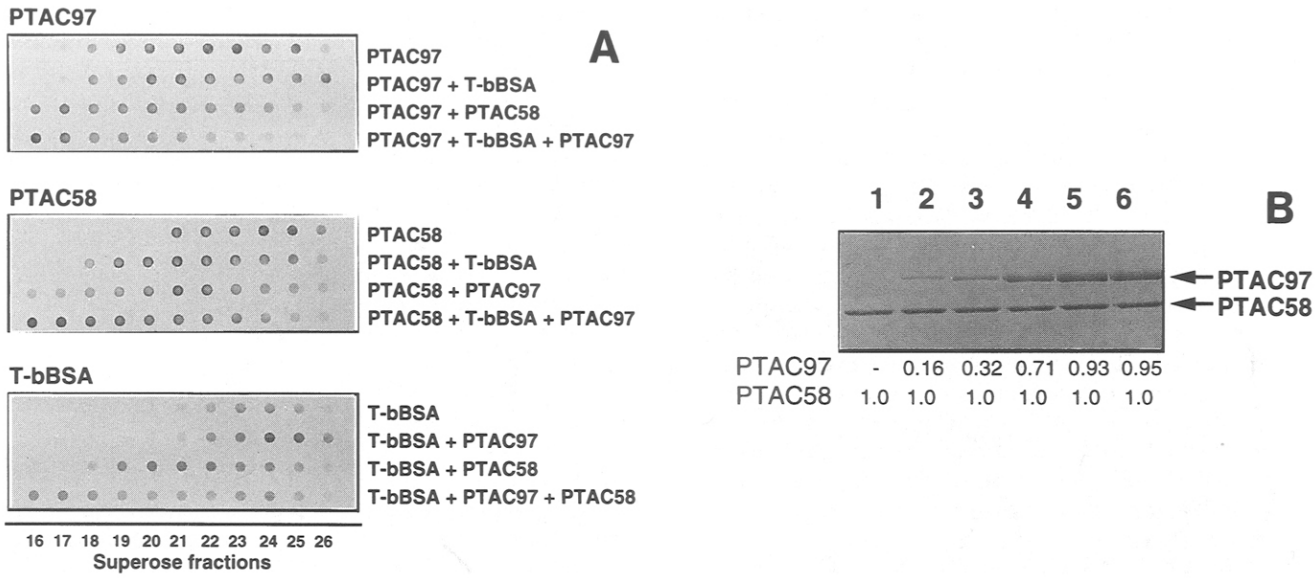


Fig. 3. PTAC97 associates directly with PTAC58 possessing NLS- binding activity. (A) T-bBSA incubated with equimolar PTAC58, HA- epitope tagged PTAC97, or both PTAC58 and HA-PTAC97, or HA- PTAC97 incubated with equimolar PTAC58 on ice for 1 h was subjected to gel filtration chromatography using a Superose 12 column equilibrated with transport buffer. Each Superose fraction was blotted onto nitrocellulose membrane and probed with anti-BSA antibodies, anti-PTAC58 antibodies, and anti-HA monoclonal antibody 12CA5, to detect T-bBSA, PTAC58, and PTAC97 respectively. The antibodies were detected with alkaline phosphatase-conjugated goat antibodies to rabbit IgG or mouse IgG (Bio-Rad) by the standard method. (B) T- bBSA trapped in Immobilized Avidin was incubated with 0 pmol (lane 1), 30 pmol (lane 2), 60 pmol (lane 3), 120 pmol (lane 4), 240 pmol (lane 5), and 480 pmol (lane 6) recombinant PTAC97 in the presence of 120 pmol recombinant PTAC58. After incubation (1 h at 4°C), proteins associated with T-bBSA were analyzed by SDS-PAGE. To know the amounts of bound proteins, the intensity of protein bands stained with Coomassie brilliant blue was quantified by densitometric scanning (Shimadzu, Model CS-930). The molar ratio of PTAC97 bound to PTAC58 indicated below each lane was estimated by dividing the intensity of protein bands with molecular mass of each protein. The results show that PTAC97 associates with PTAC58 in a 1:1 molar ratio.

and a 90 kDa subunit [6,17], and karyopherin alpha and beta subunits [18] have been reported to mediate the pore-binding step of nuclear import. One of the PTAC components, PTAC58, was found to be the mouse homologue of the 60 kDa subunit of importin [7]. During the preparation of this manuscript, PTAC97, which reconstitutes the binding-step of import in conjunction with PTAC58, was found to be the mouse homologue of karyopherin beta subunit [18] and the 90 kDa subunit of importin [17]. Since PTAC58 associates directly with T-bBSA, it is likely to be the bovine 54/56 kDa NLS-binding protein. The complex formation of the karyophile with PTAC58/PTAC97 complex reconstitutes the binding-step, indicating that PTAC97 appears to correspond to the bovine p97. In addition to these molecules, the 70 kDa heat-shock cognate protein [19–22], Ran/TC4 [23–25], and p10 [26] have been functionally demonstrated to be involved in nuclear import, which

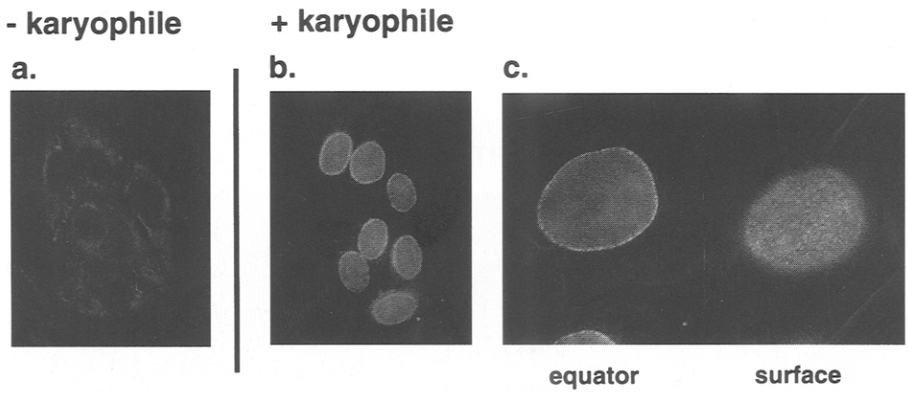


Fig. 4. Nuclear binding of PTAC58/PTAC97 complex is induced in the presence of NLS-containing karyophile. Digitonin-permeabilized Ptk2 cells were incubated (on ice, 30 min) with 12 pmol PTAC58 and HA epitope-tagged PTAC97 in the presence (panels b and c) or absence (panel a) of 6 pmol T-APC. Cells fixed with 3.7% formaldehyde were permeabilized with 0.5% Triton X-100, incubated first with anti-HA epitope monoclonal antibody 12CA5 and then with FITC-conjugated goat antibodies to mouse IgG (TAGO) to visualize the localization of PTAC97. Panel c shows magnified images of the cell. Focusing on an equatorial plane through the nucleus reveals a discontinuous peripheral nuclear stain. This pattern appears to be more punctate when focusing on the upper surface of the nucleus.

should act in concert with PTAC components in the mediated nuclear import process upon formation, pore-targeting, translocation, and/or dissociation of PTAC.

A complex of T-bBSA and PTAC58 did not accumulate efficiently at the nuclear rim in the absence of PTAC97, indicating that PTAC97 contributes to the pore-binding process of PTAC. However, it is not yet clear whether PTAC97 possesses an NPC binding site. The mechanism for how a component(s) of NPC recognizes PTAC remains to be examined. One possibility is that a component(s) of NPC recognizes a higher order (stereospecific) structure of PTAC. Another is that association of NLS induces the conformational change of PTAC58/PTAC97 complex which exposes an NPC binding site, present in primary structure of PTAC97 and/or PTAC58. Further studies of the nature of the PTAC58/PTAC97 complex in the cytoplasm of living cells, as well as how the association of a karyophile with PTAC58/PTAC97 triggers the nuclear pore-binding of PTAC in vivo are now in progress.

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