Distinct energy requirement for nuclear import and export of importin β in living cells

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Abstract Importin β can shuttle between the nucleus and cytoplasm through the nuclear pore complex (NPC). This study deals with the issue of how the energy is utilized during the NPC passage of importin β. In chilled or ATP-depleted cells, importin β was transported into the nucleus, while the nuclear export of importin β was inhibited. Further, it was found that the nuclear export inhibition of importin β is not due to nuclear retention via binding to nucleoporins or nuclear importin α. These data show that the nuclear export of importin β involves energy-requiring step(s) in living cells.

Key words: Importin; Nuclear import; Nuclear export; Nuclear pore complex

1. Introduction

Transport of molecules between the nucleus and cytoplasm proceeds through the nuclear pore complex (NPC) [1–3]. Molecules smaller than ~60 kDa in size can passively diffuse through aqueous channels of the NPC. However, most macromolecules are thought to be selectively transported by an active, receptor-mediated mechanisms.

Members of the importin β family of transport receptors mediate NPC passage of cargoes through the interaction with nucleoporins and a small GTPase Ran. Ran functions as a molecular switch by exchanging between a GDP- and a GTP-bound state [4]. This Ran GDP/GTP cycle is regulated by a nuclear guanine nucleotide exchange factor, RCC1 [5], and a cytoplasmic GTPase-activating factor, RanGAP1 [6,7]. The asymmetric distribution of these regulatory factors predicts that nuclear Ran is mainly the GTP-bound form and that cytoplasmic Ran is the GDP-bound form. This concentration gradient of RanGTP between the nucleus and the cytoplasm ensures the release site of cargoes from the transport receptors [8,9]. Importin β binds to a classical nuclear localization signal (NLS) substrate/importin α complex in the cytoplasm, and then, this ternary complex dissociates by binding RanGTP to importin β in the nucleus [8,10,11].

The passage of classical NLS-containing proteins through the NPC is generally believed to be energy-dependent. However, it is unclear how the energy is utilized during the NPC passage. On the other hand, we previously reported that importin β alone can shuttle between the nucleus and cytoplasm through the NPC, depending on its NPC binding domain [12]. In this study, we show in vivo evidence that an energy supply is required for the nuclear export, but not the import, of importin β.

2. Materials and methods

2.1. Cell cultures

BHK21 cells were incubated in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C.

2.2. Recombinant proteins

Expression and purification of recombinant green fluorescent protein (GFP)-importin β, GFP-145–449aa, and RanGTP were performed as described previously [12].

2.3. Preparation of NLS or nuclear export signal (NES) substrates

Texas red (TR)-labeled bovine serum albumin (BSA) was chemically conjugated to the synthetic peptides containing the NLS of SV40 T-antigen (CYGGPKKKRKVEDP) or the NES of Rev (CLQLPPLERLTLD) as described previously [12,13].

2.4. Cell fusion and ATP depletion

BHK21 cells were fused by the hemagglutinating virus of Japan (HVJ, Sendai virus) as described previously [14]. In ATP depletion experiments, BHK21 cells or homokaryons were preincubated for 20 min in Hanks' balanced salt solutions with 10 mM NaN_3 and 6 mM 2-deoxyglucose at 37°C. After microinjection, the cells were incubated in the drug-containing medium at 37°C.

2.5. Microinjection

Cultured cells were grown on coverslips for 48 h at 37°C prior to use in microinjection experiments. Microinjection was performed as described previously [15]. After microinjection, the cells were incubated and fixed with 3.7% formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.2) for 30 min at room temperature. The injected fluorescent-labeled proteins were detected by Axiophot 2 microscopy (Carl Zeiss, Inc.).

3. Results and discussion

The goal of this study was to determine the energy requirement for nucleocytoplasmic transport of importin β. An initial experiment involved an experiment to determine if the nuclear import of importin β is temperature-dependent or not in living cells. As reported previously, cytoplasmically injected recombinant GFP-fused importin β proteins rapidly migrate into the nucleus of mammalian BHK21 cells, which are incubated at 37°C [16]. When the BHK21 cells were preincubated

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Abbreviations: GFP, green fluorescent protein; HVJ, hemagglutinating virus of Japan; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; RanGTP, GTP-bound form of Ran; TR, Texas red

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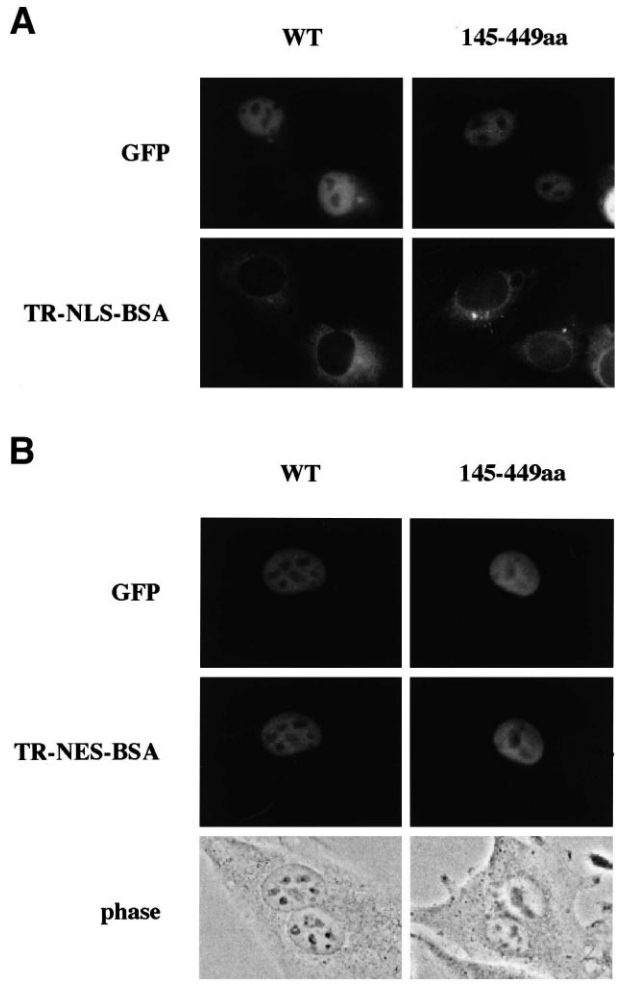

Fig. 1. Nuclear import of importin β is temperature-independent, but its nuclear export is temperature-dependent. A: After BHK21 cells had been preincubated for 15 min on ice, GFP-importin β (25 μM) or GFP-145–449aa (25 μM) together with TR-labeled NLS substrate (TR-NLS-BSA) (10 μM) was injected into the cytoplasm. Injected cells were incubated for 20 min on ice, and then fixed with 3.7% formaldehyde in PBS. B: After BHK21 homokaryons fused by HVJ were preincubated for 15 min on ice, GFP-importin β (25 μM) or GFP-145–449aa (25 μM) together with TR-labeled NES substrates (TR-NES-BSA) (10 μM) was injected into a nucleus of the homokaryons. The injected cells were incubated for 20 min on ice, and then fixed with 3.7% formaldehyde in PBS.

for 15 min on ice and the recombinant GFP-importin β proteins were then injected into the cytoplasm with T antigen-NLS substrates, importin β, but not the NLS substrates, was detected in the nucleus after a further incubation for 20 min on ice (Fig. 1A). We next examined whether the nuclear export of importin β occurs in the chilled cells. When the BHK21 cells fused with the HVJ were preincubated on ice and GFP-importin β proteins were then injected into the nucleus, the injected GFP-importin β was largely detected only in the injected nucleus after incubation for 20 min on ice (Fig. 1B). These results indicate that, while the nuclear import of importin β is temperature-independent, its nuclear export is temperature-dependent. It has previously been demonstrated that importin β shuttles between the nucleus and cytoplasm through the NPC, depending on its NPC binding domain (corresponding to amino acids 145–449) [12]. We therefore examined the issue of whether nucleocytoplasmic shuttling of the deletion mutant containing the NPC binding region (amino acids 145–449) is temperature-dependent or not. As expected, and similar to the case of wild type importin β, the deletion mutant (145–449 aa) migrated into the nucleus of chilled cells, but was not exported from the nucleus to cytoplasm under these conditions (Fig. 1A,B).

To further assess whether the nucleocytoplasmic shuttling of importin β is an active process or not, ATP depletion experiments were performed. As shown in Fig. 2A,B, the nuclear import of NLS substrates was prevented in BHK21 cells which had been pretreated with an inhibitor of ATP synthesis. In contrast, importin β and its deletion mutant (145–449 aa) migrated into the nucleus under the same assay conditions (Fig. 2A,B). Nuclear import of the NLS substrates was recovered when the ATP-depleted cells were shifted into drug-free medium (Fig. 2A,B), indicating that this inhibition is reversible and that the cells were alive and viable. In order to determine if the nuclear export of importin β occurs in the ATP-depleted cells, the BHK21 homokaryons were pretreated with the drug-containing medium. As previously reported, the nuclear export of leucine-rich NES-containing proteins was inhibited in the ATP-depleted cells [17]. Furthermore, as shown in Fig. 2C,D, as in the case of TR-NES-BSA, the nuclear export of both importin β and its deletion mutant (145–449 aa) was blocked. From these findings, it is clear that the energy requirement for the export of importin β through the NPC is quite different from that for its import.

The possibility remains that the nuclear export of importin β may be prevented as the result of its binding to nucleoporins or nuclear importin α. Since it has been shown that importin β dissociates from nucleoporins or importin α by binding of RanGTP to importin β [8,10,18], importin β proteins together with recombinant RanGTP were injected into the ATP-depleted cells, in order to exclude this possibility. As shown in Fig. 3, nuclear injected GFP-importin β was largely detected only in the injected nucleus even when co-injected with RanGTP, suggesting that the inhibition of the export of importin β from the nucleus in the ATP-depleted cells is not due to nuclear retention as a result of binding to nucleoporins or nuclear importin α.

As shown in Figs. 1 and 2, importin β migrates into the nuclei of chilled or ATP-depleted cells, suggesting that the nuclear import of importin β is independent of incubation temperature and ATP levels. Furthermore, the addition of GTPγS or apyrase does not inhibit the import of importin β, as evidenced by the digitonin-permeabilized cell-free transport assay (data not shown). These in vivo and in vitro results show that neither ATP nor GTP hydrolysis is required for the nuclear import of importin β.

In contrast, our present data show that the nuclear export of importin β involves energy-requiring step(s) in living cells. There are several possible explanations for this. Since it is likely that the nuclear import of importin β is a facilitated transport process, the apparent accumulation of importin β in the nucleus would be expected to be the result of nuclear retention, which leads to the possibility that energy may be required for the dissociation of importin β from intranuclear binding proteins. Fig. 3 rules out the possibility that the inhibition of nuclear export of importin β, which is observed in the ATP-depleted cells, is due to the binding of importin β to nucleoporins or importin α, but the possibility that nuclear

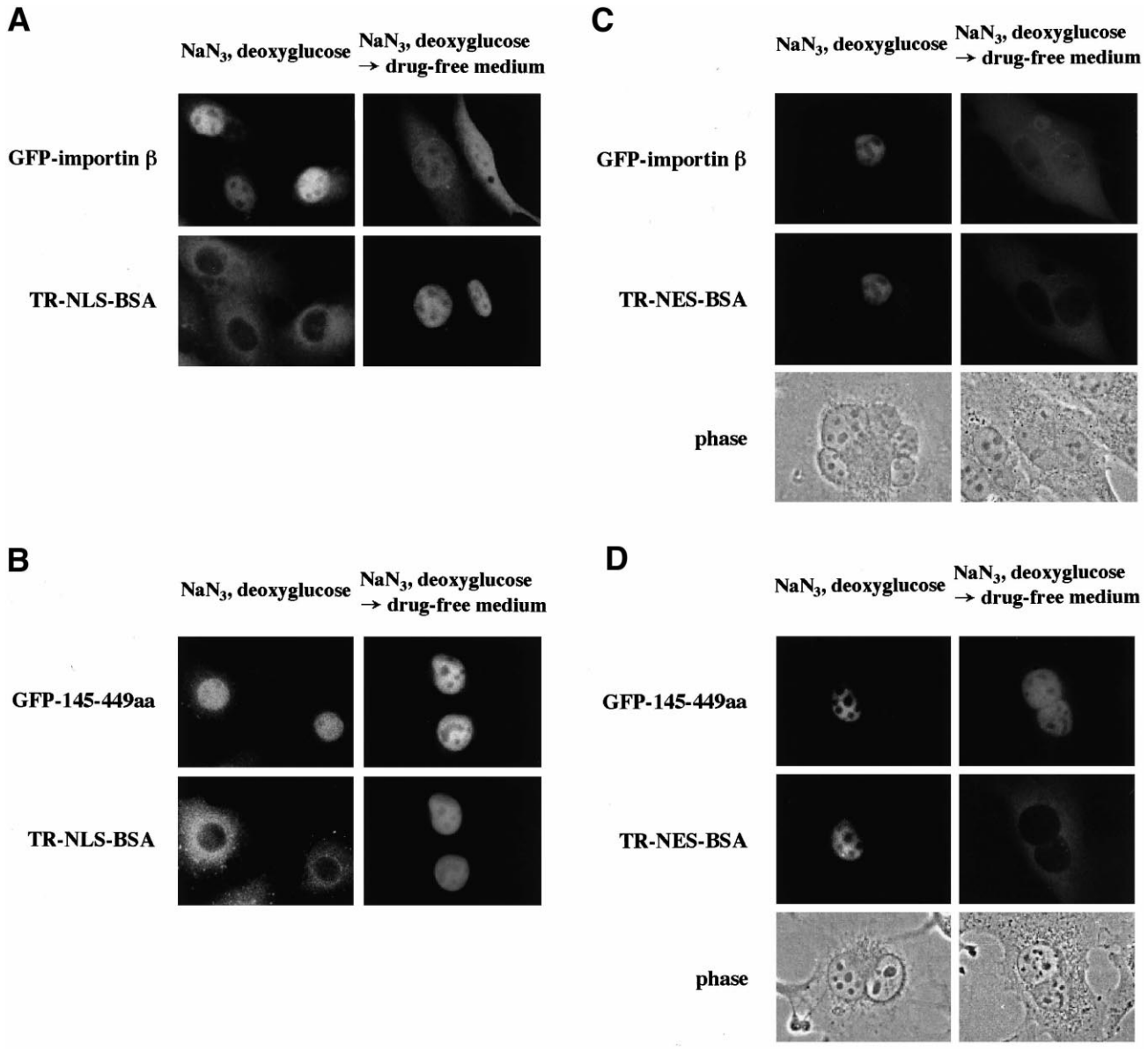

Fig. 2. Nuclear import of importin β is not inhibited in ATP-depleted cells, but nuclear export is arrested. A, B: BHK cells were preincubated for 20 min with Hanks' balanced salt solution with 10 mM NaN_3 and 6 mM 2-deoxyglucose. GFP-importin β (25 μM) (A) or GFP-145–449aa (25 μM) (B) together with TR-NLS-BSA (10 μM) was injected into the drug-treated cells. After incubation for 20 min in the drug-containing medium, the cells were fixed with 3.7% formaldehyde in PBS. To demonstrate the reversibility of the inhibition, immediately after injection the energy-depleted cells were returned to fresh drug-free DMEM supplemented with 5% FBS, and then incubated for 30 min at 37°C. C, D: After BHK21 cells were fused by HVJ, the homokaryons were treated as described above. GFP-importin β (25 μM) (C) or GFP-145–449aa (25 μM) (D) together with TR-NES-BSA (10 μM) was injected into a nucleus of the drug-treated homokaryons.

export of importin β is inhibited by the binding of intranuclear proteins other than nucleoporins or importin α cannot be excluded by these data.

Alternatively, energy could be required for the function of the NPC with respect to the NPC passage of importin β. Atomic force microscopy showed that a structural change of the NPC is induced by ATP or calcium [19,20], suggesting that the structure of NPC could well change in the case of chilled or ATP-depleted cells. Thus, the inhibition of nuclear export of importin β may be due to a structural change in the NPC as the result of energy depletion. It will be interesting to examine whether the NPC passage of importin β is mediated by some components of the NPC which are sensitive to ATP.

It has been recently reported that signal-mediated nuclear import requires neither NTP hydrolysis nor GTP hydrolysis by Ran [16,21–25]. Thus, the importin β family can be translocated through the NPC from the cytoplasm to the nucleus without NTP hydrolysis, either in the cargo-complexed form or in the cargo-free form. Although the exact mechanism of the NPC passage of importin β is not understood at present, the observations contained in this paper indicate that the nuclear export process of importin β is much more sensitive to energy depletion than the nuclear import process of the same protein. This suggests that there may exist a mechanism in cells to recruit importin β actively in the cytoplasm where the import reaction initiates. Further studies concerning the energy requirement for the nuclear export of import β will likely provide clues to the elucidation of the mech-

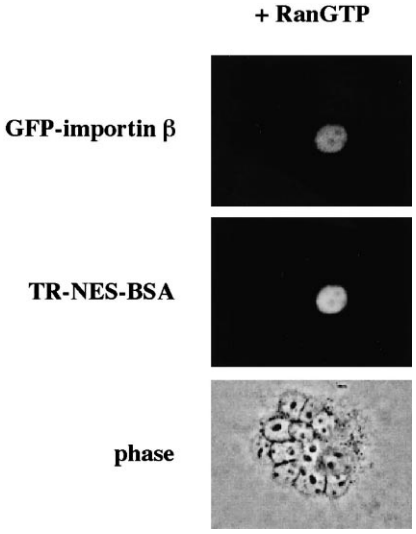

Fig. 3. Nuclear injected RanGTP does not permit the recovery of the inhibition of nuclear export of importin β in ATP-depleted cells. After BHK21 cells were fused by HVJ, the homokaryons were treated as described in Fig. 2. GFP-importin β (25 μM) together with RanGTP (17 μM) and TR-NES-BSA (10 μM) was injected into a nucleus of the drug-treated cells.

anism for the NPC passage of macromolecules, as well as the mechanism for regulation of the recycling of transport receptors.

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