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RAPID COMMUNICATION

Adenoviral E1A Protein Nuclear Import Is Preferentially Mediated by Importin α3 *in Vitro*

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Nuclear import of some viral proteins depends on importin α proteins. However, no preferences of distinct α-importins for any viral protein import have been demonstrated. We used *in vitro* import assays and observed that all ubiquitously expressed human importin α isoforms mediate nuclear translocation of adenoviral E1A. Competition with nucleoplasmin suggests that importin α3 is the most efficient import mediator of E1A. © 2001 Academic Press

Introduction. Nuclear translocation of viral proteins is crucial for their specific functions. Like for other nuclear proteins, their transport across the nuclear pore complexes is generally active if they are larger than 20–30 kDa and requires both specific signals within these molecules and distinct soluble transport factors (for review see (*5*)). Several pathways mediate the import of macromolecules into the nucleus. Members of the importin β-like receptors are usually involved. The common feature of these receptors is the presence of a RanGTP-binding domain. During import, the receptor binds its cargo in the cytosol, an area of low RanGTP, and carries it through the nuclear pore. In the RanGTP-rich nucleoplasm, the import receptor binds to RanGTP, thereby triggering the release of its cargo. In some import pathways, importin β or another family member alone is sufficient to promote import. Other pathways rely on the presence of two different importin β-like molecules. Still others need an adaptor protein that links the cargo with the receptor. Most cellular proteins use the "classical" nuclear protein import pathway that is dependent on distinct nuclear localization signal sequences (NLSs) (for review see (*6*)). Here, the proteins bind to their receptor importin β not directly, but via importin αs (also termed karyopherin α or PTAC 58). Humans have one importin β but six importin α isoforms (*8, 9, 12, 19*). The various importin α isoforms are differentially expressed in various tissues and cells. However, whereas importins α1/Rch1, α3/Qip1, α4/hSRP1γ, α5/hSRP1, and α7 are ubiquitously expressed in various cells and tissues, importin α6 has been detected at the mRNA level only in the testis (*7–9, 12, 16*). The α importins appear to differ in their substrate-specific import efficiencies, which may provide an additional step for the control of the intracellular localization of proteins (*9, 11, 12, 14, 20*). Many viral proteins translocate into the nucleus via importin β without importin α or via unknown pathways. Other viral proteins use the classical importin α/β-dependent pathway (for review see (*21*)). However, to our knowledge, no import preferences of distinct importin α isoforms for any viral proteins have been demonstrated so far. We addressed the question of whether nuclear import of viral proteins can be mediated preferentially by a distinct importin α protein, as has been observed for mammalian proteins using adenoviral E1A as a model. Although the soluble factors mediating nuclear import of E1A have not been elucidated, a NLS that is both necessary and sufficient for E1A nuclear protein import located at the carboxy terminus is known (*10*). A second E1A-NLS that has been found to function in *Xenopus* oocytes is silent in somatic cells (*15*). Here we demonstrate that all ubiquitously expressed importin α proteins can mediate nuclear translocation of an E1A-NLS fusion protein *in vitro.* However, competition experiments with nucleoplasmin suggest that importin α3 is the most potent import mediator of E1A.

Results and Discussion. To investigate E1A nuclear import *in vitro* we used a fluorescein-labeled fusion of the carboxy terminal NLS to BSA that has been characterized by Cordes *et al.* (*1*). We performed *in vitro* nuclear import assays analyzing all ubiquitously expressed importin α isoforms, namely importins α1, α3, α4, α5, and α7, as described previously (*9*). All importin α proteins tested mediated nuclear translocation of E1A-NLS-BSA without major differences in the presence of importin β (Fig. 1A). When no importin α was added to the import reaction, no labeled E1A-NLS-BSA was detected in the nuclei of permeabilized cells. When nucleoplasmin was added as a second substrate to the import reaction, a

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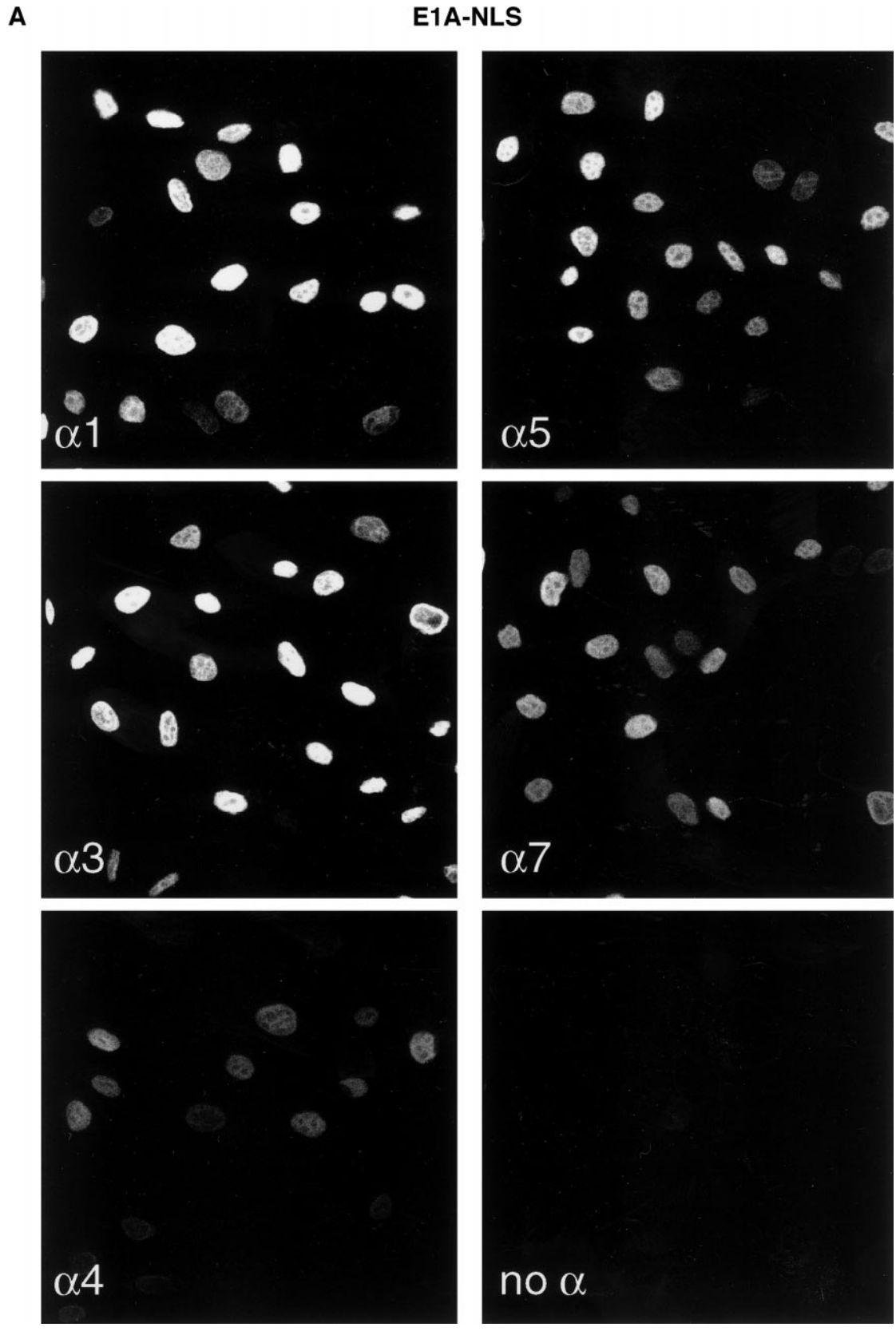

FIG. 1. Importin α-dependent nuclear import of adenoviral E1A-NLS-BSA protein in permeabilized HeLa cells. HeLa cells were grown on slides, permeabilized, and incubated with the import mixture. Reactions were stopped by fixation with 4% paraformaldehyde and coverslips were analyzed by confocal microscopy. The import reactions consisted of an energy-regenerating system, nucleoplasmin core buffer, reticulocyte lysate, and purified transport factors (RanGDP, Rna1p, RanBP1, NTF2, importin β, and the indicated importin α protein). Identical exposure times for each α-importin and negative control were used. (A) FITC-labeled E1A-NLS-BSA was added as the only substrate to the import reaction. (B) Texas Red-labeled nucleoplasmin (left) and FITC-labeled E1A-NLS-BSA (right) were both added for competition to the import reaction. α1, importin α1/Rch1; α3, importin

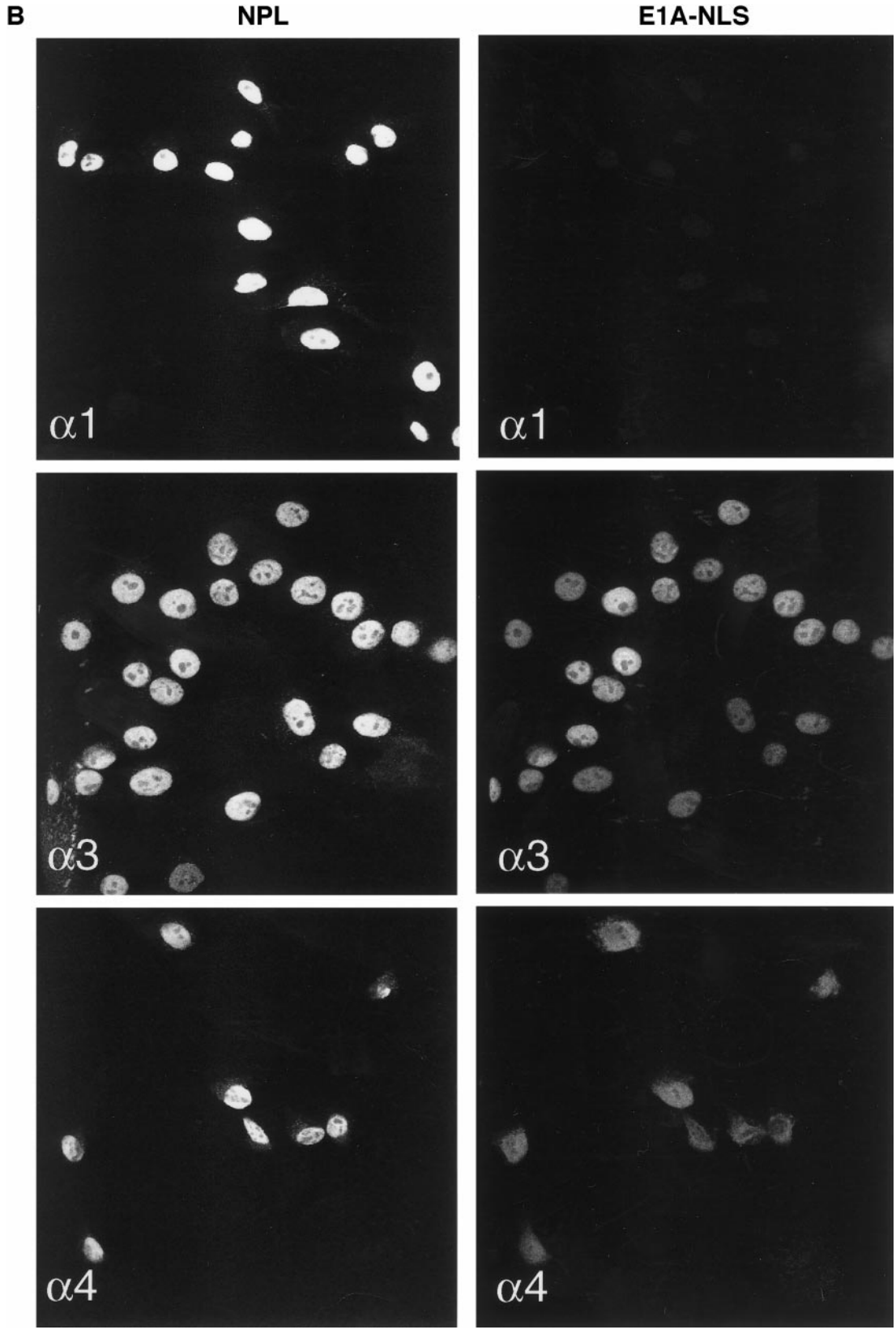

FIG. 1—*Continued*

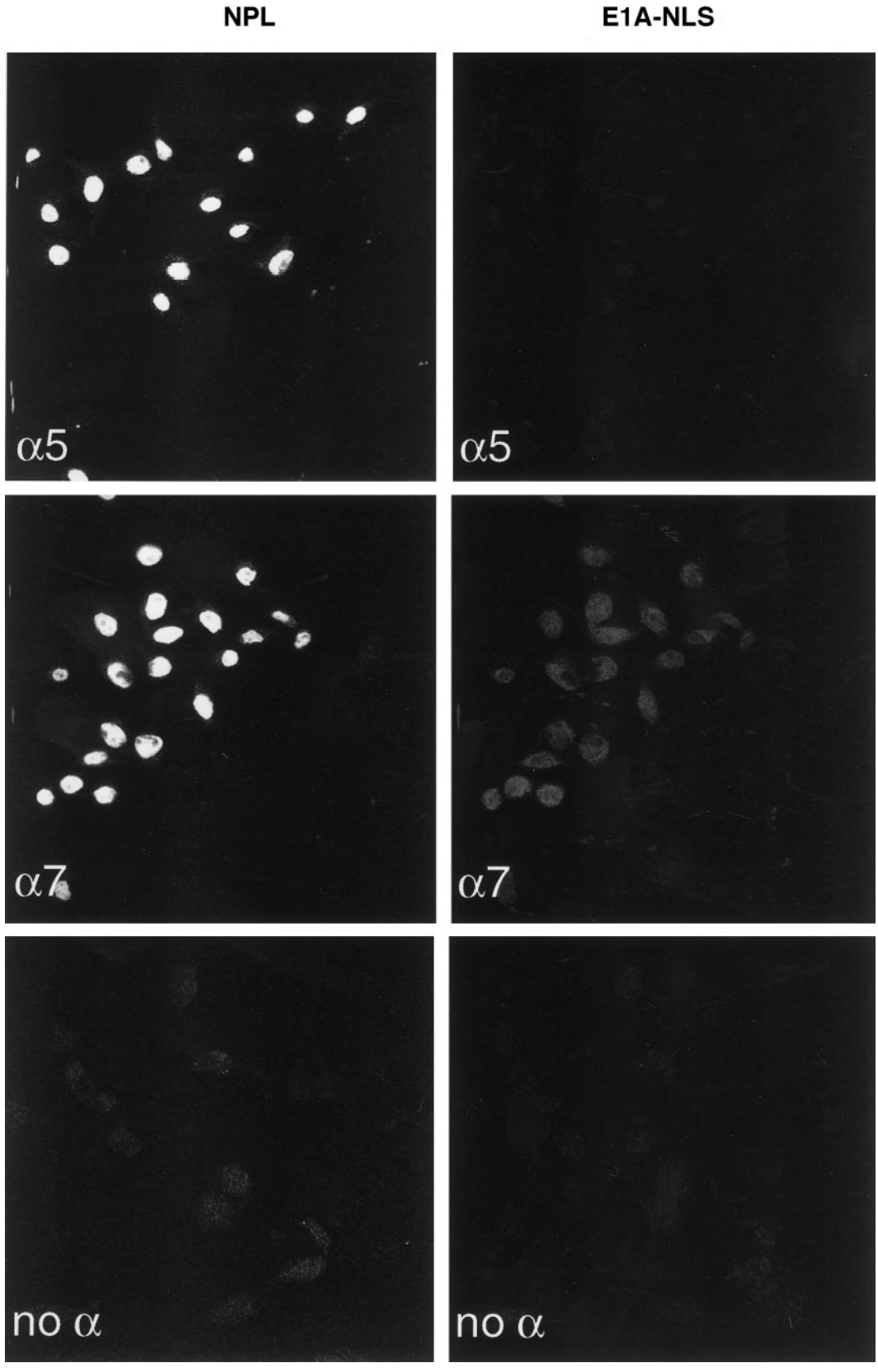

FIG. 1—*Continued*

strong inhibition of E1A nuclear accumulation for almost all importin α isoforms tested was observed (Fig. 1B). There were no differences between importins α1 and α5 and the negative control without any importin α added. Weak nuclear import of E1A in the presence of nucleoplasmin was detected for importins α4 and α7. Only importin α3 continued to mediate strong nuclear translocation of E1A. One could argue that the observed differences in E1A import efficiencies of the distinct α importins might be entirely due to varying relative affinities of nucleoplasmin for the α importins. However, we have shown previously that importin α3 is more efficient in nuclear transport of nucleoplasmin than importins α1 and α7 *in vitro* (*4, 9*). Furthermore, we also reported that nucleoplasmin shows no significant binding differences to the various importin α forms (*9*). Therefore, our data indicate that nuclear import of adenoviral E1A protein can be mediated by the classical importin α/β-dependent import pathway and that importin α3 is likely to be the most efficient import mediator for this substrate. Previously, binding studies analyzing only two of the different importin α isoforms suggested different efficiencies regarding nuclear translocation of the hepatitis δ antigen (*2*). However, to our knowledge, no substrate-specific differences of the transport efficiencies of the importin α proteins have been reported for other viral proteins. Interestingly, one of the nuclear E1A interacting proteins, namely P/CAF, is also translocated into the nucleus preferentially by importin α3 (*9*). Not all viral proteins that are imported via importin α/β necessarily display strong preferences toward a particular importin α isoform. Nuclear import of influenza viral (NP) protein has been shown to be mediated by importins α1 and α5 (*13*). Using a maltose-binding NP fusion protein described by Elton *et al.* we found that all importin αs can mediate nuclear import of NP *in vitro* (*3*). However, we could not detect a strong preference of a distinct importin α protein for NP (data not shown). Whether this result is due to the reported presence of several NLSs in the NP protein remains unresolved (*17, 18*). The specificity of E1A import suggests a special need for regulating its nuclear import.

Materials and Methods. In vitro nuclear import assays were performed as described previously (*9*). Briefly, HeLa cells were grown on microscopy slides (Roth) to 40–80% confluence, washed once in ice-cold phosphate-buffered saline (PBS), and permeabilized for 8 min with 30 μg/ml digitonin (Sigma). Permeabilized cells were incubated with 20 μl of import mixture for 8 min at room temperature. The import reactions were stopped by fixation with 4% paraformaldehyde in PBS and the slides were analyzed by confocal microscopy (MRC 1024, Bio-Rad). The import reactions consisted of an energy-regenerating system, core buffer, EGTA, 10% reticulocyte lysate, and the import factors RanGDP, Rna1p, RanBP1, NTF2, importin β, and one of the importin α proteins. Preparation and fluorescence labeling of recombinant proteins has been described (*9*).

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