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Cellular Requirements for CRM1 Import and Export

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We studied the cellular requirements for the translocation of CRM1 (exportin 1) between the nucleus and the cytoplasm. CRM1 import requires neither ATP, Ran, Ran-dependent GTP hydrolysis, nor a particular temperature. CRM1 and importin β compete with each other during their import. Thus, CRM1 is able to enter the nucleus in a manner similar to importinβ. In contrast, the *in vivo* export of CRM1 involves ATP-consuming step(s).

Key words: CRM1, importinβ, nuclear export, nuclear import.

Abbreviations: NES, nuclear export signals; NLS, nuclear localization signals; Impβ, importinβ; NPC, nuclear pore complex.

The nucleocytoplasmic transport of macromolecules occurs through the nuclear pore complex (NPC). While small molecules can passively diffuse through the NPC, the transport of molecules larger than 40–60 kDa must be facilitated by the transport machinery (*1*–*3*). The importinβ (Impβ) family (also called the karyopherin family) comprise the best studied transport receptors among the many classes of cargo carriers. This family include import receptors such as Impβ and transportin, and export receptors CRM1 and CAS (*3*, *4*). With regard to Impβ, it first binds to NLS-bearing cargo proteins in the cytoplasm either directly or *via* an adapter such as importinα (Impα). The importin-cargo complex then docks on the cytoplasmic face of the NPC. Following translocation through the NPC, the cargo and Impα are released from each other upon encountering RanGTP in the nucleus. The CAS-RanGTP:Impα and Impβ:RanGTP complexes then migrate from the nucleus back to the cytoplasm.

CRM1 mediates the export from the nucleus of numerous proteins carrying a nuclear export signal (NES) (*5*–*8*). CRM1 simultaneously associates with RanGTP and NES-bearing cargo proteins in the nucleus. The ternary CRM1-RanGTP-NES complex then interacts with the NPC and facilitates the transport of the cargo from the nucleus to the cytoplasm, after which the translocated complex dissociates through GTP hydrolysis of Ran. The empty CRM1 has been assumed to return to the nucleus for the next export cycle.

Recently, Ribbeck and Görlich (*9*) proposed that the FxFG repeats of nucleoporins form a hydrophobic permeability barrier within the NPC, and that the relatively hydrophobic nature of the transport receptors facilitates the partitioning and passage of the transport complex. Consequently, the transport receptors (with or without a cargo) are able to efficiently shuttle back and forth through the NPC. In agreement with this proposal, the translocation of the CRM1 export complex through the NPC to the cytoplasm does not require Ran-dependent GTP hydrolysis (*10*). Furthermore, free Impβ has been reported to migrate into or out of the nucleus through the NPC in a Ran-independent manner by directly binding to nucleoporins (*11*, *12*).

Although the above proposal is plausible, it is mainly based on data obtained with an *in vitro* transport assay involving permeabilized cells that lacked cellular components. It is possible, therefore, that the nucleocytoplasmic transport of transport receptors that occurs *in vivo* may be regulated in additional ways. Moreover, there appear to be differences among various importins in their translocation requirements. For example, while Impα migrates into the nucleus in an Impβ and Ran-independent manner, it probably does so *via* a different pathway from that used by Impβ (*13*). In addition, the import and export of transportin are temperature-dependent but do not need GTP hydrolysis (*14*).

Here we examined the cellular requirements for the import and export of CRM1 *in vivo* and *in vitro*, and demonstrate that CRM1 can be translocated into the nucleus in a temperature- and Ran-independent manner, the same mechanism as that used by free Impβ to migrate into the nucleus being used. However, the export of CRM1 requires ATP-consuming step(s) *in vivo*.

MATERIALS AND METHODS

Preparation of Recombinant Proteins—CRM1 was expressed by pET3a-CRM1 and purified as described previously (*15*). N-his importinβ was expressed by pQE-importin β (*16*) in *E.coli* strain BL21(DE3) Gold in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 15 h at 20°C. The *E. coli* cells were lysed in buffer A [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mg/ml of pefabloc, 1 μg/ml each of aprotinin, leupeptin, and pepstatin] containing 10 mM imidazole by sonication after 3 freeze-thaw cycles, and clarified by centrifugation at 100,000 ×*g* for 1 h. The supernatant was applied to a His-Trap Ni^+-chelating column (Amersham Pharmacia Bio-

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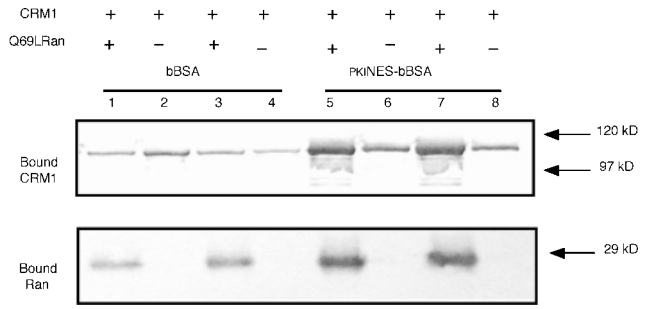

Fig. 1. **Interaction of CRM1 with immobilized PKINES in the presence of Q69LRanGTP.** CRM1 with or without Q69LRanGTP was exposed to avidin-agarose beads coated with biotinylated BSA or PKINES-BSA. The bound proteins were identified with anti-hCRM1 (upper) and anti-Ran (lower) antibodies. Lanes 1, 2, 5, and 6 in the upper panel, unlabeled CRM1; Lanes 3, 4, 7, and 8, Alexa-labeled CRM1.

tech), the trapped recombinant protein being eluted with buffer A containing 300 to 500 mM imidazole. The pooled fraction containing the His-importinβ protein was subjected to chromatography on a Hi-Trap Q column in a fast protein liquid chromatography system equilibrated with buffer A containing 10 mM imidazole and separated with buffer A containing a linear gradient of 50 to 500 mM NaCl. The separated recombinant protein was further applied to a Superdex200 column (Amersham Pharmacia Biotech) equilibrated with the transport buffer [20 mM Hepes (pH 7.3), 110 mM CH_3COOK, 5 mM CH_3COONa, 2 mM $(CH_3COO)_2Mg$, 0.5 mM EGTA, 2 mM dithiothreitol (DTT), and 1 μg/ml each of aprotinin, leupeptin, and pepstatin], and then concentrated by ultrafiltration using a Centricon YM10. Wild type RanGTP and non-hydrolysable mutant Q69LRanGTP were gifts from Dr. Yoneda, and were purified as described previously (*17–19*).

Fluorescein Labeling of Proteins—Purified CRM1 and Impβ were labeled with an Alexa 568 protein labeling kit, and FITC-BSA was prepared with a FluroReporter FITC labeling kit (Molecular Probes) following the manufacturer's instructions. All the labeled proteins were dialyzed against transport buffer at 4°C overnight, frozen in liquid nitrogen, and then stored at –80°C.

In Vitro Binding Assay—BSA or protein kinase inhibitor NES-conjugated BSA (PKINES-BSA) was biotinylated and prebound to immobilized avidin agarose beads, followed by 3 washes with the transport buffer without protease inhibitors. Unlabeled CRM1 or Alexa-labeled CRM1 (500 nM) was then incubated with the prebound BSA or PKINES-BSA substrate in the presence or absence of 2 μM Q69LRanGTP. The binding reaction was performed at 4°C for 2 h in the transport buffer containing 2 mM GTP, after then, the beads were rinsed with the transport buffer to remove the unbound proteins. The bound proteins were eluted and visualized by SDS-PAGE and immunoblotting.

In Vitro Import and Competition Assay—All the *in vitro* nuclear import and competition experiments were performed with digitonin-permeabilized Madin-Darby bovine kidney (MDBK) cells, as described previously (*13*, *20*, *21*). Pretreatment of the permeabilized cells with apyrase or wheat germ agglutinin (WGA) was performed as described previously (*22*). Preparation of the ATP regeneration system was described (*21*). The samples were observed by confocal laser scanning microscopy (Olympus Fluoview FV500).

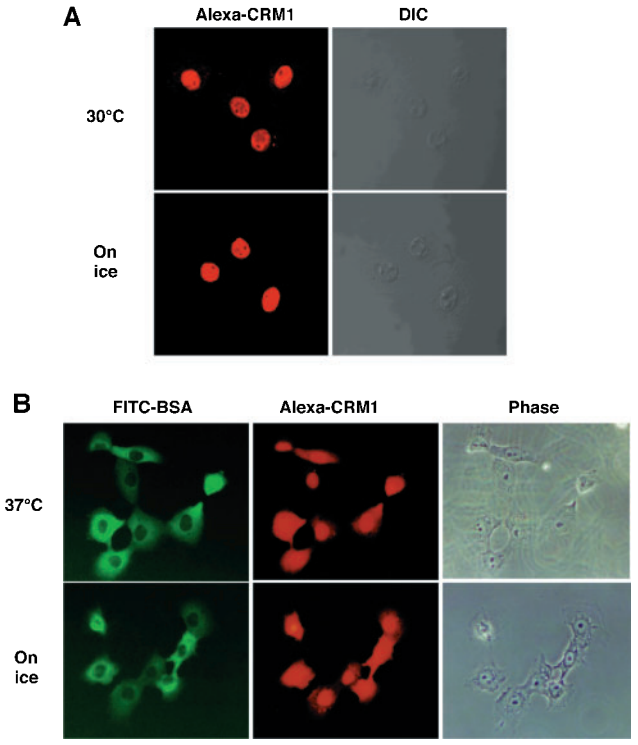

Fig. 2. **The nuclear import of CRM1 is temperature-independent.** A: The cell-free import assay with Alexa-CRM1 (1 μM) was performed either at 30°C (upper panel) or on ice (lower panel) for 30 min. B: Alexa-CRM1 (1 mg/ml) was microinjected together with FITC-BSA (1 mg/ml) into the cytoplasm of HEL cells. The cells were incubated at 37°C or on ice for 30 min before being injected.

Cell Fusion, ATP Depletion, and Leptomycin B (LMB) Treatment—Human embryonic lung (HEL) cells were fused by the hemagglutinating virus of Japan (HVJ) as described (*23*), except that they were incubated further for 1–2 h at 37°C instead of at 39.5°C after exposure to the virus. To deplete ATP, the cells were incubated in glucose-free DMEM containing 10 mM NaN_3 and 6 mM 2-deoxyglucose at 37°C for 20 min before injection (*24*). After injection, the cells were further incubated at 37°C for 30 min in the drug-containing medium. For LMB pretreatment, LMB was added to the fresh medium to 10 nM during cell fusion and the ATP depletion procedure.

Microinjection—Alexa 568–labeled CRM1 and FITC-BSA were injected together into the cytoplasm or nucleus. After microinjection, the cells were incubated at 37°C or on ice for 30 min, and then washed twice with PBS and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. For the temperature-dependency experiment, the cells were pre-incubated on ice for 10 min before injection.

RESULTS

CRM1 Migrates into the Nucleus in a Temperature-Independent and WGA-Sensitive Manner—To characterize the nuclear import of CRM1, purified recombinant

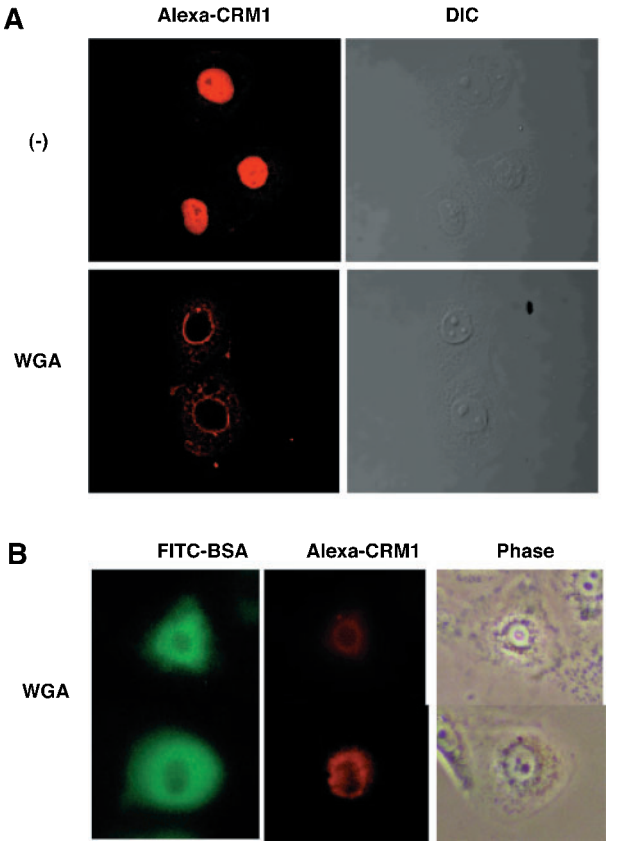

Fig. 3. **The nuclear migration of CRM1 is sensitive to WGA.** A: Permeabilized MDBK cells were pre-treated with 0.5 mg/ml WGA, and then the Alexa-CRM1 import reaction was performed in the presence of WGA at 30°C. B: Alexa-CRM1, FITC-BSA, and WGA (2.5 mg/ml) were co-injected into the cytoplasm of HEL cells.

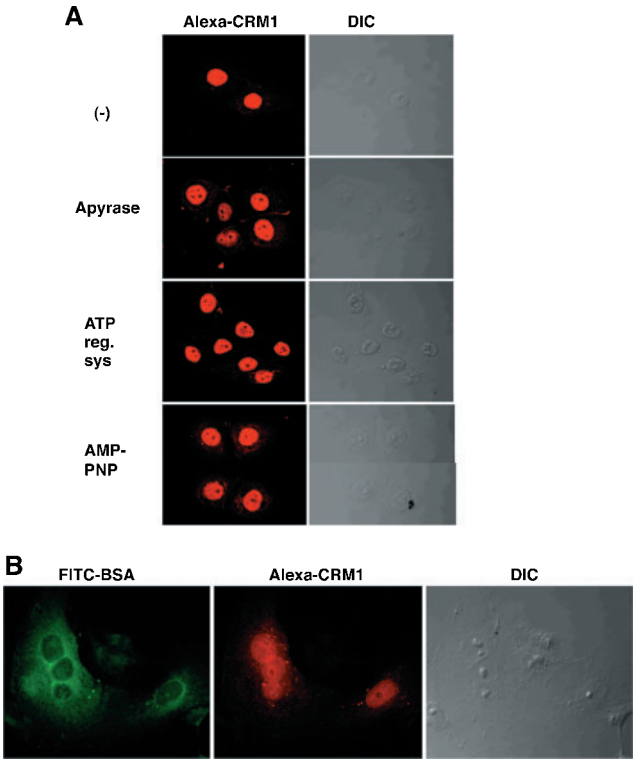

Fig. 4. **The nuclear import of CRM1 does not require ATP hydrolysis.** A: The cell-free import assay of Alexa-CRM1 was performed with cells that had been pretreated with apyrase (0.1 u/ml, 5 min at 30°C) or in the presence of an ATP regeneration system or AMP-PNP (1 mM). B: Alexa-CRM1 and FITC-BSA were coinjected into the cytoplasm of HEL homokaryons pre-treated with NaN_3 and 2-deoxyglucose.

CRM1 was labeled with Alexa 568 maleimide. Both the labeled and unlabeled CRM1 proteins were ascertained by SDS-PAGE to have the correct molecular masses (data not shown). To further confirm the functionality of our purified CRM1, we examined the ability of CRM1 to form a complex with RanGTP and the NES substrate by means of an *in vitro* binding assay. As expected, both unlabeled CRM1 and Alexa-labeled CRM1 associated with NES conjugated BSA in a RanGTP-dependent manner (Fig. 1). We then examined whether or not CRM1 can enter the nucleus by means of a cell-free import assay. As shown in Fig. 2A. Alexa-CRM1 can migrate into the nucleus in the absence of a cytosolic extract both at 30°C and on ice. To confirm this, we performed an *in vivo* study involving microinjection of Alexa-CRM1 plus FITC-BSA into the cytoplasm of HEL cells. We found that Alexa-CRM1 was imported into the nucleus within 30 min regardless of the temperature (Fig. 2B). These observations suggest that translocation of CRM1 into the nucleus is independent of temperature.

To rule out the possibility that CRM1 enters the nuclear through passive diffusion, we examined the effect of WGA on CRM1 translocation. WGA is known to block the nuclear transport of proteins through the NPC by binding to the *O*-linked GlcNAc residues of glycoproteins in the nuclear membrane. This effect of WGA does

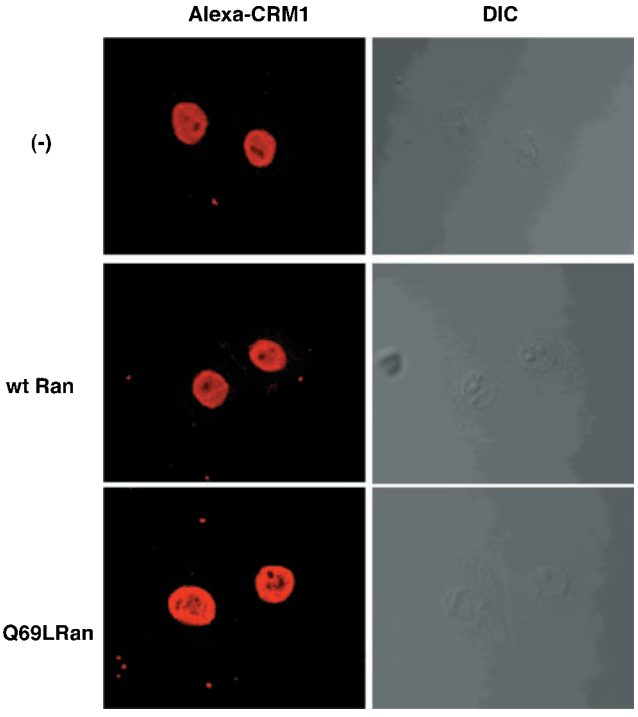

Fig. 5. **CRM1 can enter the nucleus in a Ran-unassisted manner.** The cell-free import assay of Alexa-CRM1 was performed on permeabilized cells together with wild type RanGTP or Q69LRanGTP.

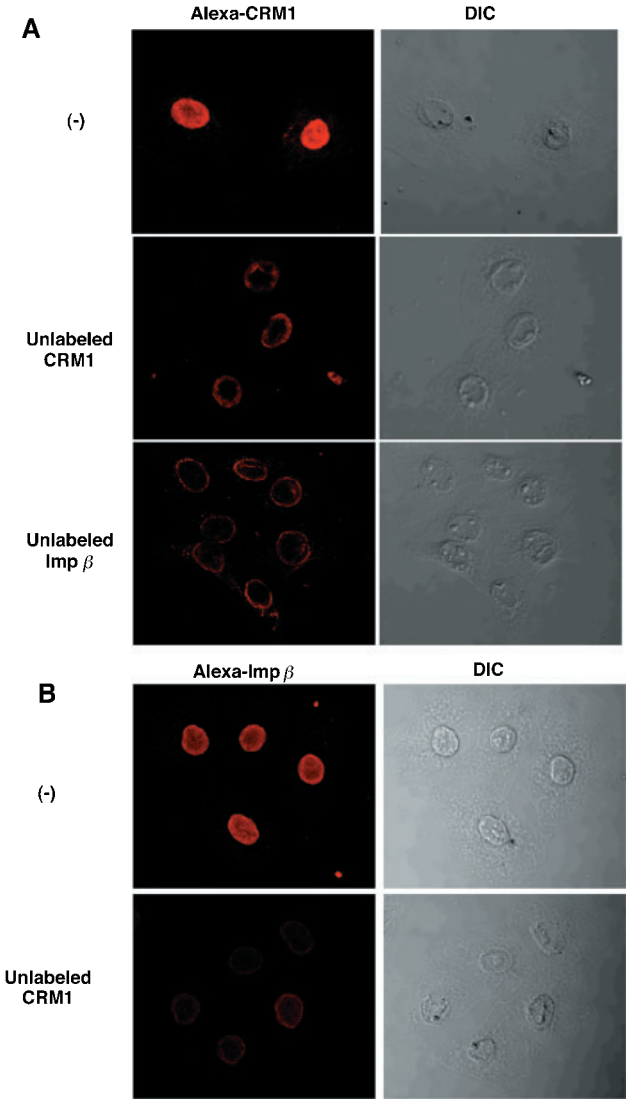

Fig. 6. **Nuclear import of CRM1 is saturable and can be competed by Impβ.** The Alexa-CRM1 (1 μM) (A) and Alexa-Impβ (1 μM) (B) import assay was performed in the presence of an excess amount (10 μM) of unlabeled CRM1 or unlabeled Impβ.

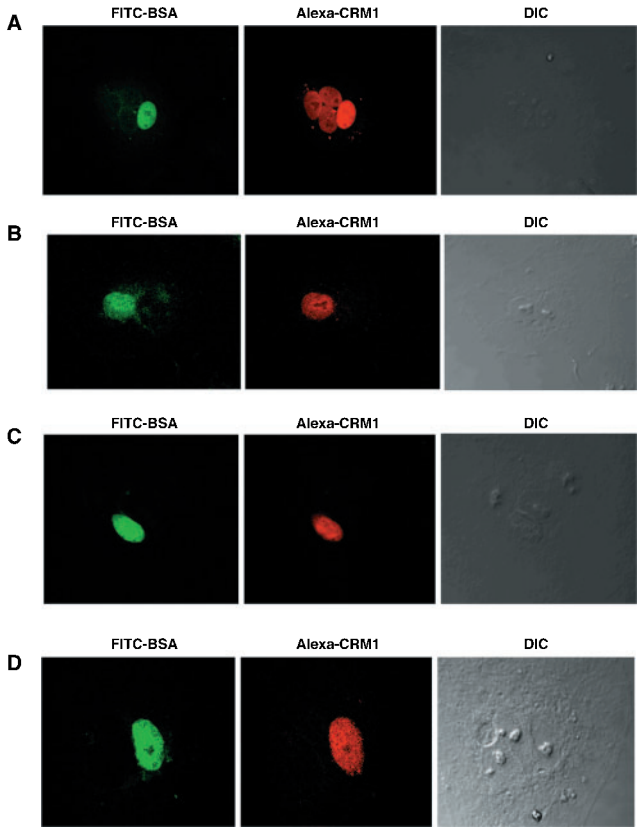

Fig. 7. **Export of CRM1 is sensitive to ATP depletion.** HEL homokaryons were pre-treated without (A) or with NaN_3 and 2-deoxyglucose (B–D), after which Alexa-CRM1 (1 mg/ml) alone (A, B, and D) or together with PKINES-BSA (2 μM) and RanQ69L (0.5 μg/ul) (C) was injected into one nucleus. In (D), the cell fusion and ATP depletion procedures were performed in the presence of LMB.

not block the passive diffusion of small molecules. As shown in Fig. 3A. the migration of CRM1 into the nucleus in the *in vitro* import reaction was completely blocked by pre-incubation of the MDBK cells with WGA. Similarly, CRM1 injected into the cytoplasm of HEL cells was not able to accumulate in the nuclei in the presence of WGA (Fig. 3B), suggesting that the nuclear import of CRM1 occurs *via* an active translocation process and not through passive diffusion.

CRM1 Import Does Not Require Ran or ATP—We then examined whether or not the nuclear import of CRM1 is energy-dependent. In the *in vitro* import assay we found that the nuclear import of CRM1 was not affected by pre-treatment with apyrase, the addition of an ATP-regenerating system, or the presence of a non-hydrolysable analog of ATP, AMP-PNP (Fig. 4A). In contrast, the nuclear import of the SV40-NLS substrate can only be achieved upon addition of an ATP regenerating system (data not shown). Furthermore, in ATP-depleted HEL homokaryons, cytoplasmically injected CRM1 accumulated in all the nuclei of the homokaryon (Fig. 4B). These data indicate that CRM1 can migrate into the nucleus in an ATP-independent manner.

We next investigated the involvement of Ran in the nuclear translocation of CRM1. We thus added wild type RanGTP or non-hydrolysable mutant Q69LRanGTP to the *in vitro* import assay mixture. However, neither form of Ran affected the movement of CRM1 into the nucleus (Fig. 5), suggesting that Ran is not involved in the nuclear import of CRM1. The import of the SV40-NLS substrate has been demonstrated to be inhibited by the addition of Q69LRanGTP (data not shown and Ref. *13*). Therefore, the import pathway for CRM1 is obviously different from the classical NLS-Impβ/α pathway.

Importinβ Competes with CRM1 on Nuclear Migration—We further examined the saturability of CRM1 import by adding 10 times more unlabeled CRM1 than labeled CRM1. We observed that most of the Alexa-CRM1 was located at the periphery of the nucleus (Fig. 6A), which indicates that the nuclear import of CRM1 was competitively inhibited. Furthermore, the excessive addition of unlabeled Impβ also greatly decreased the nuclear accumulation of CRM1 (Fig. 6A). Inversely, unlabeled CRM1 could competitively block the nuclear import

of Alexa-Impβ to a similar extent (Fig. 6B). These results indicate that CRM1 may target the same site on the NPC as Impβ or at least share some components of NPC with Impβ when they migrate into the nucleus.

CRM1 Export Is Sensitive to ATP Depletion—We investigated whether or not the CRM1 protein can shuttle between the nucleus and cytoplasm. To assess this, we injected Alexa-CRM1 into the nucleus of HEL homokaryon. Alexa-CRM1 was detected in all the nuclei of the homokaryons after 30 min incubation, which shows that CRM1 first exited the injected nucleus and then migrated from the cytoplasm into all the other nuclei (Fig. 7A).

Next, we investigated the energy requirement of CRM1 export *in vivo*. In contrast to its import, nuclear-injected Alexa-CRM1 remained in the nucleus of ATP-depleted homokaryon, which indicates that in the absence of ATP, the export of CRM1 from the nucleus is severely blocked (Fig. 7B). Since ATP depletion could reduce the *de novo* synthesis of GTP and thus have an effect on CRM1 export only indirectly by affecting RanGTP function (*24*), we injected wild type RanGTP or Q69LRanGTP, together with PKINES-BSA and Alexa-CRM1, into the nucleus of one ATP-depleted HEL homokaryon. However, neither of these conditions restored CRM1 export (Fig. 7C and data not shown), which suggests that an ATP-consuming step is apparently needed for certain steps of CRM1 export *in vivo.* To preclude the possibility that the injected CRM1 might form the cargo-CRM1-RanGTP complex in the nucleus, we treated the homokaryons with LMB throughout cell fusion and the ATP depletion procedure. Then we injected Alexa-CRM1 into the nucleus of one ATP-depleted homokaryon as above. However, this treatment with LMB did not affect the inhibition of CRM nuclear export upon ATP depletion, indicating that "free CRM1" requires ATP to exit the nucleus through the NPC.

DISCUSSION

Our results demonstrate that free CRM1 without a cargo can migrate into the nucleus in the same manner as free Impβ. This supports the hypothesis that empty CRM1 is recycled back after releasing its cargo. As with free Impβ, we found that CRM1 is able to migrate into the nucleus in the absence of other soluble factors, and that the efficiency of migration was not affected when chilled permeabilized cells or pre-cooled living cells were used (Fig. 2, A and B). In addition, like the import of free Impβ, neither RanGTP nor GTP hydrolysis is required for the nuclear accumulation of CRM1. We also found that CRM1 and Impβ inhibit each other's migration into the nucleus (Fig. 6, A and B), indicating that CRM1 and free Impβ may interact with the same NPC component with similar efficiency when being imported into the nucleus. The mutual inhibitory effect of CRM1 and Impβ on their import contrasts strongly with the one-way competitive inhibition of β-catenin by Impβ (*22*), and the inability of Impα and Impβ to inhibit one another (*13*). Thus, our observations together indicate that CRM1 is imported into the nucleus in the same manner as free Impβ.

We found that the import of CRM1 into the nucleus is not dependent on ATP as CRM1 injected into the cytoplasm of ATP-depleted homokaryons could migrate into all the nuclei in these cells. In addition, in the permeabilized cell-free import assay, pretreatment with apyrase or the addition of non-hydrolysable ATP analog AMP-PNP did not inhibit the import of CRM1 (Fig. 4A). In contrast, the export of nuclear CRM1 into the cytoplasm is dependent on ATP as the export of CRM1 injected into the nucleus of one HEL homokaryon greatly decreased upon ATP depletion (Fig. 7, B and C). Similar results with Impβ have been reported, which suggests ATP-dependent export accompanied by ATP-independent import may be a general feature of the nucleocytoplasmic translocation of the karyopherinβ family (*11*). Schwoebel *et al.* have reported a decrease in the free GTP concentration caused by the absence of ATP (*24*). However, when we co-injected CRM1, wild type RanGTP (or Q69LRanGTP), and PKINES-BSA into the nucleus of ATP-depleted HEL cells, the export of CRM1 was not restored. Furthermore, the treatment of ATP-depleted homokaryons with LMB did not alter the ATP requirement for the export of CRM1. Thus, ATP may be necessary for the translocation of CRM1 to the cytoplasm *in vivo*. The proposal by Ribbeck and Görlich (*9*) suggests that the translocation of transport receptors across the NPC does not require energy or RanGTP hydrolysis. It is also known that the formation of the CRM1-NES-RanGTP complex does not require energy. Taking these and our observations together, we propose that ATP may be required to help nuclear- but not cytoplasmically-located CRM1 move towards the NPC *in vivo*. For instance, the energy may be needed to maintain the appropriate structure of the nucleus *en route* to the NPC. It is also possible that CRM1 injected into the nucleus is retained by certain nuclear proteins and that its release is ATP-dependent. Since the hnRNP C protein has been reported to contain a nuclear retention sequence (NRS) that can override NES (*25*), it may be interesting to search for a NRS in CRM1. Further investigation of the ATP-dependent step(s) involved in the exiting of CRM1 from the nucleus will provide a greater insight into the mechanisms regulating the nucleocytoplasmic translocation of the karyopherinβ family.

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