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Purification of a Ran-interacting protein that is required for protein import into the nucleus

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ABSTRACT **Previously we reported the isolation of two cytosolic fractions (A and B) from *Xenopus* ovary that are required sequentially to support protein import into the nuclei of digitonin-permeabilized cells. Fraction A is required for recognition of the nuclear localization sequence and targeting to the nuclear envelope, whereas fraction B is required for the subsequent translocation of the bound substrate into the nucleus. The first protein required for fraction B activity to be purified was the small GTPase Ran (*ras*-related nuclear protein). Here we report the purification of the second (and final) protein required for fraction B activity. By SDS/PAGE, the purified protein appeared as a single band with an apparent molecular mass of 10 kDa, but the native protein fractionated upon gel filtration chromatography with an apparent size of 30 kDa. Peptide sequence analysis indicated that the purified protein was highly homologous to a previously identified human protein of unknown function called placental protein 15 (pp15) and to the predicted protein product of a yeast open reading frame from *Saccharomyces cerevisiae*.**

Protein import into the nucleus occurs in two stages (1, 2): docking of a nuclear localization sequence (NLS)-bearing substrate at the nuclear pore complex (NPC) followed by translocation of the docked substrate through the NPC into the nuclear interior (reviewed in ref. 3). Two fractions (A and B) can be isolated by biochemical fractionation of *Xenopus* ovarian cytosol that catalyze in turn each of these two stages of import in digitonin-permeabilized cells (4). The addition of fraction A with a fluorescent NLS-containing substrate results in the accumulation of the import substrate in a bright rim around the nucleus and also a small (but visible) amount of import. When fraction B (which has no discernible effect when added alone) is added together with fraction A, these two fractions can support import at levels comparable to unfractionated cytosol. Substrate prebound to the nuclear envelope with fraction A can be chased into the nuclear interior by the subsequent addition of fraction B, indicating that the active B component is required at a stage after docking but prior to entry into the nucleus.

The first component required for fraction B activity to be purified was Ran, a member of the Ras superfamily of small GTP-binding proteins (5). Experiments utilizing guanine nucleotide analogs indicated that Ran had to be in the GTP-bound form in order to catalyze nuclear import and that GTP hydrolysis was also required for import (though not necessarily by Ran). Melchior *et al.* (6) also reported a requirement for both GTP hydrolysis and Ran in nuclear import *in vitro*. Our results further indicated that purified Ran was by itself, however, insufficient to reconstitute full fraction B activity, indicating that a second necessary factor had been lost during Ran purification. This second factor has been designated (on the basis of activity) as the B-2 component, Ran being B-1 (3).

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Here we report the purification and characterization of the protein that supplies the B-2 activity.

MATERIALS AND METHODS

Nuclear Import Assay. The nuclear import assay was performed in digitonin-permeabilized BRL (buffalo rat liver) cells as originally described (7), with our modifications (4, 5). The import substrate was rhodamine-labeled human serum albumin coupled to peptide containing the NLS of the simian virus 40 large tumor (T) antigen (4). Standard reaction mixtures contained the import substrate (10 μg/ml), 1 mM ATP, 5 mM phosphocreatine, and creatine kinase (20 units/ml) in assay buffer [20 mM Hepes·KOH, pH 7.3/110 mM KOAc/2 mM $Mg(OAc)_2$ acetate/1 mM EGTA/2 mM dithiothreitol] and were incubated for 15 min at room temperature. Unfractionated cytosol and fraction A (ref. 4) and fraction B (ref. 5) were prepared as described. Recombinant human Ran was a generous gift of Elias Coutavas (8). Assay mixtures for B-2 activity contained recombinant Ran (100 μg/ml) and fraction A (2 mg/ml). Units of B-2 activity (see Table 1) were as described for B activity except that the import obtained just with fraction A and Ran was subtracted as background (4).

Purification of p10. All procedures were performed at 4°C unless otherwise stated. The starting material for this purification was the ovarian tissue (≈300 ml of settled ovary) obtained from 18 adult *Xenopus laevis* females (no. LM535M; Nasco, Fort Atkinson, WI). Homogenization and isolation of the 146,000 × *g* supernatant in buffer A (20 mM Hepes·KOH, pH 7.3/2 mM dithiothreitol) containing 2 mM $Mg(OAc)_2$ were performed as described (4). The high-speed supernatant (280 ml) was brought to 150 mM KOAc by dropwise addition (with stirring) of buffer A/1 M KOAc/2 mM $Mg(OAc)_2$ and loaded onto a 100-ml DE-52 column (Whatman; 4.5 cm × 6.3 cm) equilibrated in buffer A/150 mM KOAc/2 mM $Mg(OAc)_2$ at a flow rate of 300 ml/hr. The column was washed with 500 ml of the same buffer. The first 60 ml of the flowthrough was discarded and the next 570 ml of the flowthrough and wash fractions were pooled. These pooled fractions were brought to 10 mM EDTA by the dropwise addition (with stirring) of 500 mM EDTA·NaOH (pH 7.3) and incubated for 30 min at room temperature and then overnight at 4°C. Solid ammonium sulfate was added to a final concentration of 25% saturation (13.4 g/100 ml) and the mixture was centrifuged for 30 min at 10,000 rpm in a Sorvall GSA rotor. The pellet was discarded and the supernatant was loaded onto a 75-ml octyl-Sepharose column (Pharmacia; 4.5 cm × 4.7 cm) equilibrated in buffer A/1.2 M ammonium sulfate/1 mM EDTA at a flow rate of 300 ml/hr. The column was washed with 400 ml of column buffer and bound proteins were eluted with a gradient consisting of equal weights of column buffer (236 ml)

Abbreviations: NLS, nuclear localization sequence; NPC, nuclear pore complex.

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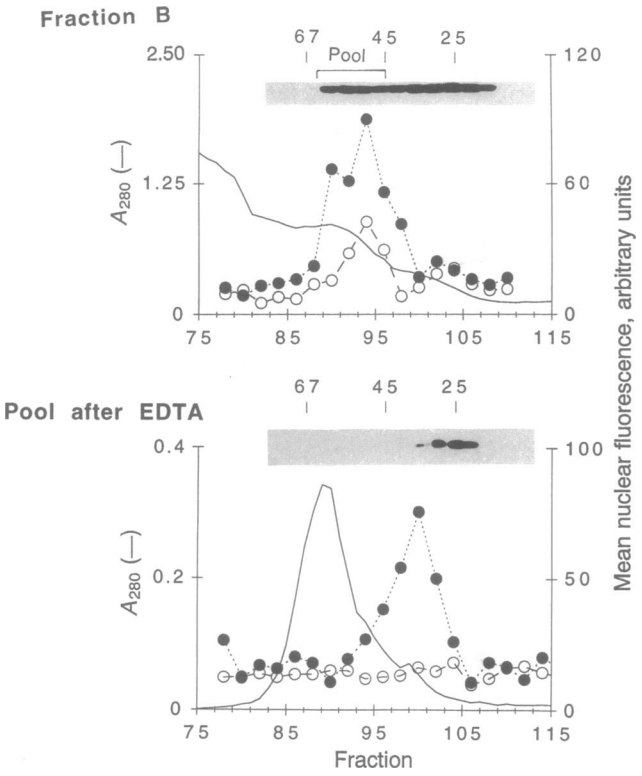

FIG. 1. Gel filtration analysis of fraction B. One and one-half milliliters of crude fraction B (protein at 30 mg/ml in assay buffer, prepared as in ref. 5) was loaded on a 150-ml Superdex-75 gel filtration column (Pharmacia; 1.5 cm × 85 cm) equilibrated in 20 mM Hepes·KOH, pH 7.3/130 mM KOAc/2 mM $Mg(OAc)_2$/2 mM dithiothreitol at a flow rate of 20 ml/hr (*Upper*). Fractions (900 μl) were collected and aliquots were assayed for import activity with fraction A alone (○) or with fraction A plus Ran (●). Other aliquots were used for SDS/PAGE followed by immunoblot analysis with an anti-Ran peptide antibody (5) (*Inset*). Fractions 90–96 in *Upper* were pooled, brought to 10 mM EDTA, and incubated for 30 min at room temperature. After concentration (Centricon-10), the pool was rerun on the same Superdex-75 column equilibrated in 20 mM Hepes·KOH, pH 7.3/130 mM KOAc/1 mM EDTA/2 mM dithiothreitol (*Lower*). Each fraction was brought to 5 mM $Mg(OAc)_2$ before assay for import activity as in *Upper*. The elution positions of the calibration standards BSA (67 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa) are indicated.

and buffer A/1 mM EDTA/20% (vol/vol) ethylene glycol (250 ml) and then with 200 ml of the ethylene glycol-containing buffer. Fractions (10 ml) were collected and aliquots were dialyzed overnight against assay buffer prior to being assayed for B-2 activity. The active fractions (eluted in the final 100 ml of the gradient plus the first 100 ml of the postgradient wash) were pooled and dialyzed against multiple changes of buffer A/20 mM KOAc. The dialyzed pool was added to 20 ml of packed phosphocellulose (Whatman; P-11 equilibrated in dialysis buffer), the resin was suspended by gentle vortexing, and the slurry was rocked back and forth for 15 min. The slurry was poured into a column, drained, and washed with 60 ml of column buffer. The flowthrough and wash fractions were pooled and loaded onto a 20-ml Q-Sepharose column (Pharmacia; 2.5 cm × 4 cm) equilibrated in dialysis buffer at a flow rate of 100 ml/hr. The column was washed with 40 ml of dialysis buffer and bound proteins were eluted with a 100-ml gradient of 20–600 mM KOAc in buffer A. Fractions (2 ml) were collected and 3 μl of each was assayed for import activity. The active fractions (eluted approximately midway through the gradient) were pooled, brought to 25% saturation with solid ammonium sulfate, and loaded onto a 1 ml octyl-Sepharose column equilibrated in buffer A/1.2 M ammonium sulfate. The resin was packed in a Dispo-column (Bio-Rad) and allowed to run under gravity flow. The column was washed with 4 ml of column buffer and bound proteins were eluted with sequential 0.5-ml aliquots of buffer A; each eluate was collected as a separate fraction. The protein-containing eluate fractions were pooled (6 ml) and concentrated to 200 μl in a Centricon-10 (Amicon). One hundred microliters was injected (in two successive runs) on a Superdex 75 HR10/30 FPLC gel filtration column (Pharmacia) equilibrated in buffer A/130 mM KOAc at a flow rate of 0.5 ml/min. Fractions (200 μl) were collected and 1 μl of each was assayed for import activity. The active fractions (32–37 in Fig. 3) were pooled, frozen in liquid nitrogen, and stored at −70°C.

RESULTS

Separation of B-1 and B-2 Activities. Gel filtration analysis of fraction B showed most of the B activity in the 50- to 60-kDa range, with a minor activity peak at 20–30 kDa (Fig. 1 *Upper*; see also ref. 5). Immunoblotting with an anti-Ran antibody showed Ran to be present in both peaks (Fig. 1 *Upper*). These and previous data (5) suggested that the peak of B activity at 50–60 kDa contained both Ran (referred to as B-1) and a Ran-associated protein (referred to as B-2).

To determine whether this cofractionation of Ran and the B-2 activity could be disrupted, the fractions corresponding to the peak of B activity at higher molecular mass were pooled, treated with an excess of EDTA to chelate the Mg^{2+} present in the column buffer, and rerun on the same column in the absence of Mg^{2+}. This treatment should result in the release of any bound guanine nucleotide from Ran (9). As a result of this treatment, the Ran was eluted exclusively as a 25-kDa species as determined by immunoblotting (Fig. 1 *Lower*). The peak of B-2 activity was also found to have shifted to a lower molecular mass, migrating slightly ahead of the Ran peak at around 30 kDa. Even though the Ran and B-2 fractions overlapped, these fractions exhibited no activity when assayed just with fraction A, indicating that the Ran was no longer active (Fig. 1 *Lower*). Later studies confirmed that this EDTA treatment irreversibly inactivated Ran's ability to support nuclear import (data not shown). These

Table 1. Purification of p10

Step	Protein, mg	Activity, units	Specific activity, units/mg	Purification, fold	Yield, %
1. 146,000 × *g* supernatant	2213	118,000	53	—	100
2. DE-52	1408	461,660	329	6	391
3. EDTA/25% $(NH_4)_2SO_4$	1392	377,660	271	5	319
4. Octyl-Sepharose	40	171,600	4,290	81	145
5. Phosphocellulose	30	153,900	5,130	97	130
6. Q-Sepharose	2.7	84,270	31,211	589	71
7. Octyl-Sepharose	0.6	38,940	64,900	1225	33
8. Superdex-75	0.063	13,563	215,300	4062	11

Units of activity are as defined in *Materials and Methods*.

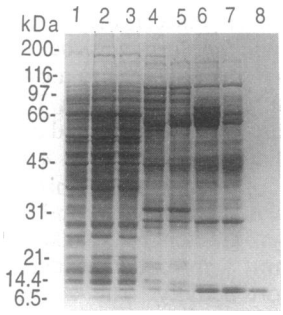

FIG. 2. SDS/PAGE analysis of the pooled active fractions from each step of the purification. The lane numbers correspond to the purification steps shown in Table 1. Protein (30 μg, lanes 1–5; 15 μg, lane 6; 10 μg, lane 7; and 1 μg, lane 8) was precipitated with trichloroacetic acid and loaded on a 7.5–15% polyacrylamide gel. After electrophoresis, proteins were stained with Coomassie blue.

results strongly suggested that Ran and the B-2 component not only were required for full fraction B activity but, in fact, formed an active (with regard to nuclear import) complex.

Purification of the B-2 Component. The active B-2 component was purified on the basis of its ability to stimulate nuclear import in the presence of fraction A and Ran (Table 1 and Fig. 2). The B-2 activity cofractionated with a single polypeptide that migrated on SDS/PAGE at 10 kDa; however, the native protein was eluted at 30 kDa upon gel filtration, possibly indicating an oligomeric structure (Fig. 3). The specific activity of the purified p10 was >4000 times that of the starting cytosol, but p10 is probably more abundant than this value suggests (Table 1). After the first DE-52 column (Table 1, step 2), an ≈4-fold increase in the total B-2

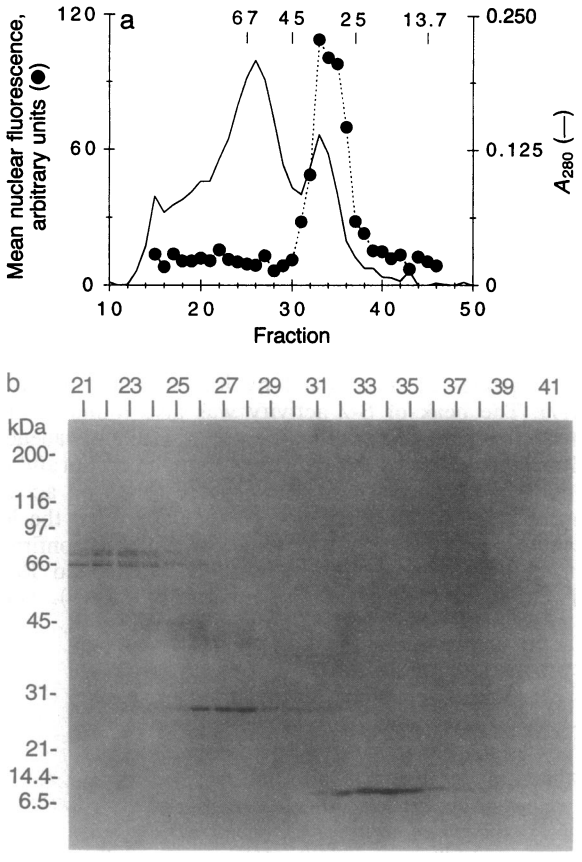

FIG. 3. Final purification of p10 on Superdex-75. (*a*) Elution position of B-2 activity. Calibration standards are as in Fig. 1, plus RNase A (13.7 kDa). (*b*) Coomassie blue-stained SDS/7.5–15% polyacrylamide gel of the same fractions.

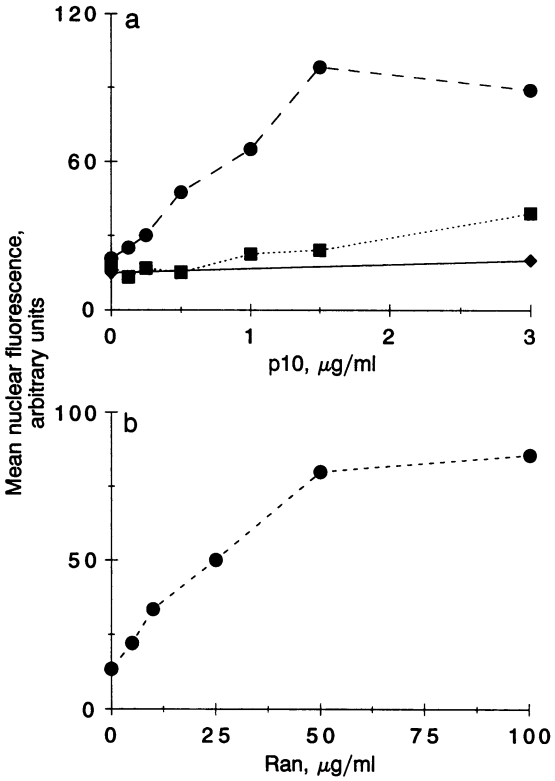

FIG. 4. Titration of the activity of purified p10. (*a*) Various concentrations of p10 were added in the presence (■, 25 μg/ml; ●, 100 μg/ml) or absence (◆) of recombinant human Ran. (*b*) Various concentrations of recombinant human Ran were added in the presence of p10 (3 μg/ml). All samples in *a* and *b* contained fraction A (2 mg/ml).

activity was observed, indicating the probable loss of an inhibitor(s), whose nature is unknown.

Analysis of p10 Activity. When Ran was not limiting in the assay (100 μg/ml), maximal B-2 activity was observed with p10 at 1.5 μg/ml, which for a 10-kDa protein would correspond to 150 nM (Fig. 4*a*). This concentration was substantially lower than the amount of recombinant Ran (50 μg/ml, 2 μM) required for maximal import (Fig. 4*b*). At present, we cannot rule out that a fraction of the recombinant Ran was inactive, which might explain why more Ran was required than p10. Alternatively, the exogenously added Ran could have been interacting with a variety of components inside the nuclei of the digitonin-permeabilized cells, effectively lowering the amount of Ran available for the import reaction (3). Ran and p10 were able to support nuclear import (with fraction A) to the same extent as fraction B, indicating that these two proteins were sufficient to supply all of the B activity (Fig. 5).

Previously, we showed that substrate prebound to the nuclear envelope with fraction A could be chased into the nucleus by the subsequent addition of fraction B (4). A similar experiment was performed with purified p10 and Ran. Incubation with fraction A and the import substrate, followed by washing to remove any unbound material, resulted in the typical fraction A staining pattern, which consists of a bright rim of substrate around the nucleus and also nucleolar fluorescence indicating a small amount of import (Fig. 6 *Left*). This small amount of import is probably due to incomplete loss of the B components from the permeabilized cells (3, 5). Together with ATP and various guanine nucleotides, Ran and p10 were then added in a second incubation. The addition of GTP resulted in a Ran- and p10-stimulated loss of the rim staining and a concomitant increase in the intranuclear fluorescence, indicating translocation of the

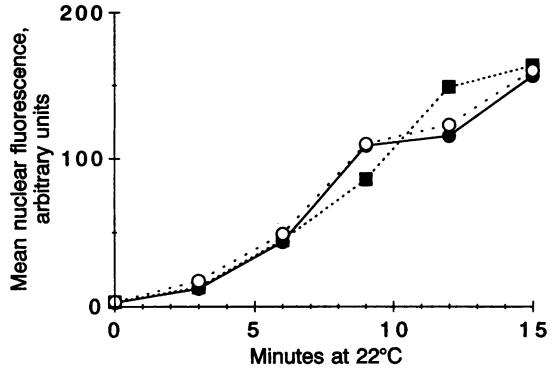

FIG. 5. Time course of import. Nuclear import was measured in the presence of *Xenopus* unfractionated cytosol (10 mg/ml) (●), fraction A (2 mg/ml) plus fraction B (2 mg/ml) (■), or fraction A plus recombinant human Ran (100 μg/ml) and purified p10 (1.5 μg/ml) (○).

substrate to the nuclear interior (Fig. 6). In contrast, the addition of the nonhydrolyzable GTP analog guanosine 5′-[β,γ-imido]triphosphate resulted in the loss of the rim staining with no concomitant increase in nuclear staining, indicating dissociation of the bound substrate rather than import (Fig. 6). When guanosine 5′-[β-thio]diphosphate, a GDP analog, was included, the addition of these two proteins had no effect (Fig. 6). Omission of either Ran or p10 in the second incubation, regardless of the guanine nucleotide present, resulted in a staining pattern identical to samples fixed after the first incubation (data not shown).

Peptide Sequence Analysis of p10. Protein sequence analysis was performed on the purified p10. The amino terminus was found to be blocked, but three segments of internal peptide sequence were obtained after proteolytic cleavage with Lys-C (Fig. 7). This peptide sequence was highly homologous to a human protein of unknown function called placental protein 15 (pp15), which is a homodimer of 14,478-Da subunits (10–12). pp15 in turn exhibits significant homology (46% identity over the region where the sequences overlap) to the predicted protein product of an open reading frame (fragment?) in the data base from *S. cerevisiae* (submitted by B. K. Haarer, A. S. Petzold, and S. S. Brown, University of Michigan, Ann Arbor). Whether these human and yeast proteins represent the true functional homologs of p10 is unknown.

DISCUSSION

We have identified a protein (p10) that is required for import of a NLS-containing substrate into the nuclei of digitonin-permeabilized cells. In the presence of fraction A, p10 and Ran were the only two proteins from the original cytosolic B fraction required to support import at levels comparable to unfractionated cytosol (Fig. 5). When added after the fraction A-mediated docking of an import substrate around the nuclear envelope had already occurred, p10 and Ran were also sufficient to catalyze translocation of the docked substrate into the nuclear interior in a reaction that required GTP hydrolysis (Fig. 6). In the presence of a nonhydrolyzable GTP analog, Ran and p10 were unable to support translocation but were able to stimulate the release of the docked substrate. We have suggested previously (3) that movement of a substrate through the NPC may be driven by a series of association–dissociation reactions; in this model, the A component would stimulate association whereas Ran and p10 would be required for the dissociation reaction. Recently, Adam and Adam (13) reported that a combination of three proteins purified from bovine erythrocyte cytosol (a 97-kDa protein plus two previously identified proteins of 54 and 56 kDa) was sufficient to support nuclear envelope binding of a NLS-containing protein. The relationship between these three proteins and the active A component is not clear (see ref. 13 for discussion).

Partial sequence analysis of p10 revealed significant homology (see Fig. 7) with pp15, a human placental protein of unknown function. As pp15 is a homodimer of 14,478-Da subunits (10–12), and as p10 fractionates as a 30-kDa species upon gel filtration, the actual mass of p10 (as opposed to its SDS/PAGE-estimated size) may be ≈15 kDa rather than 10 kDa. The 50- to 60-kDa peak of B activity observed on gel filtration (Fig. 1) is therefore likely to consist of a complex of one molecule of Ran (25 kDa) and one p10 dimer (30 kDa).

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HUM pp15   1  MGDKPIWEQI GSSFIQHYYQ LFDNDRTQLG AIYIDASCLT
S. c. ORF     MSLDFNTL AQN•T•F••N Q••T••S••• NL•RNE•M••

                                                N
                                                or
XEN p10                            ••L•• •••X••T••V ••
HUM pp15  41  WEGQQFQGKA AIVEKLSSLP FQKIQHSITA QDHQP-TPDSC
S. c. ORF     F•TS•L••AK D•••••V••• •••V••R••T L•A••AS•NGD

XEN p10                    ••D••••••• •V••••
HUM pp15  81  IISMVVGQLK ADEDPIMGFH QMFLLKNIND AWVCTNDMFR
S. c. ORF     VLV•I

HUM pp15  121 LALHNFG
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FIG. 7. Peptide sequence of p10 and alignment with potential homologs. Peptide sequence of purified *Xenopus* p10 was obtained after cleavage with endoproteinase Lys-C. The sequences of human pp15 (accession no. P13662) and the open reading frame from *Saccharomyces cerevisiae* (accession no. P33331) were obtained from the Swiss-Prot protein sequence data base. Asterisks indicate identical amino acids.

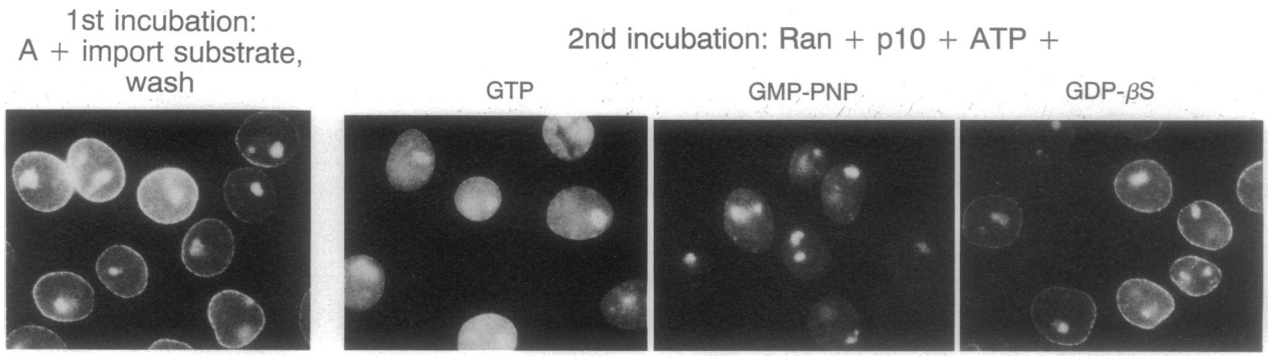

FIG. 6. Ran and p10 stimulate movement of a docked substrate. Permeabilized BRL cells were incubated for 10 min at room temperature with fraction A (2 mg/ml) and the fluorescent import substrate and then washed twice in room-temperature assay buffer. (*Left*) A sample fixed immediately after this treatment. (*Right*) Samples were then incubated for an additional 10 min in assay buffer containing Ran (100 μg/ml), purified p10 (1.5 μg/ml), 1 mM ATP, and 1 mM GTP, guanosine 5′-[β, γ-imido]triphosphate (GMP-PNP), or guanosine 5′-[β-thio]diphosphate (GDP-βS) prior to washing and fixation. All of these micrographs were exposed and printed for the same length of time.

The sequence of pp15 contains no identifiable motifs except for amino acids 29–43, which fit the consensus sequence [(LIVMSAC)-(LIVMFYWSTAGC)-(LIMSTAG)-(LIVMSTAGC)-X_2-(DN)-X_2-(LIVMWSTAC)-X-(LIVMFSTAG)-W-(DEN)-(LIVMFSTAGC)] of the so-called Trp-Asp or G_β repeat motif (reviewed in refs. 14 and 15). This motif was first identified in the β subunit of transducin (where it is repeated eight times) but has since been identified in a variety of proteins, either as a single copy or repeated a variable number of times. To our knowledge, a function for this domain has not been elucidated.

Several Ran-interacting proteins have now been described. The most extensively studied is a 45-kDa chromatin-binding protein called RCC1 (regulator of chromosome condensation) (16). RCC1 acts as a guanine nucleotide exchange factor (GEF) for Ran, and these two proteins can be isolated in a complex (9, 17). A Ran GTPase-activating protein (RanGAP1) has been purified from HeLa cells and is a homodimer of 65-kDa subunits (18). A third Ran-interacting protein, Ran-binding protein 1 (RanBP1), is a 27-kDa protein that was identified on the basis of its ability to bind GTP–Ran (but not GDP–Ran) on Western blots (8, 19). RanBP1 has been reported to enhance RanGAP1 activity and to inhibit the RCC1-stimulated exchange of Ran-bound GTP (18). Whether p10 is also involved in regulating the GTPase cycle of Ran is not known, but one intriguing possibility is that p10 functions in an analogous manner to the RabGDI protein in vesicular transport (reviewed in ref. 21). RabGDI binds a GDP–Rab in the cytosol and targets it to the appropriate membrane, whereupon membrane association is accompanied by the release of the RabGDI and exchange of the Rab-bound GDP for GTP. p10 might likewise serve to target GDP-Ran to a docked A component–import substrate complex, and association with this complex could then stimulate GDP release and GTP binding by Ran. As the only known GEF for Ran (RCC1) is located inside the nucleus, such a mechanism could serve to generate GTP–Ran on the cytoplasmic side of the nuclear envelope for use in the import reaction.

Ran is a very abundant protein (10^7 copies per HeLa cell) and is located primarily inside the nucleus; furthermore, Ran and RCC1 have been implicated in a wide variety of intranuclear events, including cell cycle regulation, DNA replication, maintenance of nuclear structure, the yeast mating response, RNA transcription and processing, and (notably) RNA export (reviewed in refs. 3 and 20). We have suggested that Ran may play a key role not just in nuclear import but in all phases of nucleocytoplasmic transport, which would explain both its abundance and why so much of it is located inside the nucleus (3, 5). The subcellular distribution of p10 is not known, and an interesting question will be whether p10 is involved in protein import only or in other phases of nucleocytoplasmic transport as well.

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