The Nuclear Import of the Human T Lymphotropic Virus Type I (HTLV-1) Tax Protein Is Carrier- and Energy-independent*

Received for publication, December 19, 2006, and in revised form, March 6, 2007 Published, JBC Papers in Press, March 6, 2007, DOI 10.1074/jbc.M611629200

Takahiro Tsuji†, Noreen Sheehy‡, Virginie W. Gautier¶, Hitoshi Hayakawa†, Hirofumi Sawa†, and William W. Hall†∥

From the †Centre for Research in Infectious Disease, School of Medicine & Medical Science, University College Dublin, Belfield, Dublin 4, Ireland and the ‡Department of Molecular Pathobiology and 21st Century COE Program for Zoonosis Control, Hokkaido University Research Center for Zoonosis Control, N18, W9, Kita-ku, Sapporo, 060-0818, Japan

HTLV-1 is the etiologic agent of the adult T cell leukemia-lymphoma (ATLL). The viral regulatory protein Tax plays a central role in leukemogenesis as a transcriptional transactivator of both viral and cellular gene expression, and this requires Tax activity in both the cytoplasm and the nucleus. In the present study, we have investigated the mechanisms involved in the nuclear localization of Tax. Employing a GFP fusion expression system and a range of Tax mutants, we could confirm that the N-terminal 60 amino acids, and specifically residues within the zinc finger motif in this region, are important for nuclear localization. Using an in vitro nuclear import assay, it could be demonstrated that the transportation of Tax to the nucleus required neither energy nor carrier proteins. Specific and direct binding between Tax and p62, a nucleoporin with which the importin beta family of proteins have been known to interact, was also observed. The nuclear import activity of wild type Tax and its mutants and their binding affinity for p62 were also clearly correlated, suggesting that the entry of Tax into the nucleus involves a direct interaction with nucleoporins within the nuclear pore complex (NPC). The nuclear export of Tax was also shown to be carrier independent. It could be also demonstrated that Tax itself may have a carrier function and that the NF-κB subunit p65 could be imported into the nucleus by Tax. These studies suggest that Tax could alter the nucleocytoplasmic distribution of cellular proteins, and this could contribute to the deregulation of cellular processes observed in HTLV-1 infection.

Human T cell lymphotropic virus type-I (HTLV-1)² is the etiologic agent of the malignant disorder adult T cell leukemia-lymphoma (ATLL) (1, 2). Whereas the pathogenesis of ATLL is unclear, the HTLV-1 regulatory protein Tax is thought to play a central role in leukemogenesis. Tax has been shown to immortalize human T cells (3) and transform fibroblast cells (4) in vitro, and transgenic animals expressing Tax have developed a range of malignancies (5–8). The mechanisms of the transformation are not fully understood, but have been shown to be related to the ability of Tax to dysregulate the transcription of genes involved in cellular proliferation, cell-cycle control, and apoptosis (9–11). Tax is a potent transcriptional transactivator not only of viral but also of cellular gene expression. The protein physically interacts with a number of cellular transcription factors, which including components of the NF-κB-Rel signaling complex, and persistent and constitutive activation of NF-κB is central to the development and maintenance of the malignant phenotype in ATLL (10–12).

Activation of NF-κB involves Tax activity in both the cytoplasm and nucleus. In the cytoplasm, Tax activates the kinase activity of IKK complex by directly interacting with IKKγ/ NEMO subunit. IκBα, which sequesters NF-κB in the cytoplasm, is phosphorylated by the Tax-IKK complex and subsequently degraded allowing the nuclear translocation of NF-κB (13). In the nucleus, Tax has also been shown to co-localize with NF-κB as well as basic transcriptional factors such as p300/CBP in nuclear speckle structures, the so-called Tax speckle structure (TSS), where active transcription of a range of cellular genes occurs (14, 15).

Consistent with both its cytoplasmic and nuclear activities, Tax has been shown to be distributed in both compartments in HTLV-1-infected and Tax-transfected cells. In initial studies, Tax was reported to be found predominantly in the nucleus and specifically accumulated in the nuclear speckled structures (16, 17). However, depending on the cell type, significant amounts of Tax have also been found in the cytoplasm (18–20). Specifically, cytoplasmic Tax was shown to co-localize in the endoplasmic reticulum, Golgi apparatus, and mitotic organizing center (MTOC), and appeared to affect both protein secretion and microtubule organization (21, 22). Recent studies employing a heterokaryon fusion system have also clearly demonstrated that Tax can effectively shuttle between the cytoplasm and the nucleus (18).

Nuclear transport of proteins occur through the nuclear pore complex (NPC), and in most cases involves an interaction between transport carriers and a nuclear localization signal (NLS) on the cargo protein (23, 24). Most transport carriers belongs to the importin (karyopherin) family and mediate transport either as monomers and heterodimers. Importin α/β heterodimers import cargo proteins containing a “classical” NLS which is generally rich in lysine residues (23). Importin β monomer imports a range of cargo proteins including the parathyroid hormone-related protein (25), the sterol regulatory

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed. Tel.: 353-1-716-1229; Fax: 353-1-716-1239; E-mail: william.hall@ucd.ie.

2 The abbreviations used are: HTLV-1, human T cell lymphotropic virus type-I; GST, glutathione S-transferase; WGA, wheat germ agglutinin; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; TSS, Tax speckle structure; NPC, nuclear pore complex; ATLL, adult T cell leukemia-lymphoma; RRL, rabbit reticulocyte lysate; DAPI, 4',6-diamidino-2-phenylindole; WT, wild type.
Nuclear Import of HTLV-1 Tax

element-binding protein 2 (SREBP-2) (26), the zinc finger protein Snail (27), as well as the viral proteins HIV-1 Rev (28, 29) and HTLV-1 Rex (30). Transportin monomer, which also belongs to the importin β family, imports cargo proteins such as hnRNP A1 protein (31), several kinds of histones (32) and ribosomal proteins (33). The import process requires metabolic energy and is propelled by a concentration gradient across the nuclear envelope of the GTP-bound form of small GTPase Ran, which is found at high concentrations in the nucleus (23). In contrast, the transport of a number of other proteins is carrier independent; these include contrast, the transport of a number of other proteins is carrier energy and is propelled by a concentration gradient across the ribosomal proteins (33). The import process requires metabolic energy and is propelled by a concentration gradient across the nuclear envelope of the GTP-bound form of small GTPase Ran, which is found at high concentrations in the nucleus (23). In contrast, the transport of a number of other proteins is carrier independent; these include contrast, the transport of a number of other proteins is carrier energy and is propelled by a concentration gradient across the ribosomal proteins (33). The import process requires metabolic energy and is propelled by a concentration gradient across the nuclear envelope of the GTP-bound form of small GTPase Ran, which is found at high concentrations in the nucleus (23). In contrast, the transport of a number of other proteins is carrier independent; these include contrast, the transport of a number of other proteins is carrier energy and is propelled by a concentration gradient across the ribosomal proteins (33). The import process requires metabolic energy and is propelled by a concentration gradient across the nuclear envelope of the GTP-bound form of small GTPase Ran, which is found at high concentrations in the nucleus (23). In contrast, the transport of a number of other proteins is carrier independent; these include}

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HeLa and COS7 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. HeLa cells were plated on 18-well printed slides (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD) for the in vitro nuclear import assays 24 h before the experiments. COS7 cells were plated on two-well chamber slides (Nalge Nunc International, Naperville, IL) for the in vitro assays 24 h before the experiments. COS7 cells were plated on two-well chamber slides (Nalge Nunc International, Naperville, IL) 24 h before transfection. Cells were transfected with 1 µg of plasmids using FuGENE6 (Roche Diagnostics, Mannheim, Germany).

**DNA Construction and Plasmids**—To create mammalian expression vectors for GFP-Tax/-Tax340/-Tax116/-Tax60/-Tax55/-Tax50, cDNA sequences encoding full-length Tax and the series of C-terminal deletion mutants were amplified by PCR and cloned into the HindIII/PstI sites of the pEGFP-C1 vector (Clontech).

The bacterial expression vectors for the SV40 T antigen NLS, GST-SV40TNLS-GFP (pGEX-SV40TNLS-GFP), GST-HA-importin β (pGEX-HA-importin β), GST-importin β (pGEX-importin β), and His₆-RanQ69L (pQE80-RanQ69L) were gifts from Dr. S. Kose and Dr. N. Imamoto (RIKEN, Saitama, Japan). To create the bacterial expression vectors for GST-Tax-GFP and GST-Tax340-GFP, the SV40-T-antigen NLS coding sequences of pGEX-SV40TNLS-GFP were swapped for the Tax and Tax1–340 coding sequences by utilizing BamHI/SmaI sites of the vector. To create bacterial expression vectors for GST-Tax340-CFP and GST-YFP-Rev, Tax1–340, and CFP encoding sequences from pECPF-C1 (Clontech) were cloned into the BamHI and SmaI sites of pGEX-2T vector (Amersham Biosciences), and YFP encoding sequences from pEYP-C1 (Clontech) and HIV-1 Rev encoding sequences were cloned into the SmaI and EcoRI sites of pGEX-2T vector. To construct bacterial expression vectors for GST-Tax, Tax encoding sequences were cloned into the BamHI site of pGEX-2T vector. The bacterial expression vectors for GST-p62FL [1–522], GST-p62N [1–265] and GST-p62C [178–252] were generated by cloning relevant sequences (41) from pcDNA3.1/His-p62 vector (a gift from Dr. N. Yaseen, Northwestern University, Chicago, IL) into pGEX-2T vector. To construct the bacterial expression vector expressing His₆-p65-YFP, p65 encoding sequencing amplified from pcDNA-p65 vector (a gift from Dr. D. Walls, Dublin City University, Dublin, Ireland) and YFP encoding sequences amplified from pEYFP-C1 (Clontech) vector were cloned into the BamHI and PstI sites of pQE80 (Qiagen) vector. QuikChange™ site-directed mutagenesis kit (Stratagene) was used for the creation of single amino acid mutations in Tax-encoding sequences.

**Expression and Purification of Recombinant Proteins**—All GST and His fusion proteins were purified from *Escherichia coli* strain BL21(DE) induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 14 h at 18 °C. Purifications were performed on glutathione-Sepharose 4B beads (Amersham Biosciences) for the GST fusions and on Ni-NTA agarose beads (Qiagen) for the His fusions, according to the manufacturer’s protocols. Tax/-TaxC23A/-TaxC29A/-TaxC36A/-TaxH41A-GFP, Tax340-GFP/-CFP, and HA-importin β were cleaved from GST by using thrombin. To obtain RanQ69L-GTP, 2 mM EDTA, 2 mM GTP, and 5 mM MgCl₂ were added to His₆-RanQ69L, which had been eluted from resin and incubated for 30 min on ice. All resultant proteins were dialyzed against transport buffer (TB; 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM EGTA, 2 mM dithiothreitol, 1 µg/ml aprotinin, leupeptin, and pepstatin A), and concentrated by ultrafiltration on Microcon (Amicon), and stored at −80 °C after snap freezing.

**In Vitro Nuclear Transport Assay**—In vitro nuclear import assays were performed essentially as described previously (34, 45). HeLa cells plated on glass slides were washed twice with ice-cold TB and permeabilized with digitonin (40 µg/ml, Sigma) in TB for 5 min on ice. Cells were then washed twice with ice-cold TB and soaked in TB for 10 min on ice. The standard reaction mixtures contained import substrates (~1 µM), an ATP regeneration system (1 mM ATP, 5 mM phosphocreatine, 20 units of creatine kinase) as a source of energy, and rabbit reticulocyte lysate (RRL, Promega) as a source of soluble import factors. The import reaction was performed for 20 min at 30 °C or on ice. For the export reactions, the cells initially subjected to the import reaction were immediately washed twice with TB and then were incubated with testing solutions for 20 min at 30 °C to examine export. The composition of each of the reaction mixtures is described in each figure legend. After the
transport reactions, the cells were washed twice with TB followed by fixation with 4% paraformaldehyde for 8 min at room temperature. The cells were washed twice with TB and mounted with 50% glycerol in TB, and examined by fluorescence microscopy. Fluorescent intensities in nuclei and in randomly chosen areas outside the nuclei were quantified by using ImageJ software (NIH).

Protein Binding Assay—Recombinant GST or GST fusion proteins were incubated with HA-importin β or GFP fusion proteins in a total volume of 100 μl in TB with 3% bovine serum albumin. After incubation for 20 min at room temperature, 10 μl of glutathione-Sepharose 4B beads were added to the reaction mixtures and incubated for 30 min at room temperature. The bead complexes were then washed four times with 500 μl of TB. Bound proteins were eluted from the beads with 25 μl of 10 mM reduced glutathione and analyzed by SDS-PAGE and immunoblotting. In immunoblotting, rabbit anti-GFP antibody (ab290, Abcam, UK) and mouse anti-HA antibody (HA-7, Sigma) were used, and the antibody detection was performed using the Superfemto chemiluminescent kit (Pierce).

RESULTS

The N Terminal 60 Amino Acids Are Important for the Nuclear Accumulation of Tax—Tax has been known to localize primarily in the nucleus in HTLV-1-infected cells (15,16,22,42–44,46). The 60 amino acids of the N terminus of Tax have been shown to be necessary for its nuclear localization, as fusion of this region permits nuclear localization of heterogeneous proteins, and deletion of the region results in the complete loss of Tax nuclear localization (42–44).

To confirm and extend these findings, we employed a GFP fusion protein system as this has been widely utilized in subcellular localization analyses of a number of proteins including Tax (19, 20, 47). DNA fragments encoding a full-length Tax or a series of C-terminal deletion mutants of Tax were inserted into the C terminus of a GFP expression vector, and their subcellular localizations were examined (Fig. 1A). As previously reported, GFP with full-length Tax localized both in the nucleus and in the cytoplasm with a speckled pattern (Fig. 1A) (20, 21). GFP with C-terminal deletions (Tax340, Tax220, Tax116, and Tax60 and as well as Tax280; Fig. 1A, and not shown) accumulated almost exclusively in the nucleus. Of note, Tax340, a mutant with the deletion of only the C-terminal 13 amino acids, completely lost its cytoplasmic localization pattern. Tax C-terminal deletion mutants shorter than Tax60 resulted in the leakage of the fusion protein into the cytoplasm (Tax55 and Tax50 as well as Tax45; Fig. 1A, and not shown). Therefore, as has been previously shown (42–44), it could be confirmed that the N-terminal 60 amino acids of Tax were the minimal region permitting the localization of the GFP fusion protein to the nucleus.

To determine which single amino acids may be critical for the nuclear localization of Tax, the subcellular localization of single amino acid mutants of GFP-Tax were examined (Table 1 and Fig. 1B). GFP with C-terminal deletion mutants (Tax340 and Tax220) and as well as Tax280; Fig. 1A, and not shown) accumulated almost exclusively in the nucleus. Of note, Tax340, a mutant with the deletion of only the C-terminal 13 amino acids, completely lost its cytoplasmic localization pattern. Tax C-terminal deletion mutants shorter than Tax60 resulted in the leakage of the fusion protein into the cytoplasm (Tax55 and Tax50 as well as Tax45; Fig. 1A, and not shown). Therefore, as has been previously shown (42–44), it could be confirmed that the N-terminal 60 amino acids of Tax were the minimal region permitting the localization of the GFP fusion protein to the nucleus.

Tax Enters the Nucleus by an Energy- and Carrier-independent Mechanism—To investigate the mechanisms by which Tax is transported into the nucleus, an in vitro nuclear import assay using digitonin-permeabilized cells was carried out (45).
Proteins larger than 40 kDa are generally transported through the NPC by an active and receptor-mediated mechanism (49). The import substrates used in these studies were full-length Tax, and the C-terminal deletion mutant Tax fused with GFP at their C terminus (Tax-GFP and Tax340-GFP) both of which are ~66 kDa in size and which would not be expected to enter the nucleus by a passive diffusion. GST-SV40TNI LS-GFP was employed as a control carrier-dependent import substrate. In the complete assay system, which contained both cytosol and energy, all three substrates were efficiently imported into the nucleus (Fig. 2A, panels a, d, and g). Wheat germ agglutinin (WGA) binds to glycosylated nucleoporins and blocks nuclear transport mediated via the NPC (50, 51). In cells treated with WGA, none of the substrates accumulated in the nucleus (Fig. 2A, panels b, e, and h). Nuclear import was also inhibited by “on-ice” incubation (Fig. 2A, panels c, f, and i). These results indicate that the nuclear import of Tax-GFP and Tax340-GFP are carried out by an active transport mechanism through the active transport channels of the NPC, and not by passive diffusion.

To further characterize the nuclear import of Tax, the effects of the depletion of energy or cytosol from import mixtures were examined. The depletion of energy markedly inhibited the nuclear import of GST-SV40TNI LS-GFP but had no effect on the nuclear import of Tax-GFP and Tax340-GFP (Fig. 2B, panels b, f, and j). Interestingly, Tax340-GFP was imported into the nucleus even in the absence of cytosol. In contrast, Tax-GFP was found also to preferentially localize to the nuclear membrane and did not have such a clear cut distribution within the nucleus compared with the C-terminal deletion mutant (Fig. 2B, panels c and g). To investigate the effect of Ran on the nuclear import of Tax, RanQ69L, a mutant Ran which is deficient in GTP hydrolysis was added to the import mixture (Fig. 2B, panels d, h, and l). RanQ69L effectively inhibited the nuclear accumulation of GST-SV40TNI LS-GFP, but not that of Tax-GFP and Tax340-GFP. These results indicate that Tax can enter the nucleus without energy or carriers including the importin β family proteins, which are known to require the GTPase activity of Ran for carrier function (23).

To determine whether the nuclear entry of Tax involves a facilitated mechanism, in vitro nuclear import assays were carried out in the presence of GST-Tax or GST-importin β as unlabeled fluorescent competitors (Fig. 2C). Both GST-Tax and GST-importin β reduced the nuclear uptake of Tax-GFP and Tax340-GFP, indicating that the nuclear import of Tax is saturable, and the import involves the specific components of the NPC which are also involved in interactions with importin β.

The Nuclear Import of Tax Involves a Direct Interaction with the FG Repeats of Nucleoporins—As it has been reported that the carrier-independent translocation of proteins into the nucleus involves a
bound fractions were analyzed by immunoblotting. In proteins were allowed to immobilize on glutathione-Sepharose 4B beads, and of GST-p62FL HA-Imp with (p62C) did not (Fig. 3 repeat region, whereas the N-terminal deletion mutant of p62 C-terminal deletion mutant of p62 (p62N) contained the FG repeat region of p62, including p62C, Nup153, and Nup214/CAN have also interacted with nucleoporins. The FG repeat containing within the NPC (23,35–38,52), we investigated whether Tax direct interaction(s) between the proteins and nucleoporins

FIGURE 3. Tax binds directly to the FG repeat region of p62 in vitro. A, scheme of p62FL and its deletion mutants, p62N and p62C, which were used in the protein binding assay. B and C, in protein binding assays, the mixtures of recombinant proteins were allowed to immobilize on glutathione-Sepharose 4B beads, and bound fractions were analyzed by immunoblotting using anti-GFP antibody. Beads, the total volume of the mixtures were increased to 300 µl, and the elution step was carried out three times. In C, recombinant GST-p62N was mixed and incubated at the indicated concentrations of HA-Impβ and Tax-GFP. FL, full-length. Impβ, importin β.

direct interaction(s) between the proteins and nucleoporins within the NPC (23,35–38,52), we investigated whether Tax can also interact with nucleoporins. The FG repeat containing nucleoporins, including p62, Nup153, and Nup214/CAN have been implicated in nuclear import and are also known to interact with several importin β family proteins (reviewed in Ref. 53). To investigate if Tax can interact with nucleoporins, recombinant GST fused with full-length p62 and both C- or N-terminal deletion mutants of this protein were purified and assayed for their binding to Tax-GFP or HA-importin β. The C-terminal deletion mutant of p62 (p62N) contained the FG repeat region, whereas the N-terminal deletion mutant of p62 (p62C) did not (Fig. 3A). In control experiments, HA-importin β was found to bind to “full-length” GST-p62FL and to GST-p62N. This was also shown not to bind to GST-p62C or GST itself (Fig. 3B). Tax-GFP and Tax340-GFP also specifically bind to GST-p62FL and GST-p62N (Fig. 3B, right). Interactions of GFP and GST or GST fusion proteins were not observed. These results clearly show that Tax interacts with the FG repeat region of p62 in vitro. Our studies also suggest that Tax340 may bind p62FL with a greater affinity than wild type Tax (Fig. 3B). However this remains to be further investigated.

To determine if Tax and importin β compete for the binding to the FG repeats of p62, in vitro competition assays were carried out (Fig. 3C). Only a 2.5-fold amount of importin β effectively inhibited the binding between Tax and p62. In contrast, as much as a 40-fold amount of Tax did not completely inhibit the binding between importin β and p62. Binding between importin β and Tax was not observed (Fig. 3C, right). This suggests that Tax and importin β may share common docking sites on the FG repeats of nucleoporins, but the affinity between Tax and p62 is considerably lower than that between importin β and p62. To determine whether the nuclear import activity of Tax is dependent on an interaction between Tax and nucleoporins, the single amino acid Tax mutants, which were found to have lost (C29A and C36A) or retained (C23A and H41A) their nuclear localization properties (Fig. 1B), were expressed as GFP fusion proteins, and their nuclear import activity and binding affinity with p62 examined. Among wild type Tax and the four mutants, the nuclear import activities in digitonin-permeabilized cells closely correlated with the binding affinity with p62 (Fig. 4). Specifically these results support the proposal that the nuclear import of Tax requires direct interactions with regions in the nucleoporins containing the FG repeats.

FIGURE 4. The nuclear import activity of Tax is dependent on the binding of Tax and the FG repeats of nucleoporin. The recombinant GFP fusions, Tax-GFP (WT), TaxC23A-GFP (C23A), TaxC29A-GFP (C29A), TaxC36A-GFP (C36A), and TaxH41A-GFP (H41A) were examined by in vitro nuclear import assay (A) and protein binding assay (B). In A, the digitonin-permeabilized HeLa cells were incubated with 10 µl of reaction mixtures containing 0.3 µM GFP fusions, ATP regeneration system, and RRL. Scale bars, 20 µm. In B, GST-p62N (0.2 µM) was mixed and incubated with GFP fusions (0.2 µM each). The reaction mixtures were then allowed to immobilize on glutathione-Sepharose 4B beads, and bound fractions were analyzed by immunoblotting using anti-GFP antibody.
Nuclear Import of HTLV-1 Tax

The Nuclear Export of Tax Is Also Carrier- and Energy-independent—A number of studies have shown that certain proteins which enter the nucleus without import carriers (transportin, β-catenin, and MAPK) are also known to exit the nucleus without export carriers (35, 54–56). This raised the possibility that Tax may also exit from the nucleus without export carriers, and to investigate this, we examined the export of Tax using digitonin-permeabilized cells. Cells were firstly incubated with import mixtures containing Tax-GFP, Tax340-GFP, and GST-YFP-Rev as control, and subsequently washed and incubated with export buffer. To identify the components required for nuclear export, the export reactions were carried out with transport buffer (Fig. 5, panels a, d, and g) or buffer containing cytosol and energy (Fig. 5, panels b, e, and h). In addition, the transport buffer containing WGA was used to confirm the integrity of the nuclear membrane structures (Fig. 5, panels c, f, and i). The export of GST-YFP-Rev was observed only in the presence of cytosolic factors and energy, consistent with previous reports that the nuclear export of the HIV-1 Rev was CRM1-dependent (Fig. 5, panel h) (57). In contrast, GST-YFP-Rev was not exported from the nucleus under cytosol- and energy-free conditions (Fig. 5, panel g). Tax-GFP and Tax340-GFP were efficiently exported under the same conditions (Fig. 5, panels a and d). Quantification of the export process demonstrated that the export efficiencies of Tax-GFP and Tax340-GFP in the presence of cytosol and energy (Fig. 5, panels b and e) were almost the same as that in the absence of cytosol and energy (Fig. 5, panels a and d). These results indicate that Tax is also able to exit the nucleus in an energy- and carrier-independent manner.

Tax Can Transport p65 into the Nucleus—Our results have demonstrated that Tax can enter and exit the nucleus without a carrier protein, and these processes are energy-independent. Moreover, the nuclear import process appears to involve a direct interaction between Tax and the FG repeat regions of nuclearoporins. Because these properties are also shared with known import receptors such as the importin β family proteins (58 – 61), we attempted to determine if Tax can also function as an import receptor. p65, an NF-κB family protein subunit, was chosen as a putative cargo of Tax, because this protein has been shown to be closely associated with Tax both in the cytoplasm and in the nucleus. Specifically, Tax has been associated with the release of p65 from the IkBα-p65 complex in the cytoplasm (13), and Tax and p65 co-localize in the nucleus (14). In addition, physical binding of Tax and p65 has been reported (62, 63). To determine whether p65 can be imported by Tax, p65 fused with YFP (p65-YFP) and Tax340 fused with CFP (Tax340-CFP) were expressed and used as substrates in the in vitro nuclear import assay. In the presence of energy and cytosol, p65-YFP was found to be distributed in both the nucleus and cytoplasm (Fig. 6A, panel a). The nuclear import of p65-YFP was inhibited by the addition of WGA or RanQ69L (Fig. 6A, panels b and c) and by the deprivation of cytosol in the import mixtures (Fig. 6B, panel a), all of which is consistent with the nuclear import of p65 being carried out by the importin α/β-mediated classical import pathway (64). Tax340-CFP was also expressed, and we confirmed as expected that the nuclear import of Tax340-CFP was a carrier- and an energy-independent process (data not shown). As shown in Fig. 6B, even without the addition of cytosolic factors and energy, p65-YFP was localized in the nucleus in the presence of Tax340. Moreover, p65-YFP migrated into the nucleus even in the presence of RanQ69L and cytosol if Tax-CFP was present in the import mixture (data not shown). These results indicate that p65 can migrate into the nucleus not only by the importin α/β import pathway, but also by a Tax-mediated pathway.

DISCUSSION

A number of studies have clearly shown that HTLV-1 Tax protein co-localizes in both the nucleus and the cytoplasm. In
Nuclear Import of HTLV-1 Tax

In the present study, we have investigated the intracellular localization of Tax and specifically the mechanisms involved in its nuclear localization. These have clearly shown that the nuclear import of Tax is carrier independent. Most proteins are transported into the nucleus by forming complexes with carrier proteins, which belong to importin families with the carrier-independent nuclear import (34, 35, 37, 39, 54). A common feature of the carrier-independent process is the direct interaction of proteins with nucleoporins in the NPC (23, 35–38, 41, 52). In the present study, we could also demonstrate a direct interaction between Tax and p62, one of the nucleoporins, which is located in the central plug of the NPC suggesting that Tax also shares this common property. It could be demonstrated using specific Tax mutants that the nuclear import of Tax correlated with the binding to p62 suggesting that this is an essential part of nuclear import. In addition, it could be shown that the nuclear import of Tax was inhibited by an excess amount of importin β, and that the binding between Tax and p62 was blocked by importin β. Taken together, the results suggest that Tax and importin β bind to a common region(s) in the nucleoporins.

Carrier- and energy-independent nuclear transport is thought to be carried out by a process similar to the so-called “facilitated diffusion,” which requires specific, but weak interactions between the protein and nucleoporins (40, 66, 67). This sort of transport mechanism involves minimal energy and is thought to be driven by Brownian motion. In the case of β-catenin, the subcellular localization of the protein is thought to be controlled by retention in either the nucleus or the cytoplasm rather than a selective increase of import or export. Specifically, the transcription factors including T-cell factor/lymphocyte enhancer factor (LEF/TCF) and BCL9 or the cytoplasmic proteins including APC and Axin function as nuclear or cytoplasmic retention factors for β-catenin (68). The nuclear localization of MAPK is also thought to be regulated by the nuclear anchor proteins (69). Consistent with this, it is possible that the nuclear localization of Tax might be related to retention caused by Tax-binding proteins in the nucleus such as p65 and CBP, which serve as a bridge between the nucleosome and Tax. Cytoplasmic localization could involve immobile structures such as the MTOC and cytoskeleton to which Tax can bind directly or indirectly. One important observation in our study was that Tax340 was imported into the nucleus efficiently without cytosol and energy, but wild type Tax was imported efficiently only in the presence of cytosol. It is likely that the C-terminal 13 amino acids, which we have shown to be associated with the cytoplasmic distribution of Tax may serve as part of a “cytoplasmic retention signal.” In the absence of cytosol, the C terminus of Tax could be sequestered by immobile structures in the cytoplasm, and in the presence of cytosol, Tax would be released from the cytoplasmic retention by the protein binding to the C terminus of Tax and permitting translocation through the NPC. It is also possible that Tax340 may have a greater binding affinity for the NPC compared with wild type Tax facilitating nuclear localization but this will require further detailed investigations. These and other studies are underway to further examine the importance of the C-terminal region in the localization of Tax.

The mechanisms of the nuclear export of Tax also remain to be defined. It was recently shown that under UV stress conditions, Tax was exported from the nucleus by an interaction with the carrier protein CRM1, on the basis that this was inhibited by leptomycin B (70). In contrast, under normal culture conditions, the nuclear export of Tax was found to be insensitive to leptomycin B (20, 70). As we demonstrated that the addition of exogenous transport factor and energy did not facilitate the nuclear export of Tax, it appears that Tax can exit the nucleus in a carrier and energy independent manner and this may account

![Image](https://example.com/image.png)
Nuclear Import of HTLV-1 Tax

for the report of the leptomycin B-insensitive nuclear export of Tax. Carrier-independent nuclear export has also been observed in a number of proteins, which have a carrier-independent nuclear import, such as transportin (54), MAPK (35), and β-catenin (55, 56).

It has recently been shown that β-catenin can also itself function as a carrier for the nuclear import of the LEF/TCF protein (71). This prompted us to investigate if Tax could also serve as a carrier for the import of cellular proteins. Several reports have demonstrated a direct interaction between Tax and the NF-κB subunit p65 (62, 63), and we could show that Tax functions as a nuclear import receptor for p65 at least under our in vitro conditions. Tax is known to have intimate interactions with p65 both in the cytoplasm and nucleus. Specifically, Tax is involved in the disassembly of the p65-IκBo complex in the cytoplasm (reviewed in Ref. 13) and co-localizes with p65 in the nucleus (14, 72). Therefore, we propose that p65 and Tax may form a complex in the cytoplasm, which can translocate to the nucleus through both a Tax carrier and an importin α/β-mediated mechanism. This proposal is supported by a recent study (62), where it was reported that the p65-CBP-Tax ternary complex is translocated into the nucleus immediately after its formation in the cytoplasm. Employing cellular fractionation studies, it could be shown that when p65, CBP and wild type Tax were co-transfected, the p65-CBP-Tax complex remained in the cytoplasm. These observations would support our hypothesis that Tax may function as an import receptor for this ternary complex.

With a carrier function, it might be expected that Tax could alter the normal nuclear/cytoplasmic distribution of cellular proteins by functioning as an exogenous transport receptor. Tax has been shown to bind with numerous cellular proteins, and fractionation of whole cell lysates from a HTLV-1-infected T cell line revealed that Tax is found in large protein complexes (~1800 kDa) in vivo (73). The ability of Tax to form protein complexes with cellular proteins together with a transport receptor-like function could lead to changes in the nucleo-cytoplasmic transport pathway of host cellular proteins.

Nuclear Import of HTLV-1 Tax are required to understand how this novel function of Tax may contribute to the pathogenesis of HTLV-1-related diseases.

Acknowledgments—We thank Dr. Y. Okada for technical advice and critical reading of the manuscript. We acknowledge Dr. S. Kose and Dr. N. Imamoto for kindly providing plasmids and helpful comments, and Dr. N. Yaseen and Dr. D. Dermot for kindly providing plasmids.

REFERENCES

1. Yoshida, M., Miyoshi, I., and Hinuma, Y. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2031–2035
2. Seiki, M., Hattori, S., Hirayama, Y., and Yoshida, M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3618–3622
3. Grassmann, R., Dengler, C., Muller-Fleckenstein, I., Fleckenstein, B., McGuire, K., Dokhemar, M. C., Sodroski, J. G., and Haseltine, W. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3351–3355
4. Tanaka, A., Takahashi, C., Yamaoka, S., Nosaka, T., Maki, M., and Hatana, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1071–1075
5. Nerenberg, M., Hinrichs, S. H., Reynolds, R. K., Khoury, G., and Jay, G. (1987) Science 237, 1324–1329
6. Grossman, W. J., Kimata, J. T., Wong, F. H., Zutter, M., Ley, T. J., and Ratner, L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1057–1061
7. Yamada, S., Ikeda, H., Yamazaki, H., Shikishima, H., Kikuchi, K., Wakisaka, A., Kasi, N., Shimotohno, K., and Yoshiki, T. (1995) Cancer Res. 55, 2524–2527
8. Hasegawa, H., Sawa, H., Lewis, M. J., Orba, Y., Sheehy, N., Yamamoto, Y., Ichinohe, T., Tsunetsugu-Yokota, Y., Kato, H., Takahashi, H., Matsuda, I., Sata, T., Kurata, T., Nagashima, K., and Hall, W. W. (2006) Nat. Med. 12, 466–472
9. Matsuoka, M. (2003) Oncogene 22, 5131–5140
10. Yoshida, M. (2001) Annu. Rev. Immunol. 19, 475–496
11. Jeang, K. T., Giard, C. Z., Majone, F., and Aboud, M. (2004) J. Biol. Chem. 279, 31991–31994
12. Sun, S. C., and Yamaoka, S. (2005) Oncogene 24, 5952–5964
13. Polonnese, J. M., Yeung, M. L., and Jeang, K. T. (2006) Immunol. Res. 34, 1–12
14. Bex, F., McDowall, A., Burny, A., and Gaynor, R. (1997) J. Virol. 71, 3484–3497
15. Bex, F., Yin, M. J., Burny, A., and Gaynor, R. B. (1998) Mol. Cell. Biol. 18, 2392–2405
16. Semmes, O. J., and Jeang, K. T. (1996) J. Virol. 70, 6347–6357
17. Nicot, C., Tie, F., and Giard, C. Z. (1998) J. Virol. 72, 6777–6784
18. Burton, M., Upadhya, C. D., Maier, B., Hope, T. J., and Semmes, O. J. (2000) J. Virol. 74, 2351–2364
19. Cheng, H., Cenciarelli, C., Shao, Z., Vidal, M., Parks, W. P., Pagano, M., and Cheng-Mayer, C. (2001) Curr. Biol. 11, 1771–1775
20. Aletantis, T., Barmak, K., Harhay, E. W., Grant, C., and Wiegand, B. (2003) J. Biol. Chem. 278, 21814–21822
21. Aletantis, T., Mostoller, K., Jain, P., Harhay, E., Grant, C., and Wiegand, B. (2005) J. Biol. Chem. 280, 17353–17362
22. Nejmeddine, M., Barnard, A. L., Tanaka, Y., Taylor, G. P., and Bangham, C. R. (2005) J. Biol. Chem. 280, 29653–29660
23. Harel, A., and Forbes, D. J. (2004) Mol. Cell 16, 319–330
24. Pemberton, I. F., and Paschal, B. M. (2005) Traffic 6, 187–198
25. Lam, M. H., Thomas, R. I., Loveland, K. L., Schilders, S., Gu, M., Martin, T. I., Gillespie, M. T., and Jans, D. A. (2002) Mol. Endocrinol. 16, 390–401
26. Nagoshi, E., Imamoto, N., Sato, R., and Yoneda, Y. (1999) Mol. Biol. Cell 10, 2221–2233
27. Yamashita, H., Sekimoto, T., Ohkubo, T., Douchi, T., Nagata, Y., Ozawa, M., and Yoneda, Y. (2005) Genes Cells 10, 455–464
28. Truant, R., and Cullen, B. R. (1999) Mol. Cell. Biol. 19, 1210–1217
29. Arnold, M., Nath, A., Hauber, J., and Kehlenbach, R. H. (2006) J. Biol. Chem. 281, 20883–20890
30. Palmeri, D., and Malim, M. H. (1999) Mol. Cell. Biol. 19, 1218–1225
31. Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F., and Dreyfuss, G. (1996) Cell 86, 985–994
Nuclear Import of HTLV-1 Tax