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A cytosolic activity distinct from Crm1 mediates nuclear export of protein kinase inhibitor in permeabilized cells

(Ran GTPase/nuclear pore complex/nucleocytoplasmic transport)

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ABSTRACT **The leucine-rich nuclear export signal (NES) is used by a variety of proteins to facilitate their delivery from the nucleus to the cytoplasm. One of the best-studied examples, protein kinase inhibitor (PKI), binds to the catalytic subunit of protein kinase A in the nucleus and mediates its rapid export to the cytoplasm. We developed a permeabilized cell assay that reconstitutes nuclear export mediated by PKI, and we used it to characterize the cytosolic factors required for this process. The two-step assay involves an import phase and an export phase, and quantitation is achieved by digital fluorescence microscopy. During the import phase, a fluorescent derivative of streptavidin is imported into the nuclei of digitonin-permeabilized HeLa cells. During the export phase, biotinylated PKI diffuses into the nucleus, binds to fluorescent streptavidin, and mediates export of the complex to the cytoplasm. Nuclear export of the PKI complex is cytosol dependent and can be stimulated by addition of the purified NES receptor, Crm1. HeLa cell cytosol treated with *N*-ethylmaleimide (NEM) or phenyl-Sepharose to inactivate or deplete Crm1, respectively, is still fully active in the PKI export assay. Significantly, the export activity can be depleted from cytosol by preadsorption with a protein conjugate that contains a functional NES. These data indicate that cytosol contains an export activity that is distinct from Crm1 and is likely to correspond to an NES receptor.**

Molecular trafficking between the nuclear and cytoplasmic compartments proceeds through nuclear pore complexes (NPCs), elaborate protein assemblies that span the double membrane system of the nuclear envelope (1). Ions and small molecules may freely diffuse through the aqueous channel of the NPC; however, the sizes of most proteins and ribonucleoprotein complexes preclude their passage by simple diffusion. These macromolecules are instead transported through a gated channel of the NPC in a process that is sensitive to temperature and energy levels, hallmarks of active transport. The specificity of nuclear transport relies on signals encoded within the proteins destined for transport. The prototypical nuclear localization signal (NLS) found in simian virus 40 (SV40) large T antigen or nucleoplasmin contains one or two clusters of basic amino acids, respectively (2). The nuclear export signal (NES) was discovered 3 years ago in protein kinase inhibitor (PKI) (3) and the HIV-1 rev protein (4), and consists of a sequence enriched in hydrophobic amino acids, particularly leucine. In addition, signals that mediate both import and export have been found in proteins that are implicated in mRNA trafficking (5).

Substantial progress has been made in analyzing the proteins that mediate nuclear transport. Biochemical and genetic analysis of nuclear import has revealed a system of cytosolic adapter proteins that facilitate molecular recognition of the NLS, docking of the protein to the cytoplasmic surface of the NPC, translocation of the protein through the NPC, and deposition of the protein in the nucleoplasm (6–8). The best-understood aspects of nuclear protein import are those involving NLS recognition and docking at the NPC, events that are carried out by the NLS receptor heterodimer and certain NPC proteins (9–13). Translocation of the transport complex through the NPC is thought to require the direct participation of the GTPase Ran (14, 15), its binding partner NTF2 (16, 17), and a number of peptide repeat-containing proteins within the NPC (refs. 18 and 19 and references therein). Proteins that regulate the GTPase cycle of Ran are also important participants in nuclear import pathways (20–23). Two of the major challenges of the nuclear import field are to chart the course of the complex as it traverses the interior of the NPC en route to the nucleoplasm, and to understand how the Ran GTPase cycle may coordinate individual transport steps (7, 24, 25).

Recent molecular analysis of nuclear export has focused on two highly conserved proteins, the GTPase Ran and a protein termed Crm1 that was originally described in fission yeast for its *c*hromosome *r*egion *m*aintenance phenotype (26). Studies both *in vivo* and *in vitro* have clearly shown that the GTP-bound form of Ran, not GTP hydrolysis *per se*, is required for nuclear protein export (27–29). Crm1 was predicted to be an export factor on the basis of its sensitivity to leptomycin B, a drug that inhibits rev-dependent RNA export (30, 31). The sequence relatedness of Crm1 to β-importins and its localization to the NPC were also consistent with a role in nuclear transport (32). Ultimately, studies from multiple laboratories using yeast, *Xenopus* oocytes, and mammalian cells established that Crm1 is a receptor for the leucine-rich NES (28, 33–36). The current model is that Crm1, NES-containing protein, and the GTP-bound form of Ran assemble into a trimeric complex in the nucleus that undergoes translocation through the NPC (8). Once in cytoplasm, the complex could undergo a disassembly reaction triggered by GTP hydrolysis, an event stimulated by the GTPase-activating protein RNA1 and the co-activator RanBP1.

We reasoned that an *in vitro* assay that reconstitutes nuclear protein export would be useful for dissecting the biochemical requirements for this process. To this end, we developed a permeabilized cell assay that reconstitutes cytosol-dependent export of PKI from HeLa cell nuclei. While Crm1 is capable of stimulating nuclear export of PKI in our assay, we made the surprising finding that depletion or inactivation of Crm1 has no measurable effect on the export capacity of the cytosol.

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Abbreviations: NEM, *N*-ethylmaleimide; NES, nuclear export signal; NLS, nuclear localization signal; PKI, protein kinase inhibitor; bPKI, biotinylated PKI; NPC, nuclear pore complex; FITC-STV-NLS, fluorescein isothiocyanate-modified streptavidin-NLS; SV40, simian virus 40; WT, wild-type.

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Significantly, the cytosolic export activity can be depleted by preadsorption with a wild-type (WT) NES, but not a mutant NES. Taken together, our data indicate that cytosol contains an export activity that is distinct from Crm1 and predicted to be either an NES receptor or a protein that performs a function linked to NES recognition.

MATERIALS AND METHODS

Preparation of Recombinant Proteins. Plasmids encoding full-length human PKI proteins (WT and NES mutant) were expressed by using the T7 RNA polymerase-based system of Studier *et al*. (37) in the *Escherichia coli* strain BL21(pLysS). The plasmids, which were originally constructed by S. S. Taylor (University of California at San Diego), were provided by M. L. Hammarskjöld (University of Virginia). The NES mutant of PKI contains two substitutions (L41A and L44A) that inactivate the export signal (3). PKI proteins were purified (10 mg/liter of culture) by using published procedures (38), with gel filtration chromatography (Sephacryl S100) as the final purification step. PKI proteins were biotinylated with a 25-fold molar excess of NHS-LC-biotin (Pierce), and the products were analyzed by time-of-flight mass spectrometry (Biomolecular Research Facility, University of Virginia) to determine the stoichiometry of the reaction products. For both WT and mutant proteins, 75% of the input PKI was modified, with 60% receiving a single biotin and 15% receiving two biotins. The plasmid encoding streptavidin fused to the SV40 NLS was constructed in pET23b and contains 53 aa of the SV40 large T antigen protein, including the NLS. The streptavidin (STV) gene was generously provided by J. E. Cronan (University of Illinois, Urbana). The STV-NLS protein was expressed in *E. coli* strain BL21(pLysS), solubilized in 8 M urea, and purified by gel filtration chromatography (Sephacryl S300) in the same buffer (4.2 mg/liter of culture). After dialysis, STV-NLS was labeled with fluorescein isothiocyanate (FITC). Recombinant Ran was prepared as described (14). Human Crm1 was purified from HeLa cell cytosol by standard column chromatography using immunoblotting as the assay. While developing the purification protocol we found that incubation with phenyl-Sepharose was an extremely efficient method for depleting Crm1 from cytosol (B. Stultz and B.M.P., unpublished data).

Microinjection Experiments. The export activity of biotinylated WT and NES mutant PKI (bPKI) was evaluated by testing whether the proteins could induce translocation of fluorescent streptavidin from the nucleus to the cytoplasm in cultured cells. Nuclei of adherent HeLa cells were co-injected with bPKI (WT or NES mutant; 2.5 mg/ml), rhodamine-conjugated streptavidin (1 mg/ml), and FITC-dextran (70 kDa; 2 mg/ml) as the injection marker. Microinjections were performed using an Eppendorf micromanipulator and Transjector mounted on a Zeiss Axiovert microscope. After injection, the cells were incubated for 1 hr at 37°C, fixed in 3.7% formaldehyde, mounted in glycerol, and viewed on a Nikon Microphot-SA fluorescence microscope equipped with a 60× objective (numerical aperture = 1.4). Images were recorded with Kodak 35-mm color film (ASA 400) using exposure times of 20–30 sec. The bPKI proteins were tested by microinjection in more than 200 cells in three separate experiments.

Permeabilized Cell Nuclear Export Assay. The nuclear export assay was carried out in suspension HeLa cells, maintained in culture as described (39). Cells were harvested, washed, and permeabilized with 50 μg/ml digitonin in transport buffer (TB; 20 mM Hepes, pH 7.4, containing 110 mM potassium acetate, 2 mM magnesium acetate, and 1 mM EGTA) made with 2 mM DTT and a protease inhibitor mixture (17). Our export assay is carried out in two phases, nuclear loading of FITC-STV-NLS (import phase) and PKI-mediated nuclear export of FITC-STV-NLS (export phase). For the import phase, FITC-STV-NLS (50 μg/ml), phenyl-Sepharose-treated HeLa cell cytosol (3 mg/ml), BSA (10 mg/ml), an energy regenerating system (17), and permeabilized HeLa cells (2×10^7 cells per ml) were combined in a total volume of 50 μl and incubated for 20 min at 30°C. Import reactions were terminated by diluting the reaction with 4 ml of ice-cold TB, and cells were collected by centrifugation. For the export phase, bPKI (WT or NES mutant; 50 μg/ml), cytosol (3 mg/ml), BSA (10 mg/ml), an energy regenerating system (17), and permeabilized cells whose nuclei were loaded with FITC-STV-NLS (5×10^6 cells per ml) were combined in a total volume of 60 μl and incubated for 15 min at 30°C. In some experiments, we included a different NLS substrate (APC-NLS; allophycocyanin coupled with SV40 NLS peptides) (40) to the export reaction. Other variations, such as the addition of purified proteins or modified cytosols, are detailed in the figure legends. Export reactions were terminated and washed as described above. The cells were fixed in 3.7% formaldehyde, washed, and applied to glass slides for examination by digital fluorescence microscopy.

Quantitation of Nuclear Export by Fluorescence Microscopy. Microscopy was performed with a Nikon Microphot SA fluorescence microscope and a 40× dry objective (numerical aperture = 0.95). Fields of cells were first randomly selected by phase-contrast optics, and the fluorescent images were captured with a Photometrics (Tucson, AZ) charge-coupled device camera (5-sec exposure) and Photometrics PMIS software (version 3.5). To quantitate nuclear export, the average pixel intensity for individual nuclei (measured in 25–50 cells) was measured over a 1-μm square area. These values were pooled and used to generate a mean and SD. The values were then scaled from 0 to 1, with 0 representing the background fluorescence in a region free of cells, and 1 representing the nuclear fluorescence after the import reaction. Typically, ≈20% of the FITC-STV-NLS is lost from the nucleus by the addition of buffer alone, possibly because of export factors that remain within the permeabilized cells after washing. The nuclear fluorescence that results from addition of buffer alone is shown for comparison in every experiment.

Immunoblot Analysis of Transport Factors. Tissue cytosols were prepared by homogenizing freshly dissected rat tissues in an equal volume of TB containing 2 mM DTT and a protease inhibitor cocktail. The anti-peptide Crm1 antibody was generated in rabbits with the peptide (C)RQADEEKHKRQMSVPGS conjugated to keyhole limpet hemocyanin and was used at a dilution of 1:5000. The Crm1 antibody used in Fig. 5 (generously provided by Brian Guzik and M. L. Hammarskjöld) was generated in rabbits against the C-terminal 380 aa of recombinant Crm1, and used at a dilution of 1:500. Monoclonal antibodies to NTF2, Ran, and β-importin (Transduction Laboratories, Lexington, KY) were used at 1 μg/ml.

Pretreatment of Cytosols. HeLa cell cytosol was depleted of Crm1 by incubation with phenyl-Sepharose at a ratio of 3:1 (vol/vol) for 30 min at 4°C. For experiments involving modification with *N*-ethylmaleimide (NEM), 10 mM NEM (final concentration) was added to samples (rendered free of reducing agents) for 30 min at 25°C. The reactions were then quenched by the addition of 20 mM DTT. Mock-treated samples were always prepared in parallel by adding 20 mM DTT to samples prior to addition of 10 mM NEM. Depletion of NES-binding proteins (Fig. 6) was carried out by mixing 3 vol of cytosol (10 mg/ml) with 1 vol of packed beads overnight at 4°C. The WT and mutant NES conjugates for these experiments were prepared by coupling synthetic peptides to BSA by using the heterobifunctional crosslinker sulfo-SMCC [sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; Pierce]. The NES-BSA conjugates were then coupled to CNBr-activated Sepharose beads (17) at a final concentration of 10 mg of protein per ml of packed beads. The WT and mutant peptide sequences were as described (3).

RESULTS

We have established a permeabilized cell assay that measures nuclear export mediated by PKI, a protein with a thoroughly characterized leucine-rich NES (3). In its cellular context, the relatively small size of PKI (75 aa) allows it to rapidly diffuse into the nucleus, where it binds to the catalytic subunit of protein kinase A and mediates its export to the cytoplasm (41). The principle of our permeabilized cell assay is that bPKI should diffuse into the nucleus, bind to a fluorescently labeled streptavidin with femtomolar affinity, and mediate its export to the cytoplasm. Digitonin permeabilization releases the proteins required for nuclear import (40) and export (28, 35). We envisioned, therefore, that nuclear export of a bPKI-streptavidin complex would display cytosol dependence and provide an important assay for discovery and characterization of nuclear export factors.

Prior to setting up the permeabilized cell assay, bPKI was tested for its ability to mediate nuclear export of fluorescently labeled streptavidin in nuclear microinjection experiments (Fig. 1). Recombinant bPKI proteins (WT or NES mutant, denoted MUT) were preincubated with rhodamine-labeled streptavidin (denoted RHOD-STV), microinjected into nuclei of adherent HeLa cells, and localized by fluorescence microscopy. Rhodamine-streptavidin was exported from nuclei when co-injected with bPKI that contains a functional NES (WT NES; Fig. 1), after which it aggregated in the cytoplasm. In contrast, rhodamine-streptavidin remained within the nuclei when co-injected with bPKI that contains a nonfunctional NES (MUT NES; Fig. 1). These data show that the nuclear export machinery specifically recognizes bPKI when it is bound to streptavidin, and it actively transports the ≈70-kDa fluorescent complex to the cytoplasm in living cells.

Our permeabilized cell export assay involves an initial import phase during which nuclei are loaded with fluorescent streptavidin. For this purpose, we engineered streptavidin to include a C-terminal fusion with 53 aa from SV40 large T antigen that spans the NLS. Nuclear import of the fluorescently labeled streptavidin-NLS protein (denoted FITC-STV-NLS) was cytosol dependent and blocked by wheat germ agglutinin (data not shown). It should be noted that the HeLa cytosol used for the import phase was first depleted of Crm1 (see below). This was done because of our concern that addition of cytosol (containing Crm1) to the import phase might mask the cytosol dependence of the export phase.

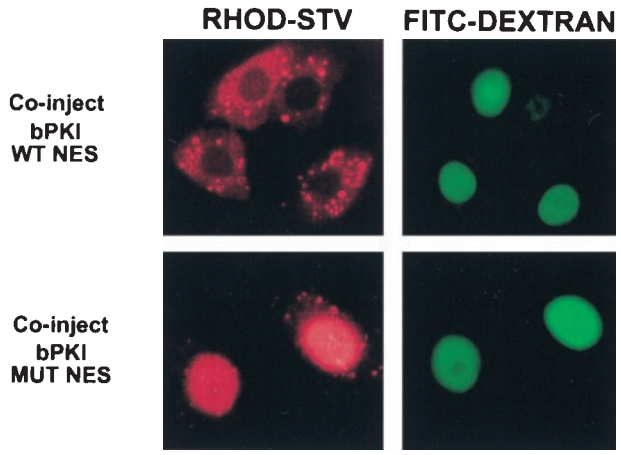

FIG. 1. NES-dependent export of a complex containing bPKI and fluorescent streptavidin. The nuclei of living HeLa cells were co-injected with bPKI proteins, rhodamine streptavidin (RHOD-STV), and a 70-kDa fluorescent dextran (FITC-DEXTRAN) to mark the injection site. The WT bPKI protein mediates export of rhodamine streptavidin from the nucleus to the cytoplasm (*Upper*). In contrast, the NES mutant bPKI protein fails to mediate nuclear export of rhodamine streptavidin (*Lower*). These data show that the biotin-modified PKI protein is fully active for export, and that streptavidin does not contain a cryptic NES.

After the initial import phase, the permeabilized cells are washed with buffer and placed on ice. In the nuclear export phase, bPKI added to the permeabilized cells diffuses into the nuclei and binds to FITC-STV-NLS. The samples are supplemented with buffer or cytosolic proteins and incubated at 30°C for 15 min. The permeabilized cells are then washed and fixed in formaldehyde, and the levels of nuclear export are determined by measuring the nuclear fluorescence in randomly selected cells. Addition of HeLa cell cytosol to the assay resulted in a substantial reduction in nuclear fluorescence (Fig. 2*A Top* and *Middle*), indicating that nuclear export of the bPKI/FITC-STV-NLS complex depends on one or more soluble transport factors. Preincubation of the cytosol at 95°C abolished the activity of the HeLa cell cytosol, indicating that heat-labile proteins mediate nuclear export of bPKI/FITC-STV-NLS in our assay. Export was blocked by low temperature (0°C) or hexokinase/glucose treatment, and export did not occur when bPKI containing a mutated NES was substituted in the assay (data not shown). We verified that both nuclear integrity and NPC function are maintained in our system by showing that a different transport substrate (APC-NLS) can be imported during the export phase of the assay (Fig. 2*B*).

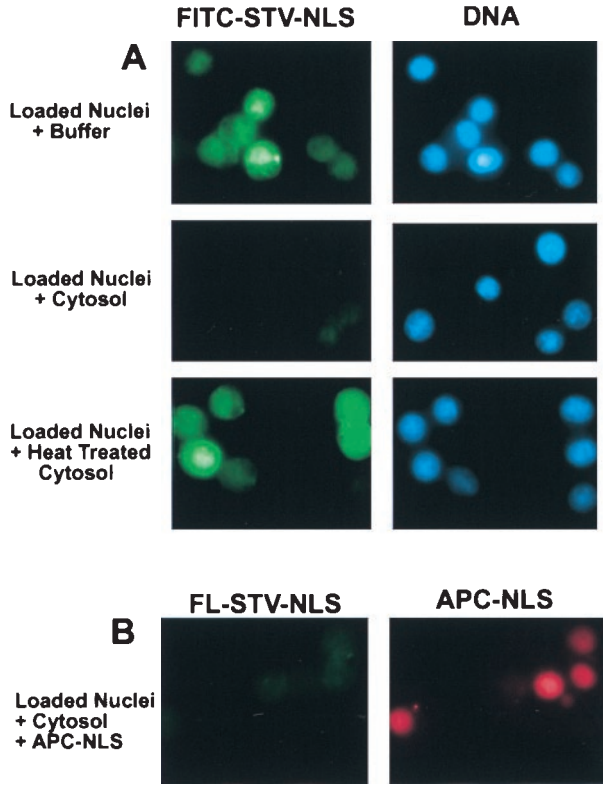

FIG. 2. A permeabilized cell assay that measures nuclear export mediated by PKI and cytosolic factors. (*A*) The nuclei of digitonin-permeabilized HeLa cells were loaded with FITC-STV-NLS, washed with buffer, and further incubated with bPKI in the presence of buffer alone (*Top*), HeLa cell cytosol (3 mg/ml; *Middle*), or heat-treated HeLa cell cytosol (3 mg/ml; *Bottom*). The cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to reveal the position of nuclei within each field. *Middle* shows that the addition of cytosol results in a significant loss of FITC-STV-NLS from HeLa cell nuclei, an effect that can be ascribed to heat-labile cytosolic proteins. (*B*) Permeabilized cells in the nuclear export assay are competent for NLS-mediated protein import. Allophycocyanin (APC) conjugated with SV40 NLS peptides (40), when added to export reactions, is imported into nuclei. This result shows that under the conditions of our assay, nuclei remain intact and NPCs are functional.

We examined the cytosol, substrate, and time dependence of the export assay. Half-maximal export of the bPKI complex was obtained with ≈1 mg/ml HeLa cytosol and ≈20 μg/ml bPKI (Fig. 3 *A* and *B*). The high concentration of bPKI required in our assay may reflect binding to endogenous or cytosolic protein kinase A. Export in our assay displays relatively fast kinetics, as ≈50% of the nuclear FITC-STV-NLS was exported within 2–3 min (Fig. 3*C*).

The recent identification of Crm1 as a receptor for nuclear export of NES-containing proteins such as NFκB (35) and NFAT (28) prompted us to examine whether it can perform a similar function for PKI. Crm1 purified from HeLa cell cytosol by column chromatography stimulated PKI export 3.7-fold (Fig. 4*A*). This indicates that Crm1 can function as an NES receptor for PKI, and that it is one of the rate-limiting components in our assay. Addition of a saturating amount of Ran (100 μg/ml), a GTPase required for both nuclear import and export (7), stimulated export only 1.3-fold in our assay. To screen for the presence of novel rate-limiting export factors, we devised methods for inactivation and depletion, respectively, of cytosolic Crm1. The first method involved treatment with NEM, an alkylating reagent that was found to potently inactivate the export activity of purified Crm1 (Fig. 4*A*). NEM treatment did not, however, affect the export activity of HeLa cell cytosol, providing evidence for an NEM-insensitive export activity. The second method involved quantitative depletion of Crm1 from HeLa cytosol by incubation with phenyl-Sepharose, a procedure that was anticipated to be effective on

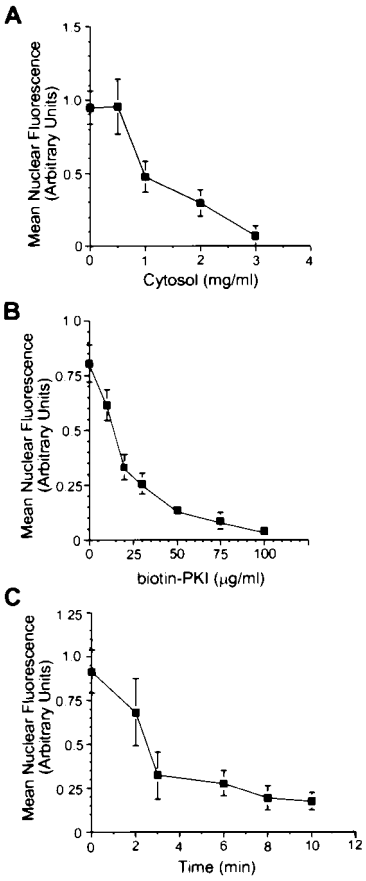

FIG. 3. Characterization of the PKI nuclear export assay. (*A*) Cytosol-dependent export of the bPKI/FITC-STV-NLS complex was observed, with maximal export occurring in the presence of 3 mg/ml HeLa cell cytosol. (*B*) Dose-response curve showing the effect of increasing the concentration of bPKI in the assay. (*C*) Time course of export promoted by 50 μg/ml bPKI in the presence of 3 mg/ml HeLa cell cytosol.

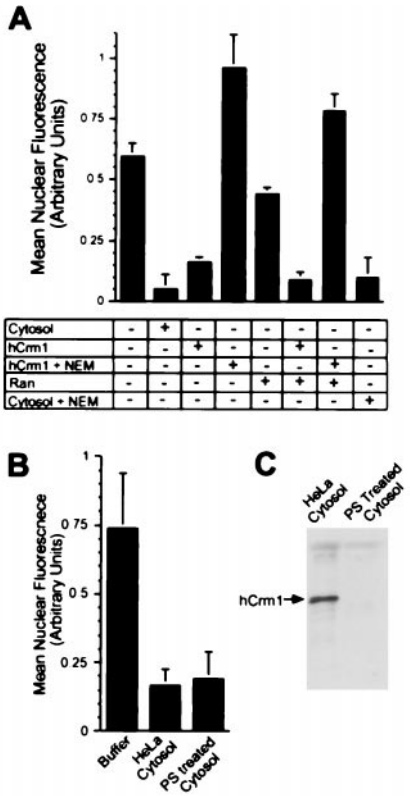

FIG. 4. Analysis of the cytosolic factors that mediate PKI export in permeabilized cells. (*A*) Addition of HeLa cytosol (3 mg/ml) or purified Crm1 (30 μg/ml) strongly stimulates nuclear export. NEM treatment of Crm1 inactivates its export activity. In contrast, NEM treatment of cytosol did not inactivate its export activity, indicating that cytosol contains an export-promoting activity that is insensitive to sulfhydryl alkylation and therefore different from Crm1. The higher nuclear signal in samples containing NEM-treated Crm1 was observed in three separate experiments. It suggests that the chemically modified Crm1 exerts a slight dominant-negative effect by reducing export promoted by factors remaining within the permeabilized cell. Note that the human Crm1 and cytosol samples in this experiment were mock-treated with NEM (see *Materials and Methods*). In this experiment, a 20% reduction in nuclear bPKI/FITC-STV-NLS was observed after the addition of Ran (100 μg/ml). In most experiments, however, the addition of Ran had no effect on export, indicating it is not a major rate-limiting factor in our assay. Note that this concentration of Ran corresponds to at least a 5-fold excess over the concentration of Ran present in HeLa cell cytosol used in our export assays. (*B*) Cytosol depleted of Crm1 stimulates PKI export in permeabilized cells. HeLa cytosol was depleted of Crm1 by incubation with phenyl-Sepharose, and the unbound fraction (PS cytosol; 3 mg/ml) was tested in the PKI export assay. (*C*) The depletion of Crm1 from cytosol was demonstrated by immunoblotting using an anti-peptide Crm1 antibody.

the basis of the high percentage of hydrophobic residues (36%) in the protein. Phenyl-Sepharose treatment of HeLa cytosol had essentially no effect on its capacity to stimulate export (Fig. 4*B*), even though it removed all Crm1 protein detectable by immunoblotting (Fig. 4*C*). The NEM inactivation and phenyl-Sepharose depletion experiments provide strong evidence that HeLa cytosol contains a rate-limiting export activity that is distinct from Crm1.

Additional evidence that the cytosolic export activity detected in our assay is distinct from Crm1 was obtained by showing that tissue extracts with extremely low levels of Crm1 can support nuclear export of PKI in our assay. Cytosolic extracts prepared from rat kidney, liver, and lung all stimulated nuclear export to about the same extent as HeLa cytosol (Fig. 5*A*). Immunoblot analysis showed that while extracts from these and five other tissues contain other housekeeping nu-

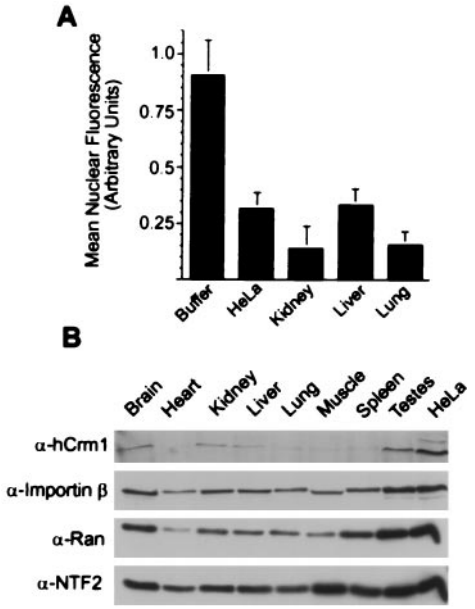

FIG. 5. Cytosols deficient in Crm1 promote nuclear export of PKI. (*A*) Cytosols prepared from rat kidney, liver, and lung stimulate export of PKI to an extent similar to that of HeLa cytosol (all tested at 3 mg/ml). (*B*) Immunoblot assays showing the relative levels of nuclear import and export factors in rat tissues. α- indicates antibody. Ran and NTF2 levels were determined on immunoblots containing 25 μg of total protein per lane, while the β-importin and Crm1 levels were determined on immunoblots containing 50 μg of total protein per lane. The robust nuclear export obtained with tissue cytosols containing low levels of Crm1 (relative to HeLa cytosol) is consistent with the presence of additional export activities.

clear transport factors (NTF2, Ran, and β-importin), the Crm1 protein was barely detectable (Fig. 5*B*). Because nuclear export is fundamentally important for the cell, these data suggest the existence of NES receptors that are distinct from Crm1.

The export activity that we detected in cytosols deficient for Crm1 could be a positive regulator of protein export, similar to the role of NTF2 in nuclear protein import (16, 17). Alternatively, the export activity could be a novel NES receptor whose biochemical properties (such as NEM insensitivity) are distinct from Crm1. One property expected of a cognate NES receptor is an ability to bind to an NES substrate. To address this issue, we preadsorbed cytosols with NES-conjugates immobilized on Sepharose beads and assayed the unbound fraction from each sample to determine whether export activity was depleted by the treatment. As expected, the export capacity of HeLa cytosol was reduced markedly by preadsorption with the WT NES conjugate (WT NES; Fig. 6), but not with the NES conjugate containing a nonfunctional NES (Mut NES; Fig. 6). Strikingly, the export capacity of cytosols lacking Crm1 was also specifically depleted by the WT NES conjugate. This result was observed with HeLa cytosol previously depleted of Crm1 by using phenyl-Sepharose (PS HeLa) and with kidney cytosol, which contains very little Crm1 protein. Taken together, our results provide unequivocal evidence for a nuclear export factor, distinct from Crm1, that is capable of discriminating between a WT and mutant NES.

DISCUSSION

The permeabilized cell assay developed 8 years ago (40) has proven to be a powerful tool for characterizing the proteins necessary to reconstitute nuclear protein import in mammalian cells. By analogy, we reasoned that a permeabilized cell assay could be used to purify and characterize proteins nec-

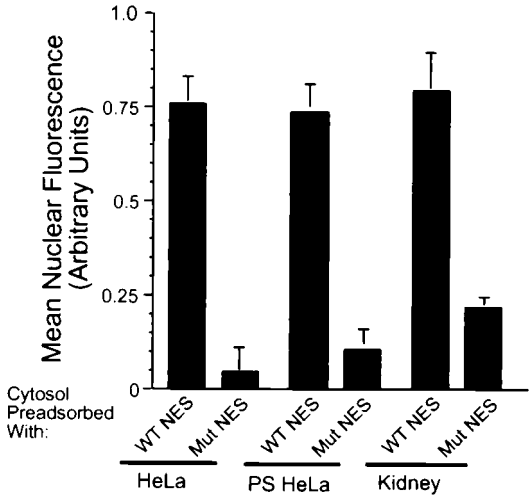

FIG. 6. Identification of a cytosolic NES-binding activity that stimulates nuclear export of PKI. HeLa cytosol (HeLa), or HeLa cytosol incubated with phenyl-Sepharose to remove Crm1 (PS HeLa), or kidney cytosol (Kidney), were each preadsorbed with peptide-BSA-Sepharose conjugates containing the WT NES or a mutant NES (Mut NES). The unbound fractions were recovered and tested in the PKI export assay. Significant depletion of export activity was observed in control cytosol (HeLa), in cytosols lacking Crm1 (PS HeLa), and in the cytosol containing an extremely low level of Crm1 (Kidney). This result indicates the presence of an export activity that can specifically recognize and bind to the WT NES.

essary for nuclear export. This logic was supported by experiments showing that cytosol could stimulate the release of an NES-containing reporter protein from the nuclei of digitonin-permeabilized cells (35). The assay that we have described here measures nuclear export of a fluorescent complex that contains PKI, a protein with a leucine-rich NES that in its cellular context mediates nuclear export of the catalytic subunit of protein kinase A (41). Nuclear export in our assay is stimulated by the addition of cytosolic proteins, an important feature that has allowed us to begin dissecting the biochemical requirements for PKI transport. We determined that Crm1 can promote nuclear export of PKI, fully consistent with its role as an NES receptor protein (28, 33–36). Careful analysis revealed, however, that cytosol contains a positively acting export activity that can be distinguished from Crm1. The export activity is also distinct from Ran, a GTPase required for protein export. The export activity that we have identified specifically recognizes and binds the WT NES, indicating it is likely to correspond to a previously undescribed NES receptor.

Gerace and coworkers have recently described a permeabilized cell assay (28) that measures nuclear export of NFAT, a transcription factor that shuttles between the nucleus and cytoplasm *in vivo* (42). The basic principle of the NFAT and PKI export assays is the same, since each assay measures nuclear efflux of NES-containing reporter proteins in a cytosol-dependent manner. There are, however, specific features that may influence which export factors are rate limiting in the two assays. For example, the PKI export assay requires a nuclear import step during which FITC-STV-NLS is loaded into nuclei of permeabilized cells. During this step of our assay, cytosolic Ran is likely to accumulate in the permeabilized cell nuclei (43), explaining why subsequent addition of recombinant Ran stimulates nuclear export only modestly in our system. In contrast, Ran is strongly rate limiting in the NFAT export assay (28). Potential advantages of the PKI export assay are that the genetically engineered version of streptavidin has no binding sites in the nucleus, export of streptavidin should not be subject to post-translational regulation, and export of the bPKI/FITC-STV-NLS complex is very rapid. The rapid

export in our system is likely due to the fact that PKI has a very efficient NES, bPKI binding to FITC-STV-NLS is predicted to be essentially irreversible ($K_d = 10^{-15}$ M), and several copies of bPKI can theoretically bind to each tetramer of FITC-STV-NLS.

Nuclear export of both PKI (Fig. 4*A*) and NFAT (28) in permeabilized cells is potently stimulated by the addition of Crm1, a recently described NES receptor. Our results show, however, that Crm1 is unlikely to be the only NES receptor in the cell. This was demonstrated by showing that inactivation or depletion of Crm1 from HeLa cytosol has no measurable effect on nuclear export of PKI. Moreover, cytosols prepared from tissues with very low levels of Crm1 support robust nuclear export of PKI. The low abundance of Crm1 in most tissues cannot be explained by an overall reduction in nucleocytoplasmic trafficking potential because tissue cytosols contain significant levels of the nuclear import factors, including Ran. Indeed, our hypothesis that Crm1 may not be the housekeeping NES receptor in all cells and tissues is supported by its highly heterogeneous expression measured by Northern blotting as well (44). The existence of more than one NES receptor would not be surprising, given the complexity of NLS receptors, with as many as five distinct but related α-importins expressed in a single species (ref. 45; see also ref. 46).

Is the cytosolic export activity identified in this study a novel NES receptor, or a positive regulator of Crm1? The latter could manifest itself in our assay by activating Crm1 that remains in the nucleus after digitonin permeabilization, and we cannot exclude the possibility that positive regulators of export may be present in our system. Nonetheless, our finding that the NES conjugate can deplete export activity from cytosol that lacks Crm1 indicates a role in NES recognition. Purification of this activity and its molecular analysis using the PKI export assay should clarify its specific function and contribute to our understanding of the export pathways used by proteins with the leucine-rich NES.

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