The 70-kD heat shock cognate protein (hsc70) facilitates the nuclear export of the import receptors

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Transport receptors of the importin β family continuously shuttle between the nucleus and cytoplasm. We previously reported that the nuclear export of importin β involves energy-requiring step(s) in living cells. Here, we show that the in vitro nuclear export of importin β also requires energy input. Cytosol, depleted of ATP-binding proteins, did not support the sufficient nuclear export of importin β. Further purification revealed that the active component in the absorbed fraction was a 70-kD heat shock cognate protein (hsc70). The addition of recombinant hsc70, but not an ATPase-deficient hsc70 mutant, to the depleted cytosol restored the export activity. In living cells, depletion of hsc70 caused the significant nuclear accumulation of importin β. These effects of hsc70 were observed in the nuclear export of importin β, but also for other import receptors, transportin and importin α. These results suggest that hsc70 broadly modulates nucleocytoplasmic transport systems by regulating the nuclear export of receptor proteins.

Introduction

Communication between the nucleus and cytoplasmic compartments of eukaryotic cells is mediated by the nuclear pore complex (NPC), which spans the nuclear envelope (Rout and Aitchison, 2001; Suntharalingam and Wente, 2003). The NPC functions as a highly selective molecular sieve. Molecules smaller than 40–60 kD (or 9 nm in diameter) are able to diffuse passively through the NPC, whereas larger molecules are translocated between the nucleus and cytoplasm by active or facilitated receptor-mediated mechanisms.

Members of the importin β family, importins or exportins (also termed karyopherins), recognize specific nuclear import or export signals of cargo molecules, and mediate nucleocytoplasmic transport by interacting with nucleoporins, components of the NPC (Görlich and Kutay, 1999; Imamoto, 2000; Bednenko et al., 2003). For example, importin β mediates the nuclear import of proteins bearing a basic NLS, which is recognized by an adaptor molecule, importin α. Transportin, an importin β–related receptor, mediates the import of proteins containing the glycine-rich M9 domain within hnRNP A1. These receptors circulate continuously between the nucleus and cytoplasm by a series of direct interactions with several nucleoporins, and translocate cargo molecules between the two compartments.

GTPase Ran regulates cargo-binding to transport receptors and confers directionality to the transport reaction of the cargo. The Ran GTPase cycle is regulated by the chromatin-associated nucleotide exchange factor, regulator of chromosomal condensation (RCC1), and the cytoplasmic RanGTPase-activating protein, RanGAP1, which results in a steep RanGTP gradient across the nuclear envelope. Importins bind to cargo molecules in the cytoplasm and are translocated through the NPC. The binding of RanGTP to importins in the nucleus cause the release of the import cargo. The importin/RanGTP complex recycles back to the cytoplasm, where RanGTP hydrolysis is stimulated by RanGAP1 and its cofactor, RanBP1. Nuclear transport factor 2 (NTF2) translocates RanGDP into the nucleus, where RanGDP is converted to RanGTP by RCC1. The binding of cargoes to exportins is regulated in a converse manner (Görlich and Kutay, 1999).

Nuclear transport in vivo is an energy-requiring process that can accumulate cargoes against a chemical gradient. This energy is produced from the chemical potential of the RanGTP gradient. However, many in vitro studies demonstrated that the NPC translocation step of transport is not coupled directly to nucleotide hydrolysis, and that a single round of import in vitro does not require any metabolic energy (Kose et al., 1997; Nakielny and Dreyfuss, 1998; Englmeier et al., 1999; Ribbeck et al., 1999). In contrast, multiple rounds of transport, which are necessary for recycling the transport receptors back to reload

cargoes, do require an input of energy. We previously presented in vivo evidence that an energy supply is required for the nuclear export, but not the import, of importin β (Kose et al., 1999). Further, the nuclear export of importin β was not restored by the nuclear injection of RanGTP in energy-depleted cells, and the importin β mutant lacking a Ran-binding domain showed an energy requirement for its nuclear export. These results led us to consider the possibility that the inhibition of the nuclear export of importin β in energy-depleted cells may not result solely from a shortage of RanGTP in the nucleus. As a result, we attempted to identify cellular factors, other than known proteins that are involved in the Ran system, that facilitate the nuclear export of importin β. Here we provide evidence to suggest that 70-kD heat shock cognate protein (hsc70) facilitates the nuclear export of import receptors, depending on its ATPase activity.

Results and discussion

To determine the energy requirement for the nuclear export of importin β, the nuclear export of GFP–importin β was monitored in the presence of cytosol with or without ATP in an in vitro transport assay (see Materials and methods). In the presence of ATP, importin β exits efficiently from the nucleus (Fig. 1 b), whereas the nuclear export of importin β was inhibited in the presence of apyrase (Fig. 1 a). A nonhydrolyzable ATP analogue, AMPPCP, did not support the nuclear export of importin β (unpublished data). Therefore, consistent with in vivo evidence that was reported previously (Kose et al., 1999), these results show that nuclear export of importin β requires energy input in an in vitro transport assay.

To identify the ATP-sensitive factor(s) required for the nuclear export of importin β, cytosol from Ehrlich ascites tumor cells initially was applied to an ATP–agarose column. As shown in Fig.1, c and d, neither the flow-through fraction, which we refer to as the depleted cytosol, nor the eluted fraction alone supported the nuclear export of importin β at the levels of starting cytosol. However, the combination of the depleted cytosol and eluted fractions reconstituted the nuclear export of importin β (Fig. 1 e); this indicated that the facilitating activity for nuclear export of importin β is present in the eluate from the ATP–agarose column.

Ran regulates the association and disassociation of importin β family receptors with cargo and nucleoporins, and is required for the recycling of importin β. The loss of export activity could be the result of depletion of Ran and its regulators by ATP–agarose. However, as shown in Fig. 2 B, Ran and its regulators (NTF2, RCC1) were present in the depleted cytosol at a level similar to that in the starting cytosol, which indicated that the Ran systems were not depleted by the ATP–agarose column.

To identify molecule(s), in addition to Ran or known regulators, that facilitate the nuclear export of importin β, the active component present in the ATP–agarose column eluate was purified using Phenyl Sepharose HP, and gel filtration on Superdex 200 (see Materials and Methods, and Fig. 2 A). When examined in an in vitro transport assay with a combination of the depleted cytosol, a fraction containing a protein with an apparent molecular mass of 70 kD (Fig. 2 A) restored the nuclear export activity of the depleted cytosol (not depicted). Two internal peptide sequences (RLIGRRFDDA and LYQSAGGMPG) were obtained from lysyl-endpeptidase fragments of the p70 protein that was excised from SDS-polyacrylamide gels. These peptide sequences were identical to the predicted amino acids 72–81 and 610–619 of the mouse hsc70. Immunoblotting of the purified p70 protein with anti-hsc70 antibodies further confirmed that this protein was hsc70 (Fig. 2 B).

To determine whether hsc70 facilitates the nuclear export of importin β, the activity of recombinant hsc70 was examined in an in vitro transport assay. As shown in Fig. 2 C, the nuclear export activity of importin β was increased significantly by the addition of recombinant hsc70 to the depleted cytosol.

Hsc70 is a constitutive member of the heat shock protein family and is implicated in a variety of cellular processes, including the folding of nascent chain polypeptides, or the translocation of proteins across membranes (Hartl, 1996). These functions generally depend on the peptide binding-release cycle, which is coupled to an ATPase cycle of hsc70. To determine whether the ATPase activity of hsc70 is necessary for facilitating the nuclear export of importin β, we constructed the ATPase-deficient hsc70 mutant protein (D10N), in which the Asn was substituted for Asp-10 (Huang et al., 1993). Hsc70 (D10N) was characterized as having no basal ATPase activity due to the perturbation of the precise position or electrostatic environment of a magnesium ion, which is critical to catalysis. This hsc70 (D10N) mutant protein did not facilitate the nuclear export of importin β (Fig. 2 C), which indicated that the ATPase activity of hsc70 is required for the export activity.

- ATP	+ ATP			
cyt.	cyt.	depleted cyt.	eluate	depleted cyt. + eluate

Imp-β
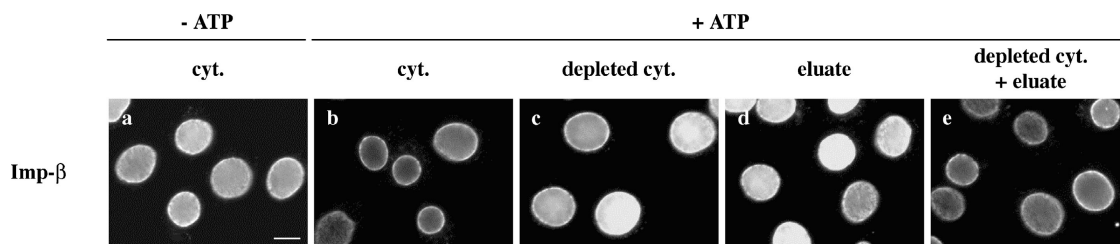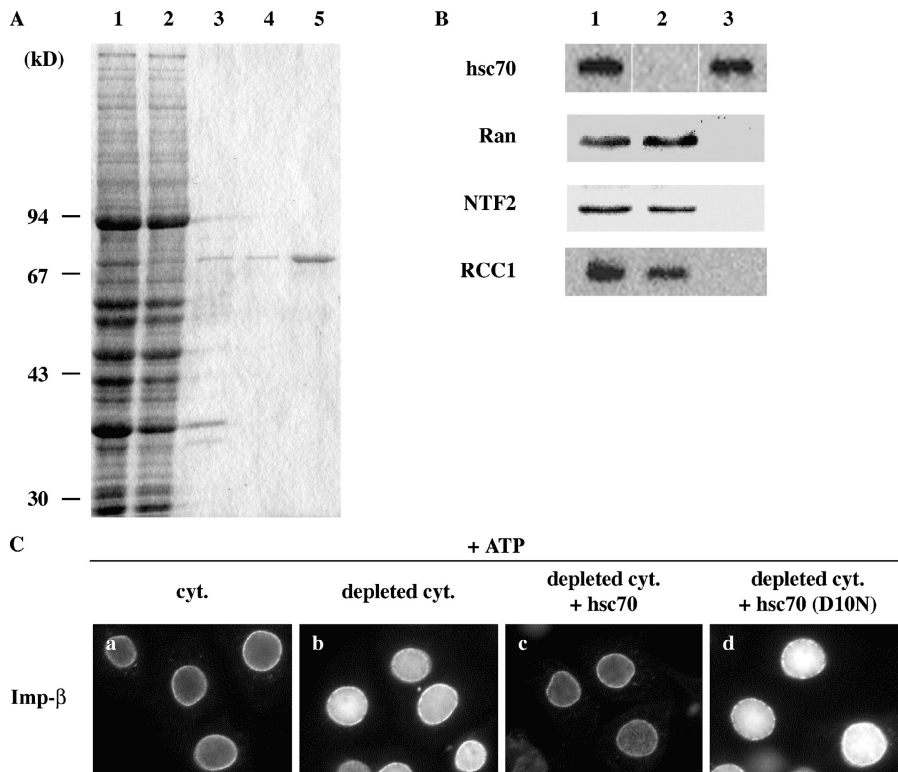
Figure 1. **Nuclear export of importin β is dependent on an energy source, and facilitated by ATP-binding proteins.** Digitonin-permeabilized cells were incubated with 0.3 μM GFP–importin β and reincubated with cytosol (cyt; a, b), the depleted cytosol (c), the eluate (e) from an ATP–agarose column, or the depleted cytosol and the eluate (e) in the presence of apyrase (a) or an ATP regeneration system (b–e). The addition of ATP-binding proteins to the depleted cytosol restored the nuclear export activity of importin β (e). Bar, 10 μm.

Figure 2. **Nuclear export of importin β is restored by the addition of hsc70 in an in vitro transport assay.** (A and B) Purification of hsc70 protein from total cytosol of Ehrlich ascites tumor cells. Total cytosol (lane 1), the flow-through (lane 2) and eluate (lane 3) from the ATP-agarose, the Phenyl Sepharose HP fraction (lane 4), and the Superdex 200 peak fraction (lane 5) were separated on 10% SDS-PAGE and subjected to Coomassie blue staining (A) or Western blotting probed with specific antibodies to indicated proteins (B). Ran, NTF2, and RCC1 were not depleted by ATP–agarose. (C) Digitonin-permeabilized cells were incubated with 0.3 μM GFP-importin β and were then reincubated with cytosol (cyt; a), or depleted cytosol (b–d) in the absence (a, b) or presence of 1 μM hsc70 (c) or hsc70(D10N) (d). Export reactions were performed in the presence of an ATP regeneration system.

Hsc70 is known to be localized in the cytoplasm and nucleus, and to shuttle between these two compartments (Mandell and Feldherr, 1990). Therefore, we examined the subcellular localization of hsc70 during an in vitro export assay. ECFP-hsc70, as well as unlabeled hsc70, facilitated the nuclear export of importin β (Fig. 3, b–d). During the export assay of importin β, a portion of the ECFP–hsc70 migrated into the nucleus of most of the cells (Fig. 3, e–g); however, the extent of nuclear migration was heterogeneous. Notably, the level of nuclear migration of

hsc70 and the export of importin β showed good correlation in this assay (i.e., a nucleus that contained more hsc70 showed lower levels of importin β). This suggests that the nuclear migration of hsc70 is important to support the export of importin β. In similar experiments, the ECFP–hsc70 (D10N) showed much more heterogeneity in its nuclear migrating activity, and the population of nuclei containing hsc70 (D10N) was decreased substantially (Fig. 3, k–m). Regardless of its nuclear migration, hsc70 (D10N) failed to support the nuclear export of importin β (Fig. 3, h–j).
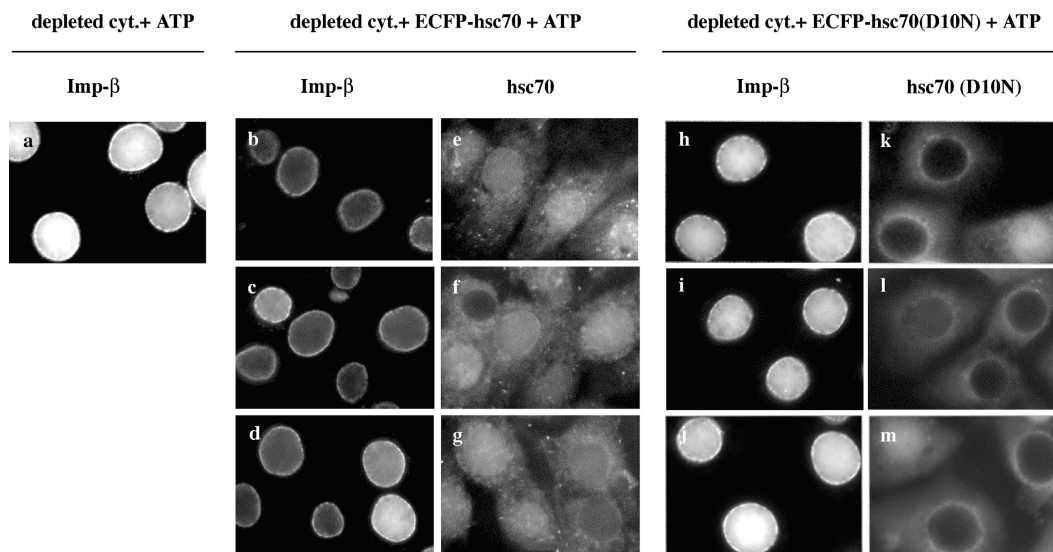
Figure 3. **Nuclear accumulation of hsc70 during in vitro importin β export assay.** Digitonin-permeabilized cells were incubated with 0.3 μM EYFP–importin β and were reincubated with cytosol (cyt; a), or depleted cytosol (b–m) in the absence (a) or presence of 1 μM ECFP–hsc70 (b–g) or ECFP-hsc70(D10N) (h–m). Export reactions were performed in the presence of an ATP regeneration system.
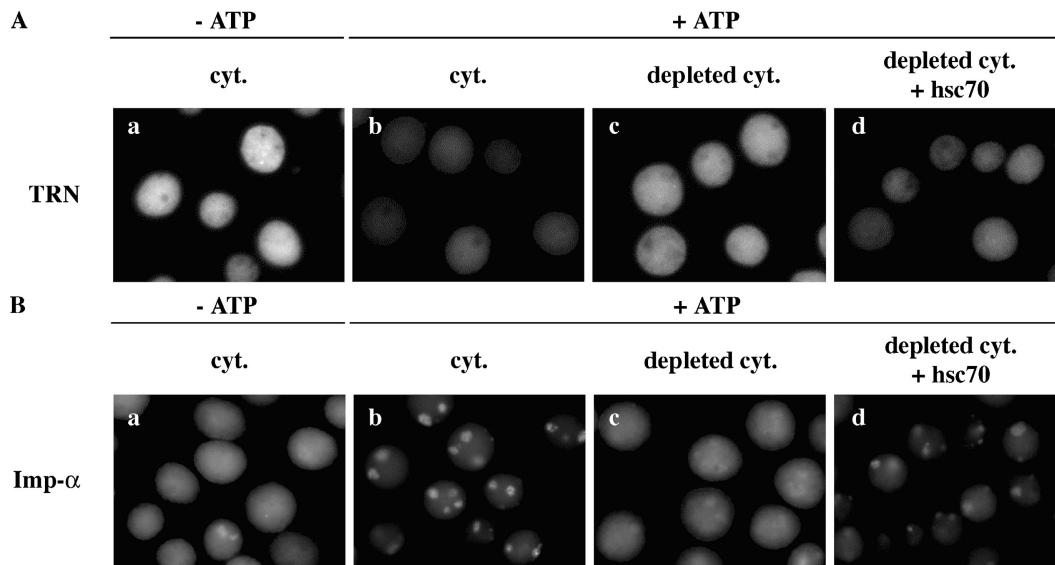
Figure 4. **Hsc70 facilitates nuclear export of transportin and importin α.** (A) Digitonin-permeabilized cells were incubated with 0.3 μM GFP–transportin (TRN) and were reincubated with total cytosol (cyt; a, b), or depleted cytosol (c and d) in the absence (a–c) or presence (d) of 0.3 μM hsc70. Export reactions were performed in the presence of apyrase (a) or an ATP regeneration system (b–d). (B) For import reactions, digitonin-permeabilized cells were incubated with 0.6 μM GFP–importin α, 0.6 μM importin β, 0.4 μM RanGDP, and an ATP regeneration system. Export reactions were performed as described above.

To determine whether the function of hsc70 is restricted to importin β, we examined the effect of hsc70 in the recycling of another import receptor, transportin (Fig. 4 A). As in the case of nuclear export of importin β, the nuclear export of transportin requires energy input and cytosol, consistent with the previous notion that export of the importin β family generally requires the Ran system (Fig. 4 A, a and b). The export activity of transportin was decreased significantly in the depleted cytosol, but was restored by the addition of hsc70, as is the case for importin β. We found that hsc70 also facilitates the nuclear export of importin α, which is known to be mediated by cellular apoptosis susceptibility gene (CAS), an export receptor of the importin β family (Fig. 4 B). These results show that the function of hsc70 is not restricted to importin β, and suggests that hsc70 may function generally in the recycling of import receptors.

To know the effect of hsc70 on the nuclear export of receptors in living cells, we depleted endogenous hsc70 by transfecting hsc70-specific small interfering RNA (siRNA) duplexes into HeLa cells. After 48–72 h transfection, the expression levels of hsc70 proteins were reduced to 10–20% of the hsc70 protein levels that were detected in untransfected control cells (Fig. 5 A). At this time point, the morphology of the cells that were depleted of hsc70 changed dramatically; some displayed cellular blebbing.

The intracellular distribution of importin β, transportin, and importin α in the control cells and in cells depleted of hsc70 were examined by immunofluorescence. In control cells, importin β (Fig. 5 B, a) and importin α (Fig. 5 D, a) were mainly cytoplasmic, whereas transportin was localized evenly between the nucleus and cytoplasm (Fig. 5 C, a). However, in hsc70 siRNA-transfected cells, importin β (Fig. 5 B, d), transportin (Fig. 5 C, d), and importin α (Fig. 5 D, d) accumulated

significantly in the nucleus. In contrast, fluorescently labeled BSA that was injected into the cytoplasm of hsc70 siRNA-transfected cells did not migrate into the nucleus, which showed that the permeability barrier of the nuclear envelope remains intact in these cells (Fig. S1; available at http://www.jcb.org/cgi/content/full/jcb.200506074/DC1). Cells that were transfected with siRNA of β-actin, an essential protein for cell survival, showed significant morphologic changes with cellular blebbing, like hsc70 siRNA-transfected cells (Harborth et al., 2001). In contrast to cells that were transfected with hsc70 siRNA, the distribution of importin β did not shift to the nucleus in cells that were transfected with β-actin siRNA (Fig. 5 B, g). Therefore, the effect of depletion of hsc70 is specific. These results provide further support for the function of hsc70 in the nuclear export of receptor proteins.

The observations in this study indicate that hsc70 facilitates the nuclear export of import receptors, importin β and transportin, and importin α. Several previous studies suggested that hsc70 is involved in nuclear protein import (Imamoto et al., 1992; Shi and Thomas, 1992; Okuno et al., 1993). Antibodies against hsc70 inhibit the basic type of NLS-mediated nuclear import in living cells, and the import activity of the basic NLS substrate is decreased when hsc70 is depleted from the cytosol in an in vitro transport assay. The results presented in this study show that the inhibition of activity of hsc70 induces the inhibition of recycling of importin β (an import receptor) and importin α (a NLS receptor), which, in turn, result in a defect in nuclear protein import.

Hsc70 is involved in several steps in nuclear transport, such as the recognition of basic-type NLS, and the NPC-translocation step of transport in yeast (Shulga et al., 1996). In mammalian cells, in an in vitro transport assay, nuclear transport can be reconstituted almost completely by using puri-
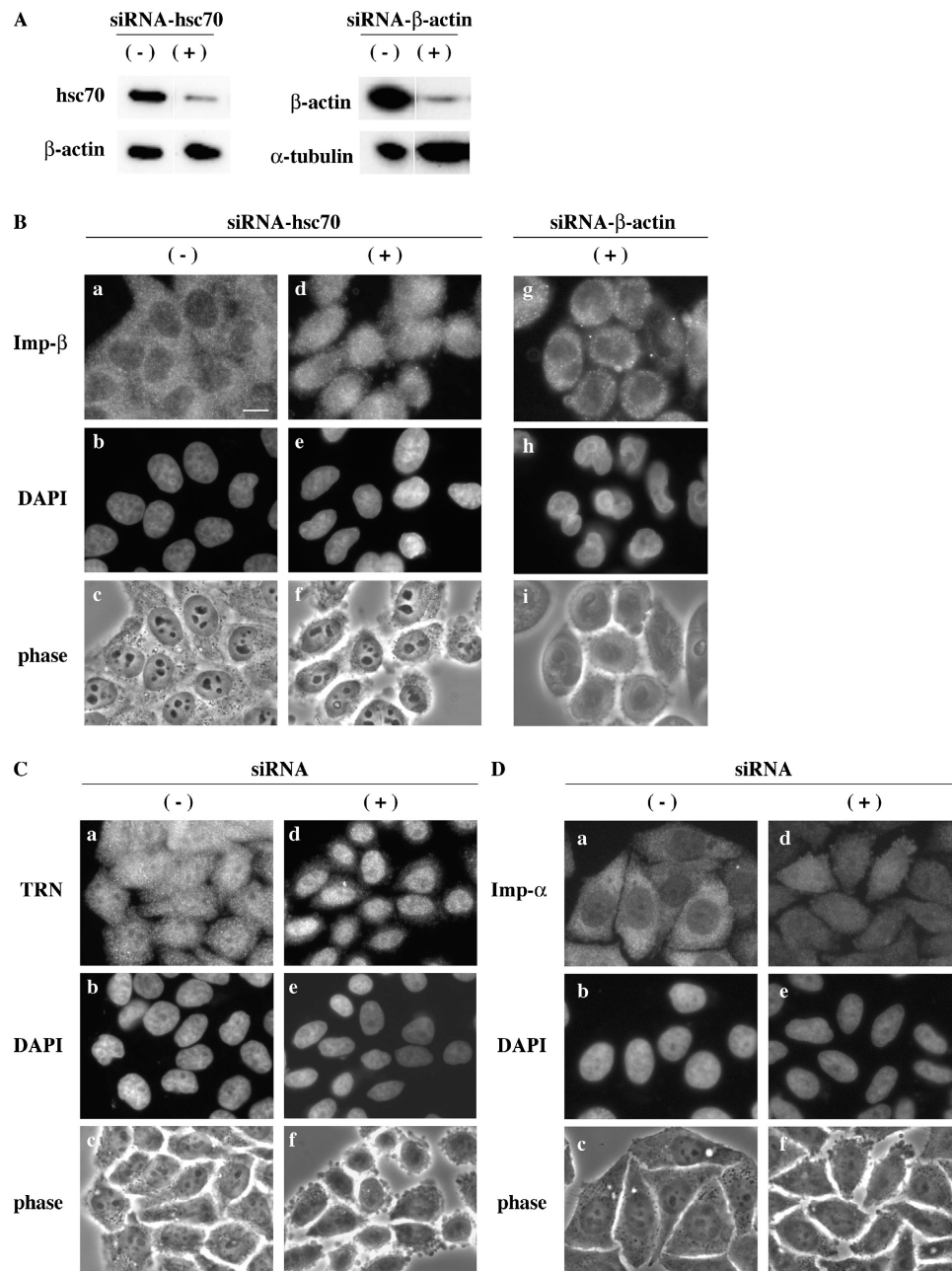
Figure 5. **Depletion of hsc70 in living cells causes the accumulation of importin β, transportin, and importin α within the nucleus.** (A) HeLa cells were treated for 48 h with (+) or without (−) siRNA targeting hsc70 (left panels) or β-actin (right panels). The expression of the indicated proteins was monitored by immunoblotting. β-Actin or α-tubulin was used as an internal control. (B–D) HeLa cells were treated for 48 h (B) or 60 h (C and D) with (+) or without (−) siRNA targeting hsc70 (B, a–f; C and D) or β-actin (B, g–i). The transfected cells were fixed with 3.7% formaldehyde and stained with specific antibodies and DAPI. "Phase" refers to the phase-contrast view. Bar, 10 μm.

fied transport mediators, such as importin α, importin β, Ran, NTF2, and RanBP1. The addition of hsc70 to the purified reconstituted system neither enhanced nor decreased the import efficiency of the basic NLS-containing import substrate (unpublished data). Further, the function of hsc70 in facilitating the nuclear export of receptors cannot be reconstituted in the purified Ran system. However, in living mammalian cells or in the in vitro transport assay driven by cytosol, the loss of function of hsc70 dramatically induced a transport defect. Such controversial results have remained a mystery, and prevented the role of hsc70 in the nuclear import in mammalian cells to

be understood. Our present results show that hsc70 facilitates the recycling of import receptors, and such a function of hsc70 is likely to be coupled with the nuclear migration of hsc70 itself (Fig. 3). Hence, import receptor(s) of hsc70 are likely to be required for hsc70 to facilitate the nuclear export of import receptors. It was reported that ATP depletion induces a rapid loss of free GTP in cells, and results in the inhibition of Ran-dependent nuclear transport (Schwoebel et al., 2002). The mechanism of nuclear import of hsc70 is unknown; however, our preliminary data indicate that hsc70 migrates into the nucleus in a Ran-dependent, but importin β– or transportin-independent

pathway. This suggests that nucleocytoplasmic shuttling of hsc70 may be inhibited in energy-depleted cells (unpublished results). Alternatively, because it is known that cofactors participate in the regulation of hsc70 for its ATPase cycle, such co-chaperones also may be required. In either case, if hsc70 functions in the nucleus to facilitate the recycling of import receptors, nuclear import mediators of hsc70 (and its co-chaperones) might play a regulatory role that could affect the activity of various transport pathways in living cells.

The precise function of hsc70 in the nuclear export of receptor proteins is not understood. Chaperone activity of hsc70 may be required for the prevention or dissociation of receptor proteins from intranuclear binding sites. Such activity could be important for nuclear quality control. Alternatively, because hsc70 associates with several nucleoporins in an ATP-dependent manner (unpublished data), it might affect the NPC translocation-step in the recycling of receptor proteins by regulating the affinity between nucleoporins and import receptors. Hsc70 facilitates the nuclear export of importin α, which is exported by an export receptor (cellular apoptosis susceptibility gene) in a Ran-dependent manner (Fig. 4 B and Fig. 5 D). We could not detect the effect of hsc70 on the nuclear import or export of β-catenin (Fig. S2; available at http://www.jcb.org/cgi/content/full/jcb.200506074/DC1), which occurs in a Ran-independent manner (Koike et al., 2004). Therefore, it is intriguing to consider the possibility that hsc70 affects the GTPase cycle of Ran, which is essential for the recycling of importins and the export mediation for exportins. For example, hsc70 has been reported to interact with RCC1 in *Xenopus* extracts (Saitoh and Dasso, 1995), which may affect the production of RanGTP in the nucleus. These or other possibilities must be examined carefully in the further studies.

We showed previously and in the present study that the nuclear export of importin β and its related receptors is an energy-dependent process. In this study, we present in vitro and in vivo evidence that hsc70 facilitates the nuclear export of the import receptors, importin β, transportin, and importin α. This function of hsc70 is dependent on its ATPase activity. We propose a novel role for hsc70 in which it is important in the recycling of import receptors in mammalian cells. Further studies regarding the function and regulation of hsc70 in connection with importin β and its related proteins likely will provide new insights into the mechanism and regulation of nuclear transport in mammalian cells.

Materials and methods

Expression and purification of recombinant proteins

Expression and purification of recombinant GFP–importin β and human untagged hsc70 protein were performed as described previously (Imamoto et al., 1992; Kose et al., 1997). For the expression of EYFP or ECFP fusion protein, DNA coding for EYFP or ECFP were amplified from the pEYFP-C1 or pECFP-C1 vectors (BD Biosciences) by PCR using synthetic oligonucleotides with appropriate restriction sites, and then cloned into the pGEX6P-1 vector (GE Healthcare). Importin β was cloned into the pGEX6P-1/EYFP vector. Hsc70 was cloned into the pGEX6P-1/ECFP vector. For the expression of an ATPase-deficient hsc70 mutant, hsc70(D10N), site-directed mutation was induced in pET3c/hsc70 or pGEX6P-1/ECFP/hsc70 vector by PCR with mutagenic oligonucleotides. Human transportin was cloned into the pGEX6P-2/hGFP vector (Yokoya et al., 1999). The expression and pu-

rification of each recombinant fluorescent protein and untagged hsc70 (D10N) protein were performed in the same manner as for GFP–importin β and untagged wild-type hsc70, respectively.

In vitro nuclear transport assay

Digitonin-permeabilized Madin-Darby bovine kidney (MDBK) cells were prepared essentially as described previously (Kose et al., 1997). After permeabilization of MDBK cells with digitonin, the cells were immersed in transport buffer (TB; 20 mM Hepes, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 1 mM EGTA, 1 mM DTT, and 1 μg/ml each aprotinin, leupeptin, and pepstatin) for 15 min at room temperature. Import reactions were performed by incubating digitonin-permeabilized cells with 12 μl of a reaction mixture containing recombinant proteins in TB under each condition indicated in the respective figure legends. For the nuclear export assay, the permeabilized cells that were incubated initially with recombinant proteins were washed immediately to remove excess substrate, and then were incubated with the reaction mixture to examine the nuclear export activity for 10 min at 18°C (for importin β and transportin) or 30°C (for importin α). After the cells were fixed in 3.7% formaldehyde in TB, fluorescent proteins were detected by epifluorescence microscopy (Olympus IX70 or BX51) using a 40×/0.75 N.A. objective. Images were captured with an ORCA-ER camera (Hamamatsu), controlled by MetaVue software (Universal Imaging Corp.).

Fractionation of Ehrlich ascites tumor cells cytosol

A total cytosolic extract of Ehrlich ascites tumor cells was prepared as described previously (Imamoto et al., 1995). For fractionation of the cytosol, the clarified extract was applied to an ATP–agarose (linked through C-8; Sigma-Aldrich) column equilibrated with TB. This column was washed with 20 mM potassium phosphate (KP) (pH 7.0), 500 mM NaCl, 1 mM DTT; the bound proteins were eluted with 20 mM KP (pH 7.0), 50 mM NaCl, 1 mM DTT containing 3 M MgCl₂. The eluate, supplemented with (NH₄)₂SO₄ at a final concentration of 1 M, was applied to Phenyl Sepharose HP (GE Healthcare), and was eluted using a liner gradient of 1–0 M (NH₄)₂SO₄ in 20 mM KP (pH 7.0), 50 mM NaCl, 1 mM DTT. The peak fractions containing p70 were applied to Superdex 200 (GE Healthcare) equilibrated with TB. Each fraction that was subjected to the transport assay was desalted with a PD10 column (GE Healthcare) equilibrated with TB, followed by concentration using Centricon-10 (Amicon).

Each fraction was separated on 10% or 12.5% SDS-PAGE, and immunoblotted with anti-hsc70 rabbit antibodies (Imamoto et al., 1992), anti-Ran rabbit antibodies, anti-NTF2 mouse monoclonal antibodies (Transduction Laboratories), or anti-RCC1 mouse monoclonal antibodies (Transduction Laboratories). The probed antibodies were detected by the standard method using AP-coupled or HRP-conjugated secondary antibodies.

siRNA experiments

siRNA preparation and transfection. Stealth siRNA duplexes for silencing of human hsc70 were designed by Block-it RNAi designer (Invitrogen) and purchased from Invitrogen. The sense sequence is 5′-UAAUUCUAAGUA-CAUUGAGACCAGC-3′. The siRNA duplexes that were used for silencing of β-actin (target sequence: AATGAAGATCAAGATCATTGC) were purchased from QIAGEN (Harborth et al., 2001). HeLa cells were transiently transfected using oligofectamine (Invitrogen) with or without a final concentration of 100 nM for the siRNAs. After 48–72 h transfection, when protein levels of hsc70 decreased to 10–20% and β-actin decreased to 10–15% of control cells, cells were subjected to microinjection or immunofluorescence studies.

Immunoblotting. Transfected HeLa cells were trypsinized, washed once in ice-cold PBS, and harvested. Cells were lysed by boiling in SDS-PAGE sample buffer. The eluted proteins were separated on 10% polyacrylamide gels and transferred to nitrocellulose; this was followed by immunoblotting with the antibodies specific to hsc70 (Imamoto et al., 1992), β-actin (Sigma-Aldrich), or α-tubulin (Sigma-Aldrich), and HRP-conjugated secondary antibodies (Bio-Rad Laboratories) using the ECL technique. Images were recorded with LAS-1000 (Fujifilm), and the intensity of bands was calculated using NIH-Image software.

Immunofluorescence microscopy. Transfected cells, grown on coverslips, were washed twice with PBS, fixed in 3.7% formaldehyde in PBS for 20 min at 37°C, and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After blocking with 3% skim milk in PBS, the cells were incubated with antibodies specific to importin β (Santa Cruz Biotechnology Inc.), transportin (Transduction Laboratories), or importin α (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, and detected with Cy3-labeled donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories) and AlexaFluor 488 donkey anti–rabbit IgG (Molecular Probes).

The fluorescent images were taken using Olympus BX51 with a 40×/0.75 N.A. objective. Images were captured with an ORCA-ER camera (Hamamatsu), controlled by MetaVue software (Universal Imaging Corp.).

Online supplemental material
Fig. S1 shows localization of Texas Red–labeled BSA injected in the cytoplasm of cells transfected with siRNA targeting hsc70. Fig. S2 shows nuclear export of β-catenin in an in vitro nuclear transport assay. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200506074/DC1.

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