Nucleocytoplasmic Shuttling of the Zinc Finger Protein EZI Is Mediated by Importin-7-dependent Nuclear Import and CRM1-independent Export Mechanisms*

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Nucleocytoplasmic translocation constitutes a foundation for nuclear proteins to exert their proper functions and hence for various biological reactions to occur normally in eukaryotic cells. We reported previously that EZI/Zfp467, a 12 zinc finger motif-containing protein, localizes predominantly in the nucleus, yet the underlying mechanism still remains elusive. Here we constructed a series of mutant forms of EZI and examined their subcellular localization. The results delineated a noncanonical nuclear localization signal in the region covering the 9th to the 12th zinc fingers, which was necessary for nuclear accumulation of EZI as well as sufficient to confer nuclear localizing ability to a heterologous protein. We also found that the N-terminal domain of EZI is necessary for its nuclear export, the process of which was not sensitive to the CRM1 inhibitor leptomycin B. An interaction proteomics approach and the following co-immunoprecipitation experiments identified the nuclear import receptor importin-7 as a molecule that associated with EZI and, importantly, short interfering RNA-mediated knockdown of importin-7 expression completely abrogated nuclear accumulation of EZI. Taken together, these results identify EZI as a novel cargo protein for importin-7 and demonstrate a nucleocytoplasmic shuttling mechanism that is mediated by importin-7-dependent nuclear localization and CRM1-independent nuclear export.

Subcellular distribution of cellular proteins is a foundation for the molecules to exert their proper functions and hence for normal biological reactions to occur. Although some proteins may diffuse freely in the cells, many are destined to localize in a specific subcellular compartment. To achieve proper subcellular localization, such a protein encodes some intrinsic signal, as well as often requires cognate cellular machinery to support its translocation. Thus, identification of such a signal and machinery is a fundamental issue to understand the molecular mechanism whereby a protein exerts its functions.

In eukaryotic cells, the nucleus and the cytoplasm are separated by the nuclear membrane. As protein synthesis takes place in the cytoplasm, nuclear proteins must cross the nuclear membrane through the nuclear pore complex (NPC) to enter the nucleus. Likewise, some nuclear proteins are exported from the nucleus to the cytoplasm, in such occasions as recycling of signal transducers, withdrawal of transcription factors, or transport of RNA molecules. Although small particles (less than around 20–30 kDa) are capable of passing through the NPC freely by passive diffusion, larger molecules require an energy-dependent mechanism mediated by specific nuclear import and export machineries to translocate between the nucleus and cytoplasm (1, 2). It is well established that many, if not all, of these processes depend on the functions of the karyopherin family proteins, which can be largely subdivided into the karyopherin α/importin α and karyopherin β/importin β/exportin subfamilies (hereafter referred to as importin α and karyopherin β subfamilies, respectively) based on their structural as well as functional characteristics (3).

Among several distinct mechanisms for nuclear import, the so-called canonical (or classical/conventional) pathway is the best characterized (1). The substrate proteins (or cargos) conveyed via this pathway possess a characteristic monopartite or bipartite nuclear localization signal (NLS) composed of one or two cluster(s) of basic amino acid residues. Those canonical NLSs are specifically recognized by one of the members of the importin α subfamily. The importin α have a binding site for importin/karyopherin B1 (hereafter referred to as importin β), a member of the karyopherin β subfamily, which can also interact with the NPC. Thus, the ternary complex formed by the cargo, importin α, and importin β docks to the NPC and then translocates into the nucleoplasm (1). After entering the nucleus, the process is terminated by binding of the GTP-bound form of Ran (RanGTP) and subsequent dissociation of the complex.

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4 The abbreviations used are: NPC, nuclear pore complex; siRNA, short interfering RNA; aa, amino acid; NLS, nuclear localization signal; NES, nuclear export sequence; HIV, human immunodeficiency virus; LMB, leptomycin B; hnRNP, heterogeneous nuclear ribonucleoprotein.
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The other mechanisms for nuclear import include those mediated by a single species of karyopherin βs (single-receptor pathways), by heterodimeric complexes of karyopherin βs (co-receptor pathways), or by direct interaction of a cargo to the NPC without any involvement of the karyopherin family proteins (4). Compared with the canonical nuclear import pathway, however, the targeting signals and molecular mechanisms for those noncanonical nuclear import pathways are not well understood, nor is the nature of the cargos governed therein.

Of note, importin-7 (also known as RanBP7) is a member of the karyopherin β subfamily (5) and functions as a nuclear import receptor in importin-α-independent, noncanonical pathways (6). Molecules homologous to mammalian importin-7 have been found in yeasts and fruit flies and are known to function in both species as a nuclear import receptor (7–10). This evolutionary conservation, together with the fact that importin-7 exists as highly abundantly as importin-β in the cells examined (5), strongly suggests physiological importance of importin-7, yet only a few cargo molecules conveyed by this import receptor have so far been identified, including ribosomal protein L23a (rpl23a), histone H1, and the HIV reverse transcriptase complex (6, 11, 12).

In active nuclear export processes, proteins with a specific nuclear export sequence (NES) form a ternary complex with an exportin/karyopherin β and RanGTP in the nucleus (13). The complex can then translocate to the cytoplasm, where GTP is hydrolyzed to GDP, and the NES-containing protein is released. The exportin-RanGDP complex diffuses back to the nucleus where GDP is exchanged to GTP by Ran-guanine nucleotide exchange factors. This process is also energy-dependent as it consumes one GTP per cycle. To date, the best known nuclear export mechanism concerns the short, leucine-rich type of NES, initially identified in the HIV Rev protein, and its binding to the exportin CRM1/Xpo1 (14). This CRM1-dependent nuclear export can be specifically and efficiently inhibited by its binding to the exportin CRM1 (14). This CRM1-decorate type of NES in a portion of the zinc finger domain, which was both necessary and sufficient for nuclear localizing activity. We also found that EZI shuttles between the nucleus and the cytoplasm, and that its nuclear export is mediated by the N-terminal region via a CRM1-independent mechanism. Finally, importin-7 was identified to be a molecule that physically interacts with EZI, and knockdown experiments using siRNA against importin-7 revealed that this nuclear import receptor is essential for nuclear localization of EZI.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The wild type (full-length; 594 aa) as well as all the mutant forms of EZI used were expressed using the eukaryotic expression vector pME18S with Myc epitope tag fused at their N termini, unless otherwise indicated. The constructs for the full-length and two kinds of deletion mutants (EZI-C and FAZF) were described previously (16). In this study we renamed EZI-C and FAZF as ΔC and ΔZF1–7, respectively, for consistency with the other mutants. The other deletion mutants were generated by standard procedures based on PCR with appropriately designed primers. The MutNLS8 mutant was generated by site-directed mutagenesis with QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The exact portions contained in each of the mutants are as follows: ΔN, aa residues 162–594; ΔZF, 1–161 plus 564–594; ΔC, 1–564; ΔZF1–7, 1–161 plus 378–594; ΔZF8–12, 1–378 plus 565–594; ΔZF1–7, 162–378; ΔZF8–12, 377–564; MutNLS8, 1–564 with the 447RHR to 447AAA substitutions; ΔZF8, 1–378 plus 454–594; ΔZF9–12, 1–452 plus 565–594; ΔZF9–12, 453–564; ΔZF10/11, 144–594; ΔZF11/12, 1–508 plus 592–594; ΔZF9/10, 1–508 plus 592–594; ΔZF9/10, 1–508 plus 592–594; ΔZF9/10, 1–508 plus 592–594; ΔZF9/10, 1–508 plus 592–594.

To express LacZ fusion products, we first subcloned a DNA fragment containing the LacZ coding region (corresponding to the C-terminal 1015 aa), derived from the pCH110 vector, into pME18S with the Myc tag. The resultant vector, pME18SmycLacZ, was designed to contain tandem BglII-Nhel-Sall cloning sites between the Myc tag and the LacZ coding region. For the ZF9 LacZ, ZF10 LacZ, ZF11 LacZ, and ZF12 LacZ constructs, synthetic oligonucleotides corresponding to each of the zinc finger motifs (ZF9, aa residues 457–483; ZF10, 485–511; ZF11, 512–539; and ZF12, 541–567) were annealed and inserted into the Nhel- and Sall-digested pME18SmycLacZ. For ZF9–12 LacZ, a fragment corresponding to the four zinc finger motifs (aa residues 453–564) was amplified by PCR and cloned into the BglII- and Sall-digested pME18SmycLacZ.
The full-length cDNA clone for mouse Mybbp1a (I.M.A.G.E. cDNA, clone ID 5363740) was obtained from Invitrogen, and the entire coding region was subcloned into the pME18S vector with FLAG tag at the N terminus. The HA-Hic-5 construct in pCG-N-BL vector was a generous gift from Dr. Mori (Showa University, Tokyo, Japan) (17). To prepare the expression construct for hnRNP A1, the entire coding region of mouse hnRNP A1 (321 aa) was amplified by PCR, cleaved with EcoRI and NotI pre-designed in the primers, and inserted between the EcoRI and NotI sites of the pcDNA3 vector with the Myc tag at the N terminus.

The full-length (wild type) cDNA for human importin-7 was amplified by PCR from a 293T cell-derived cDNA sample. The K61D mutation (changing the corresponding codon AAA to GAT) was introduced by PCR-based methods using appropriately designed primers and the wild type importin-7 cDNA as a template. The wild type and K61D mutants were each subcloned into the pME18S vector with FLAG tag at their N termini. Integrity of the constructs, in particular all the sequences derived from PCR amplifications, was confirmed by DNA sequencing.

**Antibodies**—The following antibodies were commercially obtained and used in this study: mouse anti-Myc monoclonal antibody 9E10 (sc-40), mouse anti-hemagglutinin monoclonal antibody F-7 (sc-7392), and goat anti-actin I-19 (sc-1616) were from Santa Cruz Biotechnology; mouse anti-FLAG monoclonal antibody M2 and mouse anti-importin β (clone 31H4) were from Sigma; and, goat anti-RANBP7/importin-7 (IMG-3131) was from Imgenex (San Diego).

Rabbit polyclonal anti-EZI antibody was raised and prepared as follows. A fragment of EZI cDNA corresponding to the first 430 amino acids was subcloned into the pET-32a (Novagen) and pMAL-c2X (New England Biolabs) vectors to produce His tag fusion (His-EZI) and maltose-binding protein-tag fusion (MBP-EZI) recombinants, respectively. Recombinant proteins were overexpressed in the BL-21 CodonPlus (DE3) Escherichia coli strain (Stratagene) and affinity-purified using HisBind purification kit (Merck) and amylose resin (New England Biolabs) for His-EZI and MBP-EZI, respectively. His-EZI was used for immunization (Suka-flat; Saitama, Japan), and the anti-EZI antibody was affinity-purified using MBP-EZI coupled to NHS-activated HP column (GE Healthcare).

**Cell Culture and Transfection**—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% bovine serum (Invitrogen) and antibiotics. HeLa cells and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Equitech) and antibiotics. Transient transfection into NIH3T3 cells was performed using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Briefly, 2 × 10⁶ cells were seeded in a 100-mm plate and, 24 h later, transfected with 6 μg of plasmid DNAs using 18 μl of FuGENE 6. After 48 h of culture, the cells were harvested and then subjected to co-immunoprecipitation experiments.

**Immunofluorescence Analysis**—For indirect immunofluorescence analysis to detect subcellular localization of the expressed proteins, cells on the coverslips were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with ImmunoBlock™ (DS Pharma Biomedical Co., Ltd., Osaka, Japan). To detect Myc-tagged, FLAG-tagged, and hemagglutinin-tagged proteins, cells were first incubated with the corresponding monoclonal antibodies and subsequently with the rhodamine-labeled anti-mouse IgG secondary antibody (Jackson ImmunoResearch) unless otherwise indicated. Nuclei were counterstained with Hoechst 33342 (Sigma). The coverslips were mounted on glass slides (Matsunami Glass) and then observed using Zeiss Axiostar 2 Plus microscope. Images were taken using a CCD camera (model C4742-95-12ER; Hamamatsu Photonics K.K.) with Aqua Cosmos 2.0 software, and further processed using Adobe Photoshop CS software.

To quantitatively evaluate subcellular localization of the expressed proteins, the relative staining intensities in the nucleus and cytoplasm were monitored and scored for more than 100 cells per each transfection, according to a 5-grade criterion as follows: exclusive staining in the cytoplasm; staining in the cytoplasm exceeding that in the nucleus; equivalent staining in the nucleus and the cytoplasm; staining in the nucleus exceeding that in the cytoplasm; and exclusive staining in the nucleus. The relative number of the cells for each category per total number of the cells examined were calculated and expressed in percentage. The values shown in figures represent mean ± S.D. of duplicate assays.

**Inter-species Heterokaryon Assay**—The inter-species heterokaryons composed of NIH3T3 and HeLa cells were prepared according to the procedure described by Cáceres et al. (18) with some modifications. Briefly, 4 × 10⁶ NIH3T3 cells were seeded on coverslips put in 12-well plates and transfected with constructs to be assayed for shuttling ability. Twenty four h post-transfection, the cells were overlaid with an equal number of untransfected HeLa cells for 3 h in the presence of 100 μg/ml cycloheximide (Sigma). When the CRM1-dependent nuclear export pathway was to be inhibited, the cells were treated additionally with 30 ng/ml LMB (LC Laboratories) 3 h before cell fusion. The cells on the coverslips were then covered with a solution of 50% (w/v) polyethylene glycol 1500 (Roche Applied Science) for 2 min to induce cell fusion. The solution was replaced with culture medium containing 100 μg/ml cycloheximide, as well as 30 ng/ml LMB when necessary, and further incubated for 5 h before fixation.

**Immunoprecipitation and Western Blot Analysis**—The NIH3T3 cells transfected with siRNAs were lysed in TNE buffer (20 mm Tris-HCl, pH 7.4, 150 mm NaCl, 2 mm EDTA, 1% Nonidet P-40, 1 mm phenylmethylsulfonyl fluoride, 1 mm Pefabloc SC (Roche Applied Science), and 10 μg/ml leupeptin), and the whole cell extracts were separated by SDS-PAGE and trans-
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ferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore). The membranes were subjected to Western blot analyses with anti-importin β or anti-RANBP7/importin-7 antibody, as well as with anti-β-actin antibody to confirm equal loadings.

For co-immunoprecipitation experiments, COS-7 cells were transfected with the plasmids encoding FLAG-tagged human importin-7 and Myc-tagged EZI (3 µg for each). For mock transfections, either of the constructs was replaced with the empty vector to adjust the total amount of plasmids. The cells were lysed in TNE buffer to prepare whole cell extracts, which were then used for immunoprecipitation using anti-FLAG or anti-Myc monoclonal antibody. The precipitated proteins as well as whole cell extracts were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted with anti-FLAG and anti-Myc monoclonal antibodies.

RNA interference—For RNA interference experiments, synthetic siRNA (obtained as Stealth™ select RNA interference from Invitrogen) targeting mouse importin β (target sequence, 5’-GAGUUGACGUGGCUCACAAUUA-3’), mouse importin-7 (5’-GGCGAUUGACGUGUAUCCAUUA-3’), or control siRNA without any target sequence in mouse genome (Stealth™ RNAi negative control, Medium GC duplex; Invitrogen) was transfected into NIH3T3 cells using RNAiMAX (Invitrogen) with the reverse transfection protocol according to the manufacturer’s instructions. We initially tested several experimental conditions with different time courses, and we found that the efficient and maximal level of inhibition for expression of importin β and importin-7 proteins could be achieved 72 and 48 h post-transfection, respectively, with 10 µM of the siRNA.

To examine the effect of importin knockdown on nuclear localization of EZI, cells were initially treated with the siRNAs and, after 48 h (for importin-β) or 24 h (for importin-7) of incubation, further transfected with the expression construct for the full-length (Myc-tagged) EZI. Twenty four h later, nuclear localization of EZI was examined by immunofluorescence staining as described above.

In importin-7 rescue experiments, FLAG-tagged human importin-7 was co-expressed with Myc-tagged EZI and detected with anti-FLAG monoclonal antibody. EZI was co-immunostained using rabbit anti-EZI antibody, in combination with AlexaFluor 488-conjugated anti-rabbit IgG (A21206; Invitrogen).

RESULTS

EZI Has a Nuclear Localization Signal in the Zinc Finger Domain—To clarify the mechanism that regulates the nuclear localization of EZI, we set out to perform mutational analyses of this protein. Plasmids for expressing a series of deletion mutants of mouse EZI (Fig. 1A) as Myc epitope-tagged proteins were constructed and transfected into mouse NIH3T3 cells. Distribution of the expressed proteins, either in the nucleus or the cytoplasm, or both, were analyzed by immunofluorescent staining using anti-Myc antibody, followed by quantitative evaluation. As the nuclear localization analysis in our previous study was rather qualitative (16), we first tested whether the requirement of the zinc finger domain for nuclear localization could be confirmed to be quantitatively significant. As shown in Fig. 1B, the wild type EZI, as well as two mutant forms of this molecule, ΔN and ΔC lacking the N-terminal and the C-terminal domains, respectively, showed predominant localization in the nucleus. In contrast, the ΔZF mutant lacking all the 12 zinc finger motifs failed to do so. Thus, the zinc finger domain was confirmed to be necessary for the nuclear localization of EZI.

To determine which zinc finger motif(s) is responsible for mediating nuclear localization of EZI, the zinc finger domain was dissected into two parts and separately analyzed. Thus, internal deletion mutants lacking either the N-terminal part (ΔZF1–7) or the C-terminal part (ΔZF8–12) of the zinc finger domain were generated, and subcellular localization of these proteins was examined. We found that the ΔZF1–7 mutant was capable of localizing predominantly in the nucleus, whereas that ΔZF8–12, like ΔZF, showed a clear defect in nuclear localization (Fig. 1C). Thus, the C-terminal half of the zinc finger domain, containing the 8th to the 12th zinc fingers, was suspected to be necessary for nuclear localizing activity. To test whether this subdomain is sufficient for the activity, we also employed mutants containing either the N-terminal part (ZF1–7) or the C-terminal part (ZF8–12) of the zinc finger domain. As shown in Fig. 1C, the ZF8–12 mutant was capable of localizing in the nucleus, and unexpectedly, ZF1–7 also showed the apparent nuclear localizing pattern. Nevertheless, together with the fact that ΔZF8–12 but not ΔZF1–7 has a defect in nuclear localization, we assumed that the ZF8–12 subdomain contains a signal that is functionally required for the nuclear localization of full-length EZI. In contrast, the ZF1–7 subdomain, although by itself showing a cryptic nuclear localizing activity, can be dispensable in the context of the full-length molecule.

The RHRR Sequence in the 8th Zinc Finger Does Not Confer Nuclear Localization—In parallel to the experimental approach to dissect EZI and identify its NLS, an attempt was made to predict possible subcellular localization of this molecule as well as responsible signals/sequences involved, by employing the bioinformatics algorithm PSORT II. Based on this program, the full-length EZI was predicted to be a nuclear protein with one NLS sequence, 44RHRR3490, which can be found in the 8th zinc finger domain. This led us to hypothesize that the predicted RHRR sequence was responsible for the NLS activity found in ZF8–12 as mentioned above, and this notion was examined by using two types of EZI mutants. The MutNLS8 mutant contains four alanine substitutions specifically at the RHRR sequence, whereas ΔZF8 lacks the entire ZF8 motif (Fig. 1A). Surprisingly, both of these mutants accumulated in the nucleus (Fig. 1D), indicating that RHRR is not necessarily required for nuclear localization of EZI, nor does it account for the NLS activity.

In stark contrast to the lack of contribution of ZF8, the remaining four zinc finger motifs, ZF9–12, was found to be critical for nuclear localization of EZI, as an internal deletion mutant lacking these motifs (Fig. 1A, ΔZF9–12) showed predominant cytoplasmic localization (Fig. 1D). Furthermore, the ZF9–12 mutant, composed only of these motifs, was still capa-
The Entire ZF9/10/11/12 Motifs Are Important for EZI Nuclear Localization—To further pinpoint the NLS sequence in the ZF9–12 region, we constructed the EZI/ZF9/10 and EZI/ZF11/12 mutants, each lacking two of the four zinc finger motifs in the full-length context. Both of these mutants showed a significant defect in nuclear localization (data not shown). Thus, we next deleted each of the four zinc finger motifs in the ZF9–12 region, resulting in the ΔZF9, ΔZF10, ΔZF11, and ΔZF12 mutants (Fig. 2A), and we examined their effects on nuclear localization of EZI. As shown in Fig. 2B, all of these mutants showed diffuse distribution patterns, with apparent and significant staining observed in the cytoplasm. We also tested the effect of the same deletions in the context of ZF9–12, using the mutants ZF9/11/12, ZF9/11/12, ZF9/10/12, and ZF9/10/11. Even though all of these mutants still retain the ability to localize preferentially in the nucleus, partial yet signifi-
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The N-terminal Domain of EZI Mediates Its Nuclear Export

As mentioned previously, the ZF1–7 mutant sufficiently localized in the nucleus by the activity of the cryptic NLS (Fig. 1B). Interestingly, the ΔZF8–12 mutant, which contains the N- and C-terminal domains in addition to ZF1–7, was found predominantly in the cytoplasm (Fig. 1C). This strongly suggests that the N- and/or C-terminal domains possess a function that mediates cytoplasmic retention and/or nuclear export. To test the latter possibility, we employed an interspecies heterokaryon assay system, in which murine NIH3T3 cells expressing Myc-tagged EZI were fused with human HeLa cells in the presence of cycloheximide. Staining of the hybrid cells with Hoechst 33342 dye was used to discriminate between mouse and human nuclei within a heterokaryon; murine nuclei displayed a characteristic punctate staining pattern, whereas human nuclei were stained more diffusely. In control experiments where hnRNP A1, a well known nucleocytoplasmic shuttling protein (19), was originally expressed in NIH3T3 cells, staining of the tagged protein in adjacent HeLa cell nuclei was evident (Fig. 4B, bottom panels), confirming that nuclear export of transfected proteins could be clearly detected in this experimental setting. When the Myc-tagged, full-length EZI was applied to the experiments, we observed clear accumulation of the protein in the HeLa cell nuclei (Fig. 4B, top panels), indicating that EZI can be exported out of the nucleus and thus is a nucleocytoplasmic shuttling protein.

We then tested several mutant forms of EZI to identify the region of the molecule that is critical for its nuclear export. Although the ΔC mutant was exported as the wild type (data not shown), the mutant with a deletion of the first 91 amino acids (Δ91) as well as the ones lacking a larger portion (ΔAN and Δ143) all showed a defect in shuttling activity (Fig. 4B, and data not shown). These results indicate that the N-terminal 91-aa region is necessary for nuclear export and the shuttling behavior of EZI.

Nuclear Export of EZI Is CRM1-independent—The majority of shuttling proteins described to date possess a leucine-rich type of NES similar to the one originally found in the HIV Rev protein, and this type of NES is known to be a target recognized by the exportin CRM1 (14). The amino acid sequence of the N-terminal region of EZI does not show any sign of homology to this conventional NES, implying that nuclear export of EZI is CRM1-independent. To test this notion more directly, heterokaryon assays were performed in the presence or absence of LMB, a potent and specific inhibitor of CRM1 (15). As a control, we included Hic-5, a known shuttling protein whose nuclear

FIGURE 2. The ZF9–12 region is necessary for nuclear localization of EZI. Subcellular distributions of various EZI mutants were analyzed and shown as in Fig. 1. A, schematic drawings of the constructs used to dissect the ZF9–12 region of EZI. The results of their subcellular localization are summarized on the right. WT, wild type. C = N, uniform distribution in the nucleus and cytoplasm. & deletion of each of the four zinc finger motifs in the full-length context (ΔZF9, ΔZF10, ΔZF11, and ΔZF12) resulted in significant defects in nuclear localization, indicating critical requirement for these motifs. C, comparison of the nuclear localizing ability of ZF10/11/12, ZF9/11/12, ZF9/10/12, and ZF9/10/11 with that of ZF9–12 (see Fig. 1D) revealed that lack of any of the four zinc finger motifs weakened the NLS activity to some significant extent.

FIGURE 3. All of the single zinc finger motifs comprising the ZF9–12 region are required for proper nuclear localization of EZI. The N-terminal Domain of EZI Mediates Its Nuclear Export—As mentioned previously, the ZF1–7 mutant sufficiently localized in the nucleus by the activity of the cryptic NLS (Fig. 1B). Interestingly, the ΔZF8–12 mutant, which contains the N-
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The ZF9–12 region can sufficiently mediate nuclear localization of a heterologous protein. A, schematic drawings of expression constructs containing the bacterial β-galactosidase (LacZ) coding region fused with the entire ZF9–12 region (ZF9–12 LacZ) or each of the component zinc finger motifs (ZF9 LacZ, ZF10 LacZ, ZF11 LacZ, and ZF12 LacZ). The results of their subcellular localization are summarized on the right. B, subcellular distribution of the fusion proteins were analyzed and shown with representative photographs (middle columns) and bar graphs (right columns), as in Fig. 1. The ZF9–12 LacZ fusion construct localized in the nucleus (Nuc.), whereas all the other constructs containing a single zinc finger motif failed to do so, indicating that the entire ZF9–12 region is critical for the NLS activity. Cyt., cytoplasm.

Export is CRM1-dependent (17). When cells were treated with LMB, nuclear export of EZI was still observed, albeit that the staining in the HeLa cell nuclei might appear slightly weakened (Fig. 4C). Nuclear export of Hic-5 was completely blocked by LMB treatment, showing that the drug used was active. These results suggest that the major nuclear export pathway utilized by EZI is CRM1-independent.

Nuclear Localization of EZI Does Not Depend on the Canonical Import Pathway—The results described so far have identified the responsible regions for nuclear localization and nuclear export, which together play critical roles in nucleocytoplasmic shuttling of EZI. The ZF9–12 region, identified to be responsible for the NLS activity, does not contain any sequence that appears to match the canonical NLS motif, suggesting that nuclear localization of EZI is achieved by a mechanism that does not depend on the canonical nuclear import pathway mediated by importin α/importin β heterodimers. To test this notion more directly, we asked whether blockade of the pathway by inhibiting expression of importin β could affect the nuclear localization of EZI in the cells. As shown in Fig. 5A, synthetic siRNA against mouse importin β efficiently blocked its expression in NIH3T3 cells. Accordingly, this resulted in inhibition of nuclear localization of Mybbp1a, a molecule supposed to be transported into the nucleus in a canonical NLS-dependent manner (20) (Fig. 5B). In the same experimental setting, however, nuclear accumulation of EZI was not affected at all, confirming that EZI translocates into the nucleus in a manner independent of the canonical nuclear import pathway.

Importin-7 Is Essential for Nuclear Localization of EZI—To gain more insights regarding the molecular mechanisms that mediate and regulate subcellular localization of EZI, we sought to identify molecules that might physically interact with EZI. We took an interaction proteomics approach, where proteins that formed a complex with FLAG-tagged EZI in HEK293 cells were affinity-purified using anti-FLAG antibodies and then applied to a direct nanoflow liquid chromatography system followed by tandem mass spectrometric analysis (21). This led to the identification of several candidate binding proteins, among which was a member of the karyopherin family, importin-7. None of the other known karyopherins was isolated through this screening.

To verify the potential interaction between EZI and importin-7, we next examined their physical interaction by co-immunoprecipitation experiments. COS-7 cells were transiently transfected with Myc-tagged EZI and FLAG-tagged importin-7, and whole cell extracts were prepared and subjected to immunoprecipitation using anti-Myc tag antibody. Western blot analyses using anti-FLAG tag antibody showed that importin-7 was co-immunoprecipitated with EZI (Fig. 6). We also performed the experiments in an opposite direction, i.e. anti-FLAG immunoprecipitation followed by anti-Myc Western blotting, and we confirmed that they co-immunoprecipitate (data not shown).

To explore the possible involvement of importin-7 in regulating EZI nuclear localization, we examined the effect of siRNA-mediated knockdown of this molecule in NIH3T3 cells. The extent of importin-7 knockdown was determined at the protein level by using Western blot analysis, which confirmed that expression of importin-7 was almost completely abrogated by the importin-7-specific siRNA 48 h after transfection (Fig. 7A). We then examined subcellular localization of EZI under this experimental condition. As shown in Fig. 7B, it was obvious that EZI accumulated predominantly in the cytoplasm once the expression of importin-7 was suppressed, whereas the control siRNA did not affect the nuclear localization of EZI. Control experiments using hnRNP A1, whose nuclear import has been shown mediated by transportin (22), showed that its nuclear localization was not affected by importin-7 knockdown, confirming that the effect of the siRNA targeting importin-7 was specific. We also tested the effect of two other siRNAs with
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FIGURE 4. EZI shuttles between the nucleus and the cytoplasm. A, schematic drawings of the constructs used to analyze nuclear export of EZI. The results for their nuclear export phenotypes are summarized on the right. B, N-terminal region of EZI is necessary for its nuclear export activity. Mouse NIH3T3 cells were transiently transfected with vectors encoding the full-length or mutant forms of EZI or hnRNP A1 as a control. The transfectants were co-cultured and then fused with human HeLa cells to prepare interspecies heterokaryons, as described under "Experimental Procedures." Cells were stained with Hoechst 33342 to distinguish the mouse and human nuclei; the mouse, but not human, nuclei display a characteristic speckled pattern. Arrowheads indicate nuclei derived from HeLa cells. The full-length EZI as well as hnRNP A1, a well known shuttling protein, showed translocation from the mouse nuclei to the human ones. In contrast, the ΔN143 and ΔN91 mutants did not show any accumulation in the human nuclei. WT, wild type. C, nuclear export of EZI is CRM1-independent. Heterokaryons were prepared using NIH3T3 cells expressing the full-length EZI, or Hic-5 as a control, and incubated with 30 ng/ml LMB or vehicle (0.3% EtOH) for 5 h. Treatment with LMB resulted in distinct accumulation of Hic-5 in the nucleus, indicative of blockade of the CRM1-dependent nuclear export pathway. The nuclear export of EZI, however, was not significantly affected under the same experimental condition.

FIGURE 5. Nuclear import of EZI is independent of the canonical nuclear import pathway. A, effect of siRNA for importin β expression. NIH3T3 cells were transfected with control siRNA (control) or siRNA specifically targeting mouse importin β (si-lpoβ). Seventy-two h post-transfection, cell lysates were prepared and analyzed by Western blotting using anti-importin β (lpoβ) and anti-β-actin antibodies, confirming an efficient down-regulation of importin β expression. B, inhibition of importin β expression by siRNA did not affect nuclear localization of EZI. NIH3T3 cells were transfected with siRNAs and, 48 h later, with expression constructs for full-length EZI or Mybbp1a. Cultures were continued for additional 24 h, followed by immunostaining and quantitative evaluation of subcellular distribution of the transfected proteins. The results are shown with representative photographs (middle columns) and bar graphs (right columns), as in Fig. 1. Note that nuclear localization of Mybbp1a was significantly affected in this experimental setting. Nuc., nucleus; Cyt., cytoplasm.

DISCUSSION

In this study, we addressed the signals and the mechanisms that regulate nuclear localization of the zinc finger protein EZI. Detailed mutational analyses using a series of deletion and point mutants have revealed that the functional NLS of EZI resides in its zinc finger domain, composed of the most C-terminally located four zinc finger motifs (ZF9–12). The identified NLS region does not contain any consensus sequence for the canonical NLS motif, and nuclear localization of EZI was importin β-independent, negating a functional involvement of the canonical nuclear import pathway in this event. Rather, our results have clearly shown that importin-7 can physically and functionally interact with EZI, playing an essential role in its nuclear localization. We also found that the N-terminal 91 aa of EZI contain a signal responsible for nuclear export of the molecule, and that the underlying export mechanism does not involve function of the exportin CRM1. Taken together, these results demonstrate that EZI is a novel cargo for importin-7, and we have established it as a nucleocytoplasmic shuttling protein, whose net accumulation in the nucleus is likely controlled by different target sequences, and we obtained essentially the same results (data not shown).

To further demonstrate that the observed defect of EZI in nuclear localization was indeed attributed to the loss of importin-7 expression, we carried out rescue experiments using human importin-7. Human importin-7 shares at the amino acid level a 99.4% homology with the mouse counterpart, yet the nucleotide sequence targeted by the siRNA against mouse importin-7 is not conserved in the human sequence. Within those cells where FLAG-tagged human importin-7 was exogenously expressed as judged by anti-FLAG immunostaining, nuclear localization of EZI was significantly rescued (Fig. 7C, left panels). In contrast, the K61D mutant form of human importin-7, which has a defect in binding with Ran-GTP (11), failed to revert the phenotype. Of note, the K61D mutant rather showed a dominant negative effect, in that nuclear localization of EZI was blocked even in the presence of endogenous mouse importin-7. Taken together, these results strongly suggest that importin-7 plays an essential role in mediating nuclear localization of EZI, possibly via Ran-GTP-dependent nuclear import.
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Importin-7 is an abundant protein, whose role as a nuclear import receptor is evolutionarily conserved. In Saccharomyces cerevisiae, the Sxm1 gene product is the homolog of mammalian importin-7 and importin-8 and can mediate nuclear import of the yeast homolog of the autoantigen La and the poly(A)-binding protein Pab1, as well as the exogenously introduced mammalian glucocorticoid receptor (7–9). The Drosophila melanogaster homolog of importin-7 (DIM-7), encoded by the gene moesin, has been shown to be the nuclear import machinery for activated mitogen-activated protein kinase (10). In mammals, rpl23a and histone H1 have been identified to be cargos imported by importin-7 via a single receptor pathway (by the importin-7/importin-β homodimer) and a co-receptor pathway (by importin-7/importin-β heterodimer), respectively (6, 11).

The HIV reverse transcriptase complex was also supposed to translocate into the nucleus in an importin-7-dependent manner (12), although this notion has recently been challenged in a study based on importin-7 knockdown experiments (23). Several other molecules, c-Jun, the Cdk5 activator p35, the adenovirus VII protein, and the HIV Rev and IN proteins, have also been identified very recently to be potential substrates for this nuclear import receptor (24–28), yet the entire picture of its cargo still remains obscure. Notably, in the cases of rpl23a, c-Jun, p35, VII, and Rev, not only importin-7 but also other karyopherins, such as importin-β, transportin, importin-5, and/or importin-9, have also been suggested to function as their nuclear import receptors (6, 24–27). Our present results have identified EZI, a zinc finger motif-containing protein, as a novel class of cargos for importin-7 (Fig. 5B), nuclear translocation of EZI is likely to depend on the single receptor pathway mediated by the importin-7/importin-7 homodimer, rather than the importin-7/importin-β co-receptor pathway. Alternatively, importin-7 may form a complex with another member of the karyopherin β subfamily to import EZI. Although possible involvement of other nuclear import receptors in nuclear localization of EZI cannot be excluded, it
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should be emphasized that the process is strictly dependent on importin-7 expression as revealed by the knockdown experiments (Fig. 7, B and C), and to our knowledge, this should be the first demonstration of the necessary requirement of importin-7 for nuclear translocation of any mammalian protein from cellular origin. It is of significant interest and importance to examine the possibility that other zinc finger proteins can also be a cargo for importin-7.

Many of the zinc finger proteins are known as nucleic acid binding proteins and/or transcription factors, thus having a function in the nucleus. Despite that nuclear localization of various zinc finger proteins have been reported, the underlying mechanisms are only partially elucidated. Although NLS have been found in the zinc finger domains of several zinc finger proteins, the modes of the nuclear localization appear significantly diverged among those proteins. The NLS of the mouse orphan receptor TR2 or Wilms tumor 1 (WT1) can be delimited to one specific zinc finger motif within their multiple zinc fingers (29, 30). In contrast, the nuclear localizing activity of EKLF/KLF-1, JAZ, Snail, and Zif268 cannot be ascribed to any particular zinc finger motif (31–34). In the case of EKLF, for instance, all of the three zinc fingers are necessary for efficient nuclear localization and, notably, each finger shows an incremental effect on the NLS activity (31). Regarding the nuclear import machineries, functional involvement of importin β for nuclear localization of Snail has been demonstrated by using an in vitro transport system (33), whereas physical interaction between the zinc finger domain of EKLF and importin β has been shown by glutathione S-transferase pulldown experiments although its functional significance remains unknown (35). Our results based on the knockdown experiments strongly support the idea that nuclear import of EZI does not depend on importin β, but rather on importin-7, contrary to the case of Snail and EKLF.

Despite an apparent difference in nuclear import receptors on which to depend, the characteristic feature of the NLS (ZF9–12) of EZI may show considerable similarity with that of the EKLF zinc finger domain, in that the ΔZF9–12 mutant was completely excluded from the nucleus while that of the mutants lacking any single zinc finger motif (ΔZF9, ΔZF10, ΔZF11, and ΔZF12) showed partial defects with some proteins in the nucleus (Figs. 1D and 2B). The study by Pandya and Townes (31) employing several EKLF mutants has suggested that the basic residues of the EKLF zinc finger domain could mediate interactions with DNA, leading to nuclear localization of the molecule. The ZF9–12 region of EZI contains 21 basic amino acids within the four zinc fingers, showing an equivalent level of the content compared with that of EKLF (15 residues within three fingers). Moreover, EZI can bind to DNA in vitro via its zinc finger domain (16), and our preliminary experiments employing biochemical fractionation of the transfected cells revealed that the full-length EZI, as well as the ZF9–12 LacZ fusion products, strongly associates with the chromatin fraction in vivo.5 It is thus a considerable possibility that the basic residues in the ZF9–12 might also mediate physical interaction of EZI with the chromosomal DNA, thereby contributing in part to the NLS-mediated nuclear localization. It should be noted, however, that most of the other zinc finger motifs of EZI show a similar or even higher level of basic amino acid residue content. Therefore, the basic residues could not be the sole determinant for ZF9–12 to elicit the NLS activity.

Recent progress in the identification and characterization of various nuclear export events has been adding an increasing number of proteins to the list of those shuttling between the nucleus and the cytoplasm. Surprisingly, however, only a few have been reported with respect to zinc finger proteins, and our present results have clearly established EZI as a novel member of the nucleocytoplasmic shuttling protein, as well as further demonstrating that nuclear export of this molecule does not require function of CRM1 (Fig. 4, B and C). Our knowledge on detailed molecular mechanisms and machineries involved in nuclear export events is still limited mostly to the CRM1-dependent canonical pathway, and it is not clear how EZI is transported out of the nucleus. As EZI can bind to DNA and potentially enhance the STAT3-dependent transcription (16), it might be possible that EZI functions as a transcriptional regulator, and the nuclear export is a part of the mechanism attenuating its activity within the nucleus. In the case of another zinc finger protein, JAZ, it has been shown that the protein binds to exportin 5, a known nuclear export receptor for certain classes of double-stranded RNA, in a double-stranded RNA-dependent manner and that nuclear export of JAZ is mediated by exportin 5 (36). Although the biological function of JAZ has not been clarified either, this finding suggests a possible involvement of JAZ in the processing or protecting of some RNA species. Intriguingly, our interaction proteomics approach has identified several RNA-binding proteins as candidate interacting partners with EZI.6 It is thus raising a tantalizing possibility that EZI might be involved in regulation of RNA metabolism, in such a way that it participates in the RNA-binding complex that mediates nuclear export of RNAs. Alternatively, EZI rather exits the nucleus passively along with the associated RNAs to be exported. In view of the exportin 5-dependent nuclear export of JAZ, it would be worth testing whether EZI might also be a cargo for this export receptor. Identification of such molecules that are responsible for mediating nuclear export, together with our present finding of importin-7 as the critical nuclear import receptor, should pave the way to fully understand the molecular basis and functional significance of the dynamic behavior of EZI, as well as the physiological role(s) that this molecule plays.

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5 E. Saijou, T. Itoh, and A. Miyajima, unpublished data.

6 T. Itoh, S. Iemura, T. Natsume, and A. Miyajima, unpublished results.
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