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Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis

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ABSTRACT **Nuclei of digitonin-permeabilized cells that had been preloaded with a model transport substrate in a cytosol-dependent import reaction were subsequently incubated to investigate which conditions would result in export of transport substrate. We found that up to 80% of the imported substrate was exported when recombinant human Ran and GTP were present in the export reaction. Ran-mediated export was inhibited by nonhydrolyzable GTP analogs and also by wheat germ agglutinin but was unaffected by a nonhydrolyzable ATP analog. Moreover, a recombinant human Ran mutant that was deficient in its GTPase activity inhibited export. These data indicate that export of proteins from the nucleus requires Ran and GTP hydrolysis but not ATP hydrolysis. We also found that digitonin-permeabilized cells were depleted of their endogenous nuclear Ran, thus allowing detection of Ran as a limiting factor for export. In contrast, most endogenous karyopherin α was retained in nuclei of digitonin-permeabilized cells. Unexpectedly, exogenously added, fluorescently labeled Ran, although it accessed the nuclear interior, was found to dock at the nuclear rim in a punctate pattern, suggesting the existence of Ran-binding sites at the nuclear pore complex.**

A standard *in vitro* system for protein import into nuclei consists of digitonin-permeabilized mammalian cells, an import substrate, an ATP-regenerating system, and exogenous cytosol (1). The cytosolic factors required for protein import are highly conserved, as a *Xenopus* ovary cytosol can substitute for a mammalian cytosol (2). Subfractionation of the *Xenopus* ovary cytosol yielded two distinct fractions, A and B. Fraction A is required for recognition and docking of the import substrate at the nuclear pore complex (NPC), whereas fraction B is needed for transport into the nucleus (2). The active factors in both fractions have now been purified and molecularly characterized. Fraction A consists of a complex of two subunits, termed karyopherin α and β (3, 4). Both karyopherin subunits have been generated as recombinant proteins and shown to be active (4). The karyopherin-α subunit turned out to be a member of a previously identified family of proteins of uncertain function that had been termed SRP-1 (5), Rch1 (6), hSRP (7), and NPI-1 (8). Karyopherin α also corresponds to "importin" that has recently been isolated as an important factor from *Xenopus* ovary cytosolic fraction A (9). Karyopherin α binds to the nuclear localization signal (NLS) of the transport substrate (4) and therefore is likely to correspond to the 54/56-kDa NLS receptor of bovine erythrocyte cytosol (10, 11). The β subunit of karyopherin appears to function as an adaptor for NLS–substrate karyopherin-α binding to a number of distinct nucleoporins (the collective term for NPC proteins) (3, 4). Karyopherin-β is likely to correspond to the 97-kDa protein isolated from bovine erythrocyte cytosol (11).

The active components of fraction B are the small GTPase Ran (12) and a Ran-interacting protein, p10 (13). Recombinant human Ran has been shown to substitute for endogenous Ran (12, 14). Although the precise function of Ran in nuclear transport reactions remains to be elucidated (15), it is clear that Ran plays a key role, as GTP is required and nonhydrolyzable GTP analogs inhibit not only import into the nucleus (12, 14) but also can result in undocking of import substrate (12). In fact, nuclear import appears to be entirely based on GTP and not on ATP, as nonhydrolyzable ATP analogs do not inhibit import (M. S. Moore and G.B., unpublished data).

Little is known about protein export from the nucleus. It is clear that protein transport across the NPC is bidirectional. Many proteins shuttle between the cytoplasm and the nucleus (16). Previous data suggested that protein export proceeds by default and that NLS motifs do not provide positively acting export signals (17). In contrast, other data suggested that the same NLS that mediates import also mediates export (18). If the latter were the case, then one would expect that protein export across the NPC might be mediated by some of the same factors that are required for protein import. To investigate protein export we used digitonin-permeabilized cells whose nuclei had previously imported the model substrate NLS–HSA (human serum albumin conjugated to NLS). We found that export of the NLS–HSA required Ran and GTP and was inhibited by nonhydrolyzable GTP analogs and by wheat germ agglutinin (WGA). We also found that digitonin-permeabilized cells were depleted not only of cytosolic Ran but also of the endogenous nuclear Ran explaining the requirement for exogenously added Ran in export. Although fluorescently labeled Ran accessed the interior of nuclei of digitonin-permeabilized cells, there also was punctate nuclear rim staining, suggesting the existence of Ran-binding sites at the NPC.

MATERIALS AND METHODS

Materials. Nucleotides and nucleotide analogs were from Boehringer Mannheim; fluorescein isothiocyanate (FITC)-cellite was from Molecular Probes; ^{125}I-labeled protein A was from DuPont. Recombinant human Ran and a GTPase-deficient mutant Ran (Gly-19 → Val and Gln-69 → Leu) were from Elias Coutavas, Mark Rush, and Peter D'Eustachio (19). Buffer A was 20 mM Hepes·KOH, pH 7.3/110 mM potassium acetate/2 mM magnesium acetate/1 mM EGTA/2 mM dithiothreitol/bovine serum albumin (1 mg/ml) containing leupeptin, aprotinin, pepstatin each at 1 μg/ml. The transport substrate NLS–HSA was prepared by coupling rhodamine-labeled HSA to the peptide CYTPPKKKREKV (representing the NLS of simian virus 40 large tumor antigen), as described (2).

Nuclear Export Assay. Buffalo rat liver cells grown on coverslips were digitonin-permeabilized (5 min on ice with digitonin at 35 μg/ml in buffer A) and incubated for 15 min

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Abbreviations: FITC, fluorescein isothiocyanate; HSA, human serum albumin; NLS, nuclear localization signal; NPC, nuclear pore complex; WGA, wheat germ agglutinin; GMP–PNP, guanylyl-imidodiphosphate.

in an import reaction containing NLS–HSA (20 μg/ml) as well as fraction A (2 mg/ml) and fraction B (2 mg/ml) of *Xenopus* ovary cytosol and an ATP-regenerating system (2). After being washed twice with 1 ml of ice-cold buffer A, the cells were incubated at 20°C for various time periods in a standard export reaction mixture in a final volume of 20 μl of buffer A containing 1 μl of bovine serum albumin (20 mg/ml), 1 μl of 20 mM ATP, 1 μl of 100 mM phosphocreatine, and 1 μl of creatinine phosphokinase at 400 units/ml. Where specified, the standard export reaction mixture (20 μl) also contained 1 μl of 20 mM GTP, 1 μl of 20 mM guanosine [γ-thio]triphosphate, 1 μl of 20 mM guanylyl-imidodiphosphate (GMP–PNP), 1 μl of 20 mM AMP-PNP (in this case ATP and the ATP-regenerating system were replaced by 1 μl of 20 mM GTP), 4 μl of fraction A (10 mg/ml), 2.2 μl of fraction B (18 mg/ml), 1 μl of recombinant human Ran (1 mg/ml), 1 μl of recombinant human GTPase-deficient mutant Ran (1 mg/ml), and 1 μl of WGA (10 mg/ml).

The export reaction was terminated by the addition of 1 ml of ice-cold buffer A. After being washed once with 1 ml of ice-cold buffer A, the cells were fixed for 15 min at 0°C with 3% paraformaldehyde in buffer A (without dithiothreitol). The coverslips were mounted in 10% phosphate-buffered saline and 90% glycerol containing *p*-phenylenediamine at 1 mg/ml. Fluorescence microscopy, quantitation, and photography were as described (2). Usually between 50 and 70 nuclei were scanned per coverslip.

Immunoblot Analysis. HeLa cells grown on culture dishes were washed two times in ice-cold buffer A. The cells in one dish were lysed directly in SDS sample buffer. Cells in another dish were first incubated with digitonin (35 μg/ml, 5 min on ice), and after removal of the digitonin extract, the extracted cells were lysed in SDS sample buffer. The digitonin extract was precipitated with trichloroacetic acid, and the pellet was solubilized in SDS sample buffer. An aliquot of each, representing 2.5×10^5 cells, was subjected to SDS/PAGE, and the separated proteins were transferred to nitrocellulose. The blots were probed with either an anti-Ran peptide antibody (12) or with an anti-karyopherin α (NPI-1) antibody (8). Bound IgG was detected with ^{125}I-labeled protein A and subsequent autoradiography.

Preparation of FITC–Ran. FITC–Ran was prepared by incubating 50 μl of Ran (1 mg/ml) with 5 μl of FITC–cellite (10 mg/ml) for 1 hr at 20°C. FITC–cellite was removed by centrifugation, and FITC–Ran was dialyzed against buffer A at 4°C, overnight.

RESULTS

Export of Substrate from the Nucleus Is Stimulated by Fraction B. The kinetics of import of the transport substrate NLS–HSA into nuclei of digitonin-permeabilized buffalo rat liver cells in the presence of saturating amounts of subfractions A and B of a *Xenopus* ovary cytosol and an ATP-regenerating system showed that import was linear up to 10 min and then began to level off (Fig. 1). To investigate whether the import substrate remained in the nucleus or whether it could be "exported," either via passive efflux or by a mediated process, we used the digitonin-permeabilized cells after a 15-min import reaction, washed them with ice-cold buffer to remove the nonimported substrate and soluble factors, and then incubated them again at 20°C for various time periods with an ATP-regenerating system and either buffer or other components (export reaction). At appropriate time points of the export reaction the cells were fixed, and the amount of remaining substrate in the nuclei was quantitated and compared with that at the zero time point. Over a period of 30 min there was ≈15% loss of substrate when the export reaction was done in buffer (Fig. 2). However, there was ≈70% loss of substrate when the export reaction was done in the presence

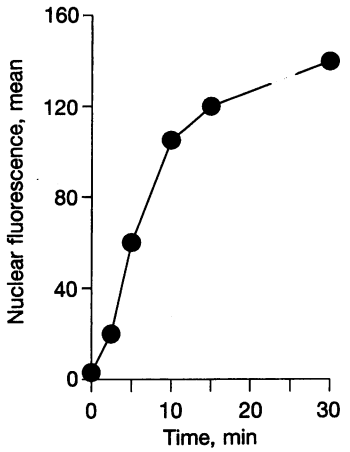

FIG. 1. Time course of import of NLS–HSA into nuclei of digitonin-permeabilized buffalo rat liver cells. *In vitro* import was done for the times indicated under standard conditions (2); quantification was as described (2).

of fraction B, suggesting that export is a mediated process (Fig. 2). Fraction B-mediated export was largely inhibited by WGA (Fig. 2). Interestingly, fraction A by itself did not stimulate export (Fig. 2). However, fraction A abolished fraction B-mediated export (Fig. 2). This result suggested that fraction B-mediated export was counteracted by fraction A- and B-mediated reimport of substrate that occurred in the presence of exogenously added fraction A. Fig. 3 shows corresponding fluorescence images at 0 min (Fig. 3*a*) and 15-min time points of various export reactions (Fig. 3 *b–f*).

Export of Substrate from the Nucleus Is Stimulated by Ran and GTP. The active components of fraction B are Ran (12) and the Ran interactive protein p10 (13), and both are required for import (13). As recombinant human Ran can substitute for endogenous Ran (12, 14) in nuclear import, we tested whether the export-stimulating effect of fraction B might be substituted by recombinant human Ran. Indeed, when the export reaction was done in the presence of Ran, there was 60% export during a 30-min reaction period (Fig. 4). Export was accelerated and reached ≈80% when the reaction was done in the presence of Ran and GTP (Fig. 4). The stimulation by added GTP suggested that the remaining endogenous GDP/GTP and the possible trace amounts of GTP introduced with the ATP-

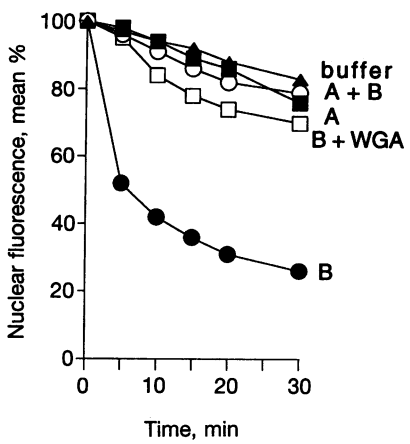

FIG. 2. Nuclear export of NLS–HSA is stimulated by fraction B. Time course of export of NLS–HSA from nuclei in an export reaction containing an ATP-regenerating system and either buffer, or fraction B, or fraction A, or fraction A and B, or fraction B plus WGA. Data were expressed as percentage of transport substrate in the nucleus at the 0-min time point of nuclear export.

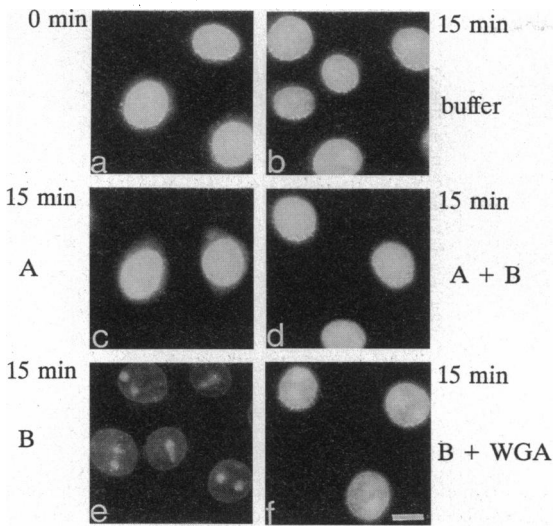

FIG. 3. Fluorescence micrographs taken at various time points of the nuclear export of NLS–HSA (see Fig. 2). *a*, 0 time point; *b–f*, 15-min time points of export reaction containing either buffer (*b*), or fraction A (*c*), or fraction A + B (*d*), or fraction B (*e*), or fraction B + WGA (*f*). (Bar = 10 μm.)

regenerating system were not sufficient. When Ran was omitted, there was no stimulation of export in the presence of GTP alone (data not shown). Together, these data indicated that exogenously added recombinant human Ran and GTP could substitute for the capacity of fraction B to export substrate from the nucleus.

When the export reaction was done in the presence of Ran and WGA, there was complete inhibition of export (Fig. 4). This complete inhibition of Ran-mediated export by WGA (Fig. 4) should be compared with the less than complete inhibition of fraction B-mediated export by WGA (Fig. 2). One possible explanation for this difference might be the presence of GlcNAc-containing proteins in fraction B competing with the nucleoporins for binding to WGA.

Nonhydrolyzable GTP Analogs or GTPase-Deficient Mutant Ran Inhibits Export. When the export reaction was done in the presence of Ran and nonhydrolyzable GTP analogs, either GMP–PNP or guanosine [γ-thio]triphosphate, there was inhibition of export (Fig. 5). These data suggested that Ran-mediated export required GTP hydrolysis. Likewise, when the export reaction contained a recombinant human Ran mutant that is GTPase deficient (19) there was inhibition of export (Fig. 5).

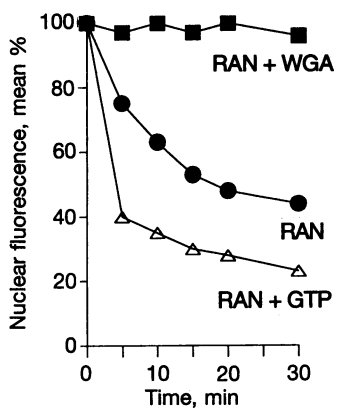

FIG. 4. Ran and GTP stimulate nuclear export of NLS–HSA, and WGA inhibits Ran-stimulated export.

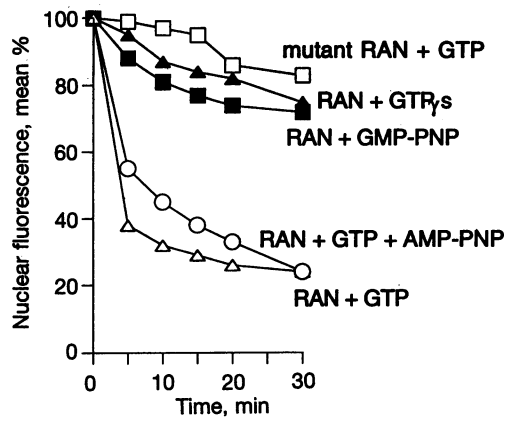

FIG. 5. Ran-stimulated nuclear export is inhibited by nonhydrolyzable GTP analogs and a GTPase-deficient Ran mutant but is not inhibited by AMP–PNP. GTPγS, guanosine [γ-thio]triphosphate.

Nonhydrolyzable ATP Analog Does Not Inhibit Ran-Mediated Export. When the ATP-regenerating system in the export reaction was replaced by the nonhydrolyzable ATP analog AMP–PNP and GTP, there was only a slight decrease in the rate, but not in the final level, of Ran-GTP-mediated export (Fig. 5). These data suggested that export is GTP and not ATP driven.

Digitonin-Permeabilization Resulted in Loss of Ran but Retention of Karyopherin α. Immunofluorescence studies of fixed cells with anti-Ran-specific antibodies have shown that Ran is more concentrated in the nucleus than in the cytoplasm (19). Why, then, is export from the nucleus dependent on exogenously added Ran? Could it be that Ran leaked out of the nucleus after digitonin-permeabilization of cells? Indeed, when the proteins of a total HeLa cell lysate or of a total lysate of digitonin-extracted HeLa cells were separated by SDS/PAGE, transferred to nitrocellulose, and then probed with an anti-human Ran-peptide antibody (13), the digitonin-permeabilized HeLa cells were found to have lost essentially all their Ran (Fig. 6*A*). In contrast, probing identical blots with anti-karyopherin-α (NPI-1) antibodies showed that after digitonin-permeabilization much of karyopherin α was retained in the nucleus (Fig. 6*B*), in agreement with previously reported cell-fractionation data (8).

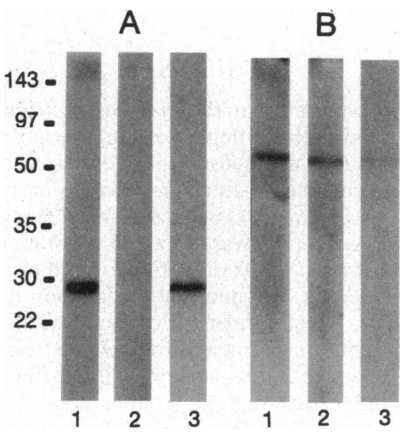

FIG. 6. Immunoblot of total lysate either of HeLa cells (lanes 1) or of digitonin-extracted HeLa cells (lanes 2) and of the digitonin-extracted material (lanes 3) with anti-Ran and anti-karyopherin-α antibodies. Each lane contains the proteins from an equivalent of 2.5 × 10^5 HeLa cells separated by SDS/PAGE, transferred to nitrocellulose, and probed with anti-Ran peptide antibodies (*A*) or with anti-karyopherin-α antibodies (*B*). Bound antibodies were detected with ^{125}I-labeled protein A and subsequent autoradiography.

Exogenous Ran Docks at the Nuclear Envelope and Enters the Nucleus. Does the exogenously added Ran that is required for export reenter the Ran-depleted nuclei during the export reaction? To address this question recombinant human Ran was labeled with FITC and then incubated in the export reaction containing either WGA or no WGA. After a 15-min incubation the cells were fixed and analyzed by fluorescence microscopy (Fig. 7). In the absence of WGA, FITC-labeled Ran was found within the nucleus (Fig. 7). Surprisingly, however, it also was found docked at the nuclear envelope in a punctate fashion, suggesting that it bound to NPCs (Fig. 7). WGA inhibited both binding of FITC–Ran to the nuclear rim, as well as its uptake into the nucleus (Fig. 7).

DISCUSSION

Fidelity of the *in vitro* Export Reaction. As in *in vitro* export system we used digitonin-permeabilized cells whose nuclei had been preloaded with the fluorescently labeled model substrate NLS–HSA during a 15-min *in vitro* import reaction at 20°C. After washing cells at 0°C to remove nonimported substrate and soluble factors and reincubating cells at 20°C for various time periods with an ATP-regenerating system and either buffer or other components, the loss of NLS–HSA from nuclei was quantified and taken as a measure for nuclear export. We first observed that there was only a slow rate of export (up to 20%) when only buffer was present during a 30-min export reaction. However, there was a much faster rate of export (up to 70%) when the *Xenopus* ovary cytosolic fraction B was present during the export reaction. In control experiments, digitonin-permeabilized cells that had undergone a 15-min mock import reaction without NLS–HSA and a subsequent 30-min mock export reaction with buffer or fraction B retained an apparently intact and transport-competent nuclear envelope, as nuclei remained capable of importing NLS–HSA in a strictly cytosol-dependent fashion (data not shown). Together these data suggested that the *in vitro* export in this system occurs by a mediated process rather than nonspecific leakage and that it reproduces with fidelity export events *in vivo*. The slower rate of export in buffer suggested a missing export-limiting component(s) that was at least partially supplied by fraction B, thereby increasing the rate of export.

An Import–Export Cycle. Although the *Xenopus* ovary cytosolic subfraction B stimulated export, the cytosolic subfraction A, when added alone, did not stimulate export. Surprisingly, when fraction A was added together with fraction B, the export-stimulating effect of fraction B was abolished. The activities of these two cytosolic subfractions have previously been defined in the import reaction with fraction A (containing karyopherin α and β) being involved in docking of NLS–HSA to the NPC (2–4) and fraction B (containing Ran and p10) being involved in translocation of the import substrate across the NPC into the nucleus (12, 13). If one considers that import and export of NLS–HSA substrate across the NPC may require the same signals and some of the same transport factors (15) for transport in either direction, then the observed annulment of fraction B-stimulated export by fraction A could be rationalized as fraction A-dependent (and fraction B-mediated) reimport of transport substrate. Thus, the plateau in import seen in the import reaction (Fig. 1) at saturating concentration of fraction A and B could be interpreted as import and export of substrate having reached equilibrium. In this model inhibitors of import would be expected to inhibit export as well. Indeed, we show here that WGA, a well known inhibitor of protein import (2), also inhibited protein export (Figs. 2 and 4).

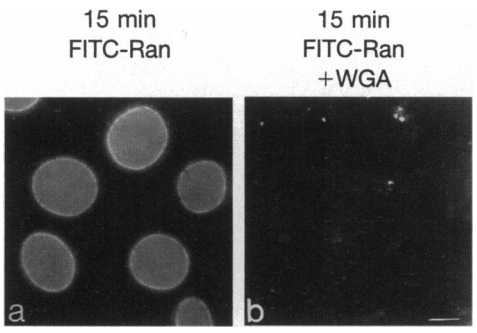

FIG. 7. FITC-labeled Ran yields staining at the nuclear rim and the nuclear interior and both are inhibited by WGA. Digitonin-permeabilized buffalo rat liver cells were incubated for 15 min at 20°C with buffer A, an ATP-regenerating system, and FITC-labeled Ran (100 μg/ml) in the absence (*a*) or in the presence of WGA (*b*). (Bar = 10 μm.)

Ran and Protein Export from the Nucleus. We found the fraction B-mediated export activity to be efficiently substituted for by recombinant human Ran and GTP (although we have not yet tested whether p10 would affect export as well). Nonhydrolyzable GTP analogs inhibited export, indicating that GTP hydrolysis was required for export. Likewise, a recombinant human Ran mutant that was GTPase deficient also abolished export. As nonhydrolyzable ATP analogs do not interfere with import (M. S. Moore and G.B., unpublished data), it was not surprising that AMP–PNP also did not interfere with export. Thus, bidirectional transport of proteins across the NPC appears to be entirely GTP-driven. The requirement for Ran in both protein import (12, 14) and export strongly supports the notion of a common import/export pathway across the NPC (15).

Much of the cellular Ran is known to be located in the nucleus (19). The requirement for Ran in the import assay could be readily rationalized as a result of losing the cytoplasmic pool of Ran during digitonin-permeabilization. However, it was difficult to rationalize a requirement for Ran in export. The solution to this puzzle came with the demonstration that, in addition to the cytoplasmic pool, the nuclear pool of Ran was depleted during digitonin-permeabilization (Fig. 6). Why nuclear Ran is not retained in the nucleus during digitonin-permeabilization remains to be investigated. It may be unanchored after digitonin-permeabilization and, being small, it could simply diffuse out of the nucleus during the permeabilization and washing steps.

We showed that fluorescently labeled Ran can readily reenter the nucleus. The most surprisingly result of these experiments, however, was that exogenously added fluorescently labeled Ran also appeared to dock at the NPCs, based on punctate nuclear rim staining in fluorescence microscopy (Fig. 7). This result suggested the existence of Ran-binding sites at the NPC.

Using immunoblot analysis, we also found that, in contrast to Ran, the α subunit of karyopherin remained in the nucleus after digitonin-permeabilization (Fig. 6), most likely because this α subunit is too large to diffuse out of the nucleus. Therefore karyopherin would not be expected to be limiting in our export assay. Nevertheless, we believe that karyopherin α, serving as the transport substrate-binding protein (4), is required for export as well. If karyopherin α is, indeed, exported in our export system, it, together with the nucleoporin-bound karyopherin β (3, 4), could serve to mediate another round of import. Such reimport might explain the observed slow phase of Ran and GTP-mediated export that follows an initial fast phase (Figs. 4 and 5).

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