Nuclear Import of Sterol Regulatory Element–binding Protein-2, a Basic Helix-Loop-Helix–Leucine Zipper (bHLH-Zip)–containing Transcription Factor, Occurs through the Direct Interaction of Importin β with HLH-Zip

Emi Nagoshi,* Naoko Imamoto,* Ryuichiro Sato,† and Yoshihiro Yoneda*‡§

*Department of Anatomy and Cell Biology, Osaka University Medical School, †Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, and ‡Institute for Molecular and Cellular Biology, Osaka University, Osaka 565-0871, Japan

Submitted March 1, 1999; Accepted May 3, 1999
Monitoring Editor: Marc Mumby

The sterol regulatory element–binding protein-2 (SREBP-2) is produced as a large precursor molecule attached to the endoplasmic reticulum membrane. In response to the sterol depletion, the N-terminal segment of the precursor, which contains a basic helix-loop-helix–leucine zipper domain, is released by two sequential cleavages and is translocated to the nucleus, where it activates the transcription of target genes. The data herein show that released SREBP-2 uses a distinct nuclear transport pathway, which is mediated by importin β. The mature form of SREBP-2 is actively transported into the nucleus when injected into the cell cytoplasm. SREBP-2 binds directly to importin β in the absence of importin α. Ran-GTP but not Ran-GDP causes the dissociation of the SREBP-2–importin β complex. G19VRan-GTP inhibits the nuclear import of SREBP-2 in living cells. In the permeabilized cell in vitro transport system, nuclear import of SREBP-2 is reconstituted only by importin β in conjunction with Ran and its interacting protein p10/NTF2. We further demonstrate that the helix-loop-helix–leucine zipper motif of SREBP-2 contains a novel type of nuclear localization signal, which binds directly to importin β.

INTRODUCTION

Eukaryotic cells can be subdivided into various membrane-bound compartments, each of which provides an optimal environment for specific biochemical reactions. As a result, the specialized systems have evolved, which permit the transport of macromolecules from one compartment to another. Because the nucleus is the central apparatus that coordinates all cellular activity, via gene expression, DNA replication, and ribosome assembly, proteins that are involved in these nuclear events must be selectively transported into the nucleus. At the same time, tRNAs and mRNAs are synthesized in the nucleus and are subsequently exported to the cytoplasm. The nuclear pore complex (NPC), which provides the gateway for this nucleocytoplasmic traffic, has recently been reviewed (Davis, 1995; Fabre and Hurt, 1997). Small molecules up to ~9 nm diameter, which corresponds to a globular protein of ~60 kDa, are able to pass through the aqueous pore by passive diffusion, whereas larger molecules are selectively transported via an energy- and signal-dependent mechanism. Proteins that are actively transported between nucleus and cytoplasm have specific signals for import, termed nuclear localization signals (NLSs), or for export, termed nuclear export signals. Previous studies have concluded that multiple transport pathways specified by distinct signals exist in cells, and this area has been reviewed by several groups (Corbett and Silver, 1997; Nigg, 1997; Yoneda, 1997; Mattaj and Englmeier, 1998; Ohno et al., 1998). Among the enormous amount of nucleocytoplasmic traffic, the nuclear import pathway,

© 1999 by The American Society for Cell Biology

2221
Thus, importin α acts as a receptor molecule for karyophiles. Ran represents one extreme example of importin β-family members, only importin β uses an adapter, whereas the others directly bind to their cargoes. However, recent reports have suggested that importin β is capable of binding directly to some nuclear proteins and to mediate their import. The ability of importin β to function without an adapter was first demonstrated for a fusion protein containing the importin β binding domain of importin α (IBB domain) (Görlich et al., 1996; Weis et al., 1996). It has been also shown that the yeast mRNA binding protein Nab2p interacts directly with human importin β and is imported into the nucleus of human cells, whereas the yeast homologue of importin β (Kap95p) is not able to mediate the import in human cells (Truant et al., 1998). In another example, Jäkel and Görlich (1998) reported that ribosomal proteins are directly imported by at least four importin β-family import receptors, namely importin β, transportin, RanBP5, and RanBP7. Interestingly, the importin β binding domains of these substrates share no obvious sequence similarities. This raises questions about how diverse substrates are imported by importin β, and what is the underlying mechanism by which a single receptor is able to recognize and carry distinct cargoes.

Import into the nucleus occurs not only as a continuous flux but also as temporally controlled events. For example, a transcription factor, NF-κB, is transported into the nucleus after its cytoplasmic masking protein, IkB, is phosphorylated and then undergoes degradation (Beg et al., 1992). The nuclear accumulation of the NF-AT family of proteins is triggered by the dephosphorylation of critical serine residues, allowing the two basic NLSs to be exposed on the molecular surface (Shibasaki et al., 1996; Beals et al., 1997). STAT1 is a transcription factor, which is translocated from the cytoplasm to the nucleus when cells are stimulated by interferon-γ. Interferon-γ stimulation leads to the tyrosine phosphorylation of STAT1, which enables it to form a nuclear pore-targeting complex with NPI-1 (a family of importin α) and importin β (Sekimoto et al., 1996, 1997).

The other manner in which nuclear transport is regulated is based on an anchoring–releasing mechanism. Membranous organelles as well as the plasma membrane are involved in the cytoplasmic anchoring of certain signaling molecules. One extreme example of
show that importin SREBP-2. A variety of in vivo and in vitro experiments
mechanism. significantly less is known about the overall nuclear import
mechanism. However, SREBPs possess no consensus
the mature form occurs via a sterol-independent
1995). Therefore, it is probable that nuclear import of
(Sato et al., 1996) and subcloned into the Salt–NolI sites of pGEX-6P-3 (Pharma-
cia, Piscataway, NJ). A FLAG tag with BglII and BamHI sites at the ends was generated by annealing two synthetic oligonu-
lot, which is followed by a membrane attachment do-
main of ~80 amino acids with two transmembrane
segments, and a C-terminal regulatory domain of
~590 amino acids. The precursor SREBPs are attached
to the endoplasmic reticulum membrane and the outer
nuclear envelope in a hairpin manner with their N-
and C-terminal domains projecting into the cyto-
plasm. The middle attachment domain projects into
the endoplasmic reticulum lumen. When the choles-
terol content of cells is reduced, the N-terminal do-
main of SREBP is released from the membranes by
sequential proteolytic cleavages at two sites, desig-
nated Site-1 and Site-2 (Rawson et al., 1997; Sakai et al.,
1998). The cleaved N-terminal fragment, referred to as
the mature form of SREBP, travels to the nucleus,
where it activates the transcription of genes involved
in cholesterol and fatty acid metabolism. When cells
accumulate cholesterol, the activity of the Site-1 pro-
tease is reduced, and the SREBP remains bound to
the membranes. As a result, the transcription of the target
genes is decreased. This regulation assures a steady
supply of cholesterol and fatty acids by preventing
their overaccumulation. It has been reported that
when the cDNAs that terminate before the first trans-
membrane domain of SREBPs are transfected into
cells, the SREBPs constitutively accumulate in the nu-
clei independently of the intracellular sterol content
(Sato et al., 1994; Wang et al., 1994; Yang et al.,
1994, 1995). Therefore, it is probable that nuclear import of
the mature form occurs via a sterol-independent
mechanism. However, SREBPs possess no consensus
sequence with previously identified NLSs, and signif-
icantly less is known about the overall nuclear import
mechanism.

This paper reports a study of the molecular mecha-
nism of the nuclear import of the mature form
SREBP-2. A variety of in vivo and in vitro experiments
show that importin β interacts directly with SREBP-2
and mediates import in a Ran-dependent manner. In
addition, we show that the HLH-Zip motif of the
SREBP-2 contains a novel type of NLS, which directly
binds to importin β.

MATERIALS AND METHODS

Construction of Plasmids

The XhoI–NolI fragment encoding an active form of human SREBP-2
(1150 amino acids 1–481) was extensi-
from pSREBP2(1–481) (Sato et al.,
1996) and subcloned into the Salt–NolI sites of pGEX-6P-3 (Pharma-
cia, Piscataway, NJ). A FLAG tag with BglII and BamHI sites at the ends was generated by annealing two synthetic oligonu-
lotides (5′-GATCTGACTACAAGGACGACGATGACAAGG-3′ and
5′-GATCCCTTGTACATGCTGCTCTGTAGTCA-3′) and
inserting them into the BamHI site of the above construct. The resultant
construct is referred to as pGEX FL-SREBP2. To generate a His-
tagged SREBP-2(1–481) expression vector, pRSETA-SREBP2, the
BamHI–NolI fragment from the pGEX FL-SREBP2 was inserted into the
BamHI–PvuII sites of pRSETA (Invitrogen, San Diego, CA) after
blunting the NolI site. To construct the expression vector for the
His-tagged SREBP-2(1–370) mutant, the fragment encoding amino
acids 1–370 of SREBP-2 was amplified using specific primer pairs
with BamHI and EcoRI at the ends and subcloned into the BamHI–
EcoRI sites of the pRSETA. To generate the plasmids which encode
for the His-tagged green fluorescent protein (GFP) chimera, the
expression vector pRSETA-GFP* was engineered from the pRSETA
by inserting an amplified GFP into the BamHI site and introducing
NolI and EcoRV cutting sites in the multicloning site using two
oligonucleotides (5′-CCGGCGGAGAGATGGATGATG-3′ and
5′-AATTCGATATCGATGATGATG-3′). To construct
pRSETA GFP-SREBP2, the expression vector encoding for the His-
tagged SREBP-2 protein fused with GFP at the N terminus, the
BamHI–NolI fragment from pGEX FL-SREBP2 was inserted into the
same restriction sites of pRSETA-GFP*. To produce the expression
vectors encoding the His-tagged GFP chimera of SREBP-2 deletion
mutants, pRSETA GFP-SREBP2(1–403), pRSETA GFP-SREBP2(1–
370), pRSETA GFP-SREBP2(1–317), and pRSETA GFP-SREBP2(343–
403), each appropriate fragment from SREBP-2 was amplified using
the specific primer pairs with BamHI and EcoRI at the ends and
subcloned into the BamHI–EcoRI sites of the pRSETA-GFP*. The
expression vector encoding GST-GFP-SREBP2(343–460), pGEX
GFP-SREBP2(343–460), was constructed by inserting an amplified
fragment into the BamHI–EcoRI sites of the pGEX-6P-2-hGFP, which
carrries the S65A/Y145F humanized GFP gene at the N terminus of
the multicloning site (kindly provided by Dr. S. Kuroda, Institute of
Scientific and Industrial Research, Osaka University). The expres-
sion vector encoding His-tagged mouse importin α (PTAC58),
pRSETA-PTAC58, was constructed by ligating the encoding full-
length m-importin α (Imamoto et al., 1995b) to pRSETA in frame.

Expression and Purification of Recombinant
Proteins

To express GST-FLAG-SREBP-2, Escherichia coli strain BL21, which
had been transformed with pGEX FL-SREBP2, was grown in Luria-
Bertani medium containing 100 µg/ml ampicillin at 37°C to a
density of 1.2 (OD600). Expression was induced by the addition of 1 mM
isopropyl-β-D-thiogalactopyranoside and incubated for 14 h at
20°C. Cells were harvested by centrifugation and resuspended
in high-salt buffer (30 mM Tris-HCl, pH 8.0, and 500 mM NaCl)
containing 1 mM PMSF, 1 mM DTT, and protease inhibitor mixture
(1 µg/ml each aprotinin, leupeptin, and pepstatin), using 1/25 vol
of the original cell culture. After two freeze–thaw cycles, PMSF was
again added to the cell suspension to a final concentration of 1 mM,
and cells were lysed by sonication. After the extract was clarified by
centrifugation, glycerol was added to the supernatant to a final
concentration of 10%, and the extract was incubated with glutathi-
one-Sepharose (Pharmacia) at 4°C. The recombinant protein-bound
Sepharose was washed exhaustively with cleavage buffer (50 mM
Tris-HCl, pH 7.0, 100 mM NaCl, and 1 mM DTT) containing pro-
tease inhibitor mixture and incubated with Prescision Protease
(Pharmacia) at 5°C for 4 h. Partially purified recombinant FLAG-
SREBP-2, which is cleared from the GST moiety but associated with

Vol. 10, July 1999 2223
Further purification was performed as follows. The flow-through was collected, eluted with a PD10 column (Pharmacia) equilibrated with transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, and 0.5 mM EGTA) containing 2 mM DTT and protease inhibitor mixture, followed by concentration by ultrafiltration using Centricon 30 (Amicon, Beverly, MA).

Peak fractions containing FLAG-SREBP-2 (eluted between 300 and 350 mM NaCl) were pooled and desalted with a PD10 column (Pharmacia) equilibrated with transport buffer containing 2 mM DTT and protease inhibitor mixture and then concentrated. Note that the partially purified FLAG-SREBP-2 had the same activity relative to nuclear import as the purified FLAG-SREBP-2 when examined in digitonin-permeabilized cell transport assays, as well as by microinjection.

GST–FLAG–SREBP-2 protein was eluted from the protein-bound glutathione beads with elution buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 20 mM glutathione) containing 1 mM DTT and protease inhibitor mixture and purified on a Mono S column (1 ml; Pharmacia) at a flow rate of 0.5 ml/min with a linear gradient of 0.02–1.0 M KCl in 10 mM potassium phosphate, pH 6.7, 1 mM DTT, and protease inhibitor mixture. Pooled fractions containing GST–FLAG–SREBP-2 were dialyzed against transport buffer containing 2 mM DTT and protease inhibitor mixture.

GFP-SREBP-2(343–460) protein was purified from E. coli strain BL21 transformed with pGEX-GFP-SREBP2(343–460) using glutathione-Sepharose in the same manner as for partially purified FLAG–SREBP-2.

His-SREBP-2(1–370) protein was expressed in E. coli strain BL21(DE3)pLysS transformed with pRSETA SREBP2(1–370) and thione-Sepharose in the same manner as for partially purified BL21 transformed with pGEX GFP-SREBP-2(343–460) using glutathione-Sepharose. Murine IgG1 monoclonal anti-penta His antibody was purchased from Qiagen.

Monoclonal anti-penta His antibody was purchased from Qiagen. Monoclonal anti-human transportin antibody was purchased from Transduction Laboratories (Lexington, KY).

Microinjection

HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Gaithersburg, MD) supplemented with 5% FBS and plated on coverslips 36–48 h before use. Proteins were injected through a glass capillary into the cytoplasm of HeLa cells, which were grown on coverslips. After incubation for 30 min at 37°C or on ice, the cells were fixed with 3.7% formaldehyde in PBS. To examine the localization of injected FLAG–SREBP-2, fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, incubated with 3% skim milk in PBS for 20 min, and then incubated with 30 µg/ml monoclonal anti-FLAG M2 antibody for 1 h at room temperature. The mouse antibody was detected with Cy3-labeled goat anti-mouse IgG (Amersham, Arlington Heights, IL). To examine the localization of injected His–SREBP-2(1–370), fixed and permeabilized cells were subjected to indirect immunofluorescence using rabbit anti-human SREBP-2 at a 1:200 dilution and Cy3-labeled goat anti-rabbit IgG (Amersham). All samples were examined by Axioskop microscopy (Carl Zeiss, Thornwood, NY).

In Vitro Transport Assay

Transport assays were performed essentially as described previously (Adam et al., 1990; Imamoto et al., 1995c). Briefly, HeLa cells were plated at a density of 1 × 10⁵ cells/ml on an eight-well multistep slide (6040805; ICN, Costa Mesa, CA) 36–48 h before use. The cells grown on slides were rinsed twice in an ice-cold transport buffer and permeabilized for 5 min in ice-cold transport buffer containing 40 µg/ml digitonin (nacalai tesque, 123-50; diluted from a 20 mg/ml stock solution in DMSO), 2 mM DTT, and protease inhibitor mixture. After removing the digitonin-containing buffer, the slides were washed twice and immersed in an ice-cold transport buffer containing 2 mM DTT and protease inhibitor mixture for 5 min. The slides were then blotted to remove excess buffer, and 10 µl of reaction mixture per single well was applied to the cells. Import reactions were performed by incubating the slides for 20 min at 30°C unless otherwise indicated. After incubation, the cells were fixed with 3.7% formaldehyde in transport buffer for 15 min at room temperature. For wheat germ agglutinin (WGA) treatment, permeabilized cells were incubated with 0.5 mg/ml WGA (E.Y. Laboratories, San Mateo, CA) in transport buffer containing 2 mM DTT and protease inhibitor mixture for 5 min on ice before the import reaction. All reaction mixtures contained 2% BSA, 2 mM DTT, and protease inhibitor mixture in transport buffer. As described in the respective figure legends, each reaction mixture contained an import substrate combined with cytosol or a combination of recombinant transport factors at the indicated amounts in the presence or absence of a ATP regeneration system (1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase) and 0.5 mM GTP. Total cytosol from Ehrlich ascites tumor cells was prepared as described previously (Imamoto et al., 1995c). For the competition experiments, biotinylated BSA, which was chemically coupled to a synthetic peptide containing the SV40 T-antigen NLS (PKKKRKVE) was ligated to the Bmi1–Smad sites of pGST–GFP (Tachibana et al., 1996). GST–NLS–GFP fusion protein was expressed and purified to homogeneity using glutathione-Sepharose following the manufacturer’s recommendations. Aliquots of each recombinant protein were frozen in liquid nitrogen and stored at −80°C.

Antibodies

Rabbit anti-importin β polyclonal antibodies were prepared as described previously (Kose et al., 1997), as were rabbit anti-importin α (PTAC58; mouse Rch1) polyclonal antibodies (Sekimoto et al., 1997). A polyclonal antibody (RS004) against human SREBP-2 was produced by injecting rabbits with a fusion protein encoding six histidines followed by amino acids 1–481 of human SREBP-2. The fusion protein constructs were cloned into a PET28(a) vector (No-vagen, Madison, WI), expressed in E. coli, and purified by Ni²⁺–Sepharose affinity chromatography. Murine IgG1 monoclonal anti-importin β (Imamoto et al., 1995b) was purchased from Kodak (Rochester, NY). Monoclonal anti-penta His antibody was purchased from Qiagen.

To examine the import of FLAG–SREBP-2, the fixed cells were rinsed in PBS, permeabilized with 0.2% Triton X-100 in PBS for 5
min at room temperature, and subjected to indirect immunofluorescence using a monoclonal anti-FLAG M2 antibody (see Microinjection). The import of GST-NLS-GFP and GFP-SREBP-2 (343–460) was detected by Axiophot microscopy after fixation.

**Immunoprecipitation**

Total cytosol from Ehrlich ascite tumor cells was prepared in transport buffer containing 2 mM DTT and protease inhibitor mixture as described above and preclarified by incubation with protein G-Sepharose (Pharmacia). Five-microliter aliquots of protein G-Sepharose beads were incubated with 6 μg of monoclonal anti-FLAG M2 antibody for 1 h at room temperature; the unbound antibody was then removed. The resultant anti-FLAG antibody-coupled beads were incubated on a turntable for 2 h at 4°C with 90 μl of clarified cytosol in the presence or absence of 10 μg of partially purified recombinant FLAG-SREBP-2. After incubation, the beads were pelleted and washed three times in transport buffer containing 200 mM NaCl. The bound fraction was eluted by boiling in SDS-PAGE sample buffer and analyzed by 12.5% SDS-PAGE followed by Coomassie blue staining and immunoblotting using the antibodies described in the figure legends. The protein bands were visualized by an ECL Western blotting detection kit (Amersham).

**Solution Binding Assay Using Cytosolic Extract**

Binding assays were performed in transport buffer containing 2 mM DTT and protease inhibitor mixture. To preclar the cytosol, purified GST and glutathione-Sepharose were incubated with the cytosol for 1 h at 4°C, and the resin was removed by centrifugation. Ten micrograms of purified GST-SREBP-2, GST-NLS-GFP, or GST were incubated with 90 μl of clarified cytosol for 30 min on ice and centrifuged at 15,000 rpm for 20 min to remove any insoluble material. The supernatants were then incubated with 15 μl of glutathione-Sepharose on a turntable for 1 h at 4°C. After incubation, the beads were washed five times with binding buffer. The bound fractions were analyzed by 10% SDS-PAGE and immunoblotting using the monoclonal anti-penta His antibody.

**Immunoblotting**

Proteins were separated on 10 or 12.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. After blocking with 3% skim milk in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween 20), the blots were probed with rabbit polyclonal antibodies: anti-importin α (diluted 1:100), anti-transportin (diluted 1:100), or mouse monoclonal antibodies: anti-penta His (diluted 1:5000), or mouse monoclonal antibodies: anti-PTAC58 (diluted 1:5000), or mouse monoclonal antibodies: anti-transportin (diluted 1:100) and anti-penta His antibody (diluted 1:1000). The probed antibodies were detected by standard methodology using alkaline phosphatase–coupled secondary antibodies unless otherwise indicated.

**RESULTS**

**SREBP-2 Is Actively Imported into the Nucleus by Soluble Mediator(s)**

The mature form SREBP-2 is an N-terminal fragment (~480 amino acids) with an apparent molecular mass of ~60 kDa, which is referred to herein as SREBP-2. This molecule lacks the classical basic-type NLSs. To understand the mechanism of the nuclear import of SREBP-2, we prepared various epitope-tagged recombinant N-terminal 481-amino-acid fragments. As shown in Figure 1A, when FLAG-tagged SREBP-2 was injected into the cell cytoplasm, the SREBP-2 accumu-
lated in the nucleus 30 min after injection. The import was found to be temperature dependent and sensitive to the coinjection of WGA (Finlay et al., 1987; Yoneda et al., 1987).

Next, to examine factor requirements for the import, we subjected the FLAG-tagged SREBP-2 to an in vitro cell-free transport assay. Nuclear accumulation of FLAG-SREBP-2 was reconstituted in the presence of cytosol and an ATP regenerating system and inhibited by WGA and energy depletion (Figure 1B). Note that sterol depletion had no effect on either the in vivo or in vitro assays (our unpublished results). These results indicate that the nuclear import of SREBP-2 is an active process, which is mediated by soluble factor(s), and that this import is not dependent on cellular sterol levels, which is consistent with the previous findings obtained by transfection experiments (Sato et al., 1994; Wang et al., 1994; Yang et al., 1994, 1995).

The Nuclear Import of SREBP-2 Is Not Identical to the Basic NLS-mediated Import

To characterize the nuclear transport pathway of SREBP-2, we tested whether the excess amount of the basic-type NLS conjugates are able to compete with the SREBP-2 import in vitro. We used a chimeric protein consisting of GST fused with SV40 T antigen NLS and GFP (GST-NLS-GFP) as a typical basic-type NLS-bearing transport substrate. BSA conjugated with peptides containing the NLS of SV40 T antigen (T-BSA) was used as an unlabeled competitor. As expected, the import of GST-NLS-GFP was completely inhibited in the presence of a 10-fold excess of unlabeled competitor. In contrast, the nuclear import of FLAG-SREBP-2 was only slightly inhibited even in the presence of a 30-fold excess of T-BSA (Figure 2A). This result suggests that the nuclear import machinery of SREBP-2 is not identical to that of the basic NLS-mediated import.

However, the possibility remains that both SREBP-2 and basic NLS-bearing proteins are imported by the importin α/β heterodimer but independently bind to different sites of importin α.

To exclude the possibility, we examined whether SREBP-2 is capable of interacting with importin α in the cytosol using immobilized recombinant GST-SREBP-2 followed by immunoblotting with antibodies that are specific for importin α family members. GST-NLS-GFP was used as a positive control. As shown in Figure 2B, no interaction of GST-SREBP-2 with the

Figure 2. (A) Basic NLS-containing substrate cannot compete with the nuclear import of SREBP-2 in permeabilized cells. Digitonin-permeabilized cells were incubated with 10 μl of a reaction mixture containing 4 pmol of GST-NLS-GFP or FLAG-SREBP-2 in the absence (−) or presence of 40 pmol (+ 10× T-BSA), 80 pmol (+ 20× T-BSA), or 120 pmol (+ 30× T-BSA) of T-BSA. All reaction mixtures contained 4 mg/ml cytosol and an ATP regeneration system. After incubation for 20 min at 30°C, the cells were fixed, and the localization of GST-NLS-GFP and FLAG-SREBP-2 was examined by fluorescent microscopy. All photographs for the same substrate were taken with the same exposure time, and the images were obtained by scanning photographic negatives using Adobe (Mountain View, CA) Photoshop version 5.0 under the same conditions. (B) SREBP-2 does not interact with importin α. Cytosol (90 μl) was incubated with GST (G), GST-NLS-GFP (T), or GST-SREBP-2 (S). GST fusion proteins were then captured on glutathione-Sepharose beads. The beads were then washed, and the bound proteins were eluted by boiling in SDS sample buffer. Eluates were separated on 10% SDS-PAGE and analyzed by immunoblotting using anti-mouse importin α polyclonal antibodies. Cytosol (2 μl) was loaded directly onto SDS-PAGE (C).
Rch1 family of importin α was observed. Neither of the two other families of importin α, NPI-1 and Qip1, interacted with SREBP-2 (our unpublished results). These collective findings indicate that the nuclear import of SREBP-2 occurs in a manner that is independent of importin α.

**SREBP-2 Directly Binds to Importin β**

We next studied the involvement of small GTPase Ran in the nuclear import of SREBP-2 by using a G19VRan mutant, which is deficient in GTPase activity and remains in the GTP-bound state, even in the presence of cytoplasmic RanGAP1 (Carey et al., 1996). Because the GTP-bound Ran triggers the release of cargoes from the importin β family import factors, the addition of G19VRan-GTP has been shown to block several nuclear import pathways that are mediated by the importin β family import factors (Sekimoto et al., 1996; Kose et al., 1997). By coinjecting G19VRan-GTP into the cell cytoplasm, the nuclear import of SREBP-2 was strongly inhibited (Figure 3). This predicts that the nuclear import of SREBP-2 would be mediated by the importin β family molecule and dependent on the Ran GTPase cycle.

In this regard, we attempted to examine the issue of whether the importin β family transport factors interact with SREBP-2. As shown in Figure 4A, one major protein of ~94 kDa was coprecipitated with FLAG-SREBP-2 from the Ehrlich ascites tumor cell cytosol by the anti-FLAG antibody. Immunoblotting in conjunction with importin β-specific antibodies showed that the communoprecipitated fraction actually contained importin β. No significant interaction of transportin with SREBP-2 was detected, as evidenced by the transportin-specific antibody. By using the solution binding assay with GST-SREBP-2 as in Figure 2B, we were able to confirm that SREBP-2 efficiently interacts with importin β (Figure 4B).

The finding that SREBP-2 interacts with importin β but not with importin α raises two possibilities: first, that SREBP-2 binds directly to importin β, and second, that SREBP-2 requires an adapter molecule other than importin α to form a complex with importin β. To address these issues, we tested whether recombinant GST-importin β is able to bind directly to His-tagged recombinant SREBP-2. The *E. coli* lysate, which contained a His-tagged importin α, was used as a positive control material. Each lysate was incubated with immobilized GST-importin β, and the bound proteins were analyzed by immunoblotting with anti-His tag antibody. The results clearly show that His-SREBP-2 is able to bind directly to importin β, and that His-importin α binds to GST-importin β (Figure 4C, left panel). By using the purified recombinant FLAG-SREBP-2 and GST-importin β, we confirmed that SREBP-2 directly binds to importin β independently of an adapter protein (Figure 4C, right panel). Moreover, as shown in Figure 4C, left panel, it was confirmed that neither importin α nor transportin binds to SREBP-2.

**Importin β Mediates the Nuclear Import of SREBP-2 in a Ran-dependent Manner**

To test whether the GTP-bound state of Ran causes the dissociation of the SREBP-2–importin β complex, immobilized GST-importin β, which had been prebound to His-SREBP-2, was incubated with buffer containing Ran-GDP or Ran-GTP (G19VRan-GTP), and the eluate and bound fractions were examined by immunoblotting using anti-His tag antibody. As shown in Figure 4D, SREBP-2 was released after incubation with Ran-GTP but not with Ran-GDP.

We examined the issue of whether importin β mediates the nuclear import of SREBP-2 by using the in vitro transport assay. As shown in Figure 5, in the presence of importin β alone, FLAG-SREBP-2 was targeted to the nuclear rim of the permeabilized cells. With the further addition of Ran and p10/NTF2, the efficient nuclear accumulation was completely reconstituted. These results clearly indicate that the nuclear import of SREBP-2 is mediated by importin β in conjunction with Ran and p10/NTF2.

**An HLH-Zip Domain Is Required for the Nuclear Import of SREBP-2**

To determine the regions of SREBP-2 that are required for the binding to importin β, we constructed the subsets of SREBP-2 deletion mutants (shown in Figure 6A) and performed binding assays using *E. coli* lysate expressing each SREBP-2 deletion mutant and GST-importin β as described in Figure 4C. As shown in Figure 6B, residues 1–403 of SREBP-2 bound as efficiently as the full-length protein (1–481). Further C-terminal deletion of 33 amino acids (1–370) severely abolished binding activity. The N-terminal deletion of 342 residues (343–403) did not reduce but, rather, increased the binding activity. These results indicate that residues 371–403 of SREBP-2 are necessary and, at most, residues 343–403 are sufficient for binding to importin β.
The issue of whether the importin β binding domain (343–403) confers import was examined by means of in vivo and in vitro assays. For this, we produced GFP fused with SREBP-2(343–460) mutant protein instead of the (343–403) mutant, because GFP-SREBP-2(343–403) protein is sufficiently small (30 kDa) to diffuse through the NPC. When injected into the cytoplasm, purified GFP-SREBP-2(343–460) accumulated efficiently in the nucleus (Figure 7A). The import was completely blocked when WGA was coinjected (our unpublished results). In the permeabilized cells, GFP-SREBP-2(343–460) docked at the nuclear rim as the result of the exogenous addition of importin β alone and was imported into the nucleus with further addition of Ran and p10/NTF2 (Figure 7B). These results indicate that GFP-SREBP-2(343–460) was sufficient to reproduce the nuclear import of the mature form SREBP-2(1–481) in vivo and in vitro. On the other
hand, recombinant SREBP-2(1–370) was not imported into the nucleus (Figure 7A). Collectively, these results suggest that the minimum domain that is sufficient for SREBP-2 import lies within residues 343–460, probably residues 343–403 of SREBP-2, which contains an HLH-Zip motif but not the preceding basic domain, which consists of the cluster of basic amino acids (see Figure 6A and DISCUSSION). This is the first evidence that the HLH-Zip region functions as an NLS.

**DISCUSSION**

The present study describes the characterization of the nuclear import of the mature form of SREBP-2 by using in vivo microinjection experiments and in vitro transport assay. The results show that SREBP-2 is actively imported via SREBP-2–importin β complex formation in a Ran-dependent manner. Furthermore, it was found that the HLH-Zip is responsible for the binding to importin β and hence acts as a novel NLS. These findings extend our understanding relative to the nuclear import mechanisms of bHLH-Zip–containing transcription factors and the versatility of importin β.

We have shown that a saturable amount of basic NLS-containing substrate does not completely prevent the import of SREBP-2 in permeabilized cells (Figure 2A). It is noteworthy, however, that a slight (~30%) decrease in SREBP-2 import was observed in the presence of a 10-fold excess of the competitor but that no more decrease was observed on increasing dose of the competitor. This observation is somewhat surprising, because basic NLS-bearing substrates also use importin β via importin α-family adapters. Several possible explanations exist for explaining this phenomenon. First, the recycling of
importin \( \beta \) may occur more efficiently than importin \( \alpha \), and, as a result, a significant amount of free importin \( \beta \) may remain unoccupied by importin \( \alpha \) even in the presence of a large amount of cargoes for importin \( \alpha \). Because it has been clearly shown that importin \( \beta \) alone can shuttle between the nucleus and the cytoplasm (Kose et al., 1997, 1999), whereas importin \( \alpha \) is exported via a specific export receptor, CAS (Kutay et al., 1997), it is reasonable to speculate that importin \( \beta \) returns to the cytoplasm more efficiently than importin \( \alpha \) without limitation by export carrier molecules. Alternatively, considering the fact that this experiment was performed using the total cytosol as a source of transport factors, unknown modifying factor(s), which may regulate importin \( \beta \)-mediated import, are present.

In the immunoprecipitation experiment using the cell extract, SREBP-2 significantly bound to importin \( \beta \), whereas no significant binding to other importin \( \beta \) family members was observed (Figure 4A). Even transportin, a closely related homologue of importin \( \beta \), failed to interact with SREBP-2 (Figure 4, A and C). Therefore, although the possibility that other members of importins also mediate the SREBP-2 import cannot be completely excluded, it is probable that importin \( \beta \) is the principal import receptor for SREBP-2. As mentioned in INTRODUCTION, importin \( \beta \) has been shown to carry nuclear proteins in two ways: 1) via the importin \( \alpha \)-family adapters and 2) via direct interaction with the nuclear protein. Cargoes that are carried by importin \( \beta \) appear to be divided into two groups by virtue of receptor selectivity: 1) karyophiles, which can be imported by several importin \( \beta \)-related receptors, and 2) molecules, which are transported exclusively by importin \( \beta \). The former includes ribosomal proteins, whereas the latter includes SREBP-2 as well as importin \( \alpha \) family proteins. It has been suggested that, during evolution, an importin \( \alpha \)-independent common ancestor gave rise to the importin \( \alpha \)-dependent importin \( \beta \) molecule together with importin \( \alpha \), and, at some point afterward, importin \( \alpha \) was divided into several groups (Malik et al., 1997). Therefore, the former group might consist of evolutionary old karyophiles, which were already in existence before importin \( \beta \) diverged, whereas the latter might be newer, having appeared at the stage of evolution of importin \( \beta \). Homologues of SREBP are consistently observed only in higher eukaryotes, ranging from the fly (Drosophila melanogaster) (Rosenfeld and Osborne, 1998) to mammals (human, mouse, rat, and hamster).

The bHLH-Zip motifs are found in a large number of eukaryotic transcription factors. The basic region of bHLH-Zip proteins binds to specific sequences in DNA, and the adjacent HLH-Zip region mediates homo- and heterodimerization (for review, see Ferré-D’Amaré and Burley, 1995). We have demonstrated herein that the HLH-Zip domain is responsible for binding to importin \( \beta \) and the nuclear import of SREBP-2 (Figures 6 and 7). Three SREBPs, designated SREBP-1a, -1c, and -2, are known to exist in the nucleus (Hua et al., 1993; Tontonoz et al., 1993; Yokoyama et al., 1993). The mature forms of these SREBPs are most highly conserved (\(-71\% \) identical) in the bHLH-Zip region, whereas they are varied in other regions. Therefore, it is possible that SREBP-1a and -1c are also recognized and imported by importin \( \beta \) by virtue of the conserved HLH-Zip domain. Furthermore, it would be worthwhile to determine whether importin \( \beta \) binds to dimerized HLH-Zip of SREBP-2. If this is the case, homo- or heterodimerization with the proper partner would be required, not only for the productive binding to target DNA sequences but also for efficient nuclear import, leading to the highly precise regulation of lipid metabolism.

SREBP-2 represents the first example of a protein that contains an NLS within the HLH-Zip motif. c-Myc and USF2, both of which contain bHLH-Zip motifs that are structurally very similar to SREBP-2, have two NLSs, one within the basic region and the other upstream of the basic region (Dang and Lee, 1988; Luo and Sawadogo, 1996). Either of the two NLSs consists of basic amino acids, implying that the nuclear import of these proteins is importin \( \alpha / \beta \) dependent. In fact, Saphire et al. (1998) have demonstrated that the importin \( \alpha / \beta \) heterodimer mediates the nuclear import of c-Myc basic NLS-containing substrate in vitro. Conversely, both the basic region and the upstream domain of SREBP-2 are dispensable for its nuclear localization. One possible explanation for the difference in the importance of the basic region arises from structural aspects. All bHLH-Zip–containing proteins including SREBPs bind to palindromic sequences containing a so-called E box (CANNTG). Unlike other E box–binding proteins, SREBPs have an atypical tyrosine, instead of a conserved arginine in the basic regions, allowing them to recognize an asymmetric target sequence called the sterol regulatory element (SRE; 5’-ATCACCCAC-3’) as well as an E box sequence (Párraga et al., 1998). Therefore, it is possible that such an unusual structure in the basic region might affect not only the DNA-binding properties but also the affinity for importin \( \alpha \).

Recent studies have revealed that each member of the import receptor can import a variety of cargoes rather than a single specific class of karyophiles. To date, three classes of importin \( \beta \)-binding sequences have been identified: the IBB domain of importin \( \alpha \) (Görlich et al., 1996; Weis et al., 1996), the beta-like import receptor binding domain on ribosomal proteins (Jäkel and Görlich, 1998), and the RGG repeat of yeast Nab2p (Truant et al., 1998). This study points to the fact that importin \( \beta \) recognizes a wider variety of
signals than has previously been expected. These importin β-binding signals, including HLH-Zip of SREBP-2, share little homology with one another except for some basic residues. How does importin β carry these different classes of cargoes into the nucleus? The binding site in importin β for ribosomal proteins has been shown to be distinct from that for importin α (Jäkel and Görlich, 1998). We also have collected some preliminary data that indicate that the SREBP-2 binding site and the importin α binding site in importin β are not identical (our unpublished results). Thus, does importin β sort out various cargoes via the use of its own different regions? Or is there a regulatory mechanism in cells for cargo selectivity? Further studies, including structural analysis, will be required to address these questions.

ACKNOWLEDGMENTS

We thank Dr. S. Kuroda (Institute of Scientific and Industrial Research, Osaka University) for the gift of pGEX-6P-2-hGFP. This work was supported by grant-in-aid for scientific research on priority areas 07282103, grant-in-aid for scientific research (B) 08458229, grant-in-aid for scientific research (C) 09680692, and grant-in-aid for Center-of-Excellence research 07CE2006 from the Japanese Ministry of Education, Science, Sports and Culture.

REFERENCES


system is essential for transport into and out of the nucleus. EMBO J. 16, 6535–6547.


