Human T cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus that causes human adult T cell leukemia. The viral oncoprotein Tax1, encoded by the HTLV-1 genome, is the molecular determinant for transformation of T lymphocytes (1). Tax1 modulates the activity of the transcriptional factor NF-κB to promote T cell proliferation, and it also inactivates tumor suppressor p53. These molecular bases are thought to be crucial for Tax1-induced transformation of T lymphocytes. Moreover, vast amounts of experimental data have shown that Tax1 regulates a wide variety of cellular factors in addition to NF-κB and p53, which may play distinct roles at various stages of oncogenesis (4). Persistent activation of NF-κB is one of the characteristic features of adult T cell leukemia. Tax1 is essential to establish proliferative growth of T cells at least in the early stage of infection, and Tax1 immortalizes primary human T cells in a manner highly dependent on its ability to activate NF-κB (2). Tax2, the Tax protein from HTLV-2, shares a significant amino acid sequence homology with Tax1 and is able to activate NF-κB and to transactivate the long terminal repeat of both HTLV-1 and HTLV-2 by enhancing the activity of CREB (5). Although the viral genome can be isolated from some hairy cell leukemia patients, HTLV-2 infection is not causally linked to T cell leukemia (5). Furthermore, unlike Tax1, Tax2 rarely transforms primary cells although it can immortalize primary human T cells. The underlying mechanism for the differential effect of Tax1 and Tax2 in transformation of T cell remains poorly understood.

The precise mechanism of Tax activation of NF-κB remains elusive. It has been demonstrated that Tax1 induces activation of NF-κB via several distinct mechanisms. First, Tax1 modulates the 1κB kinase complex, the key regulator of NF-κB signaling, by directly stimulating the activity of the catalytic subunits, IKKα and IKKβ, through interaction with the non-catalytic subunit, IKKa or NEMO (6–11). Indeed, Tax1 fails to activate IκB kinases in NEMO/IKKγ-deficient cells (12), indicating that IKKγ is one of the key targets for Tax1-mediated activation of IKK. Second, Tax1 was shown to modulate the activity of upstream kinases of IKK, which include mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (MEKK1) and NF-κB-inducing kinase (13, 14). It has been recently shown that Tax1 stimulates the activity of transforming growth factor β-activated kinase 1 (Tak1), an upstream kinase of IKK, and promotes interaction of Tak1 with IKKγ (15). Depletion of Tak1 abrogates the activation of IKK induced by Tax1, suggesting a critical role of Tak1 in this activation process. Furthermore, Tax1 was shown to interfere with the noncanonical pathway of NF-κB signaling by inducing processing of NF-κB2/p100 through up-regulation of IKKa in T cells (16). These evidences implicate that Tax1-mediated activation of NF-κB involves a complex process. The mechanism of the persistent activity of NF-κB by Tax1 is yet to be addressed.

In T lymphocytes, activation of TCR elicits tyrosine phosphorylation cascades and induces membrane lipid raft recruitment and activation of signaling molecules such as ZAP70,
phosphatidylinositol 3-kinase, and PKCθ (17, 18). The lipid rafts are glycosphingolipid- and cholesterol-enriched, detergent-resistant microdomains that play crucial roles in signaling transduction (19–20). The lipid rafts are assembled in the Golgi and can be recycled between the plasma membrane and Golgi (21). Carma1, PKCθ, and Bcl10 are the key players that direct the plasma membrane lipid raft recruitment of the IKK complex, leading to transient activation of IKK and nuclear translocation of NF-κB upon T cell activation (22–33). Aside from TCR-directed signaling events, tumor necrosis factor α (TNFα) also induces lipid raft translocation of IKK, together with the IKK-associated chaperone protein, Hsp90 (34). It remains largely unclear whether Tax1-mediated activation of NF-κB is involved in lipid raft translocation of IκB kinases. It has been shown that cytoplasmic Tax1 mediates activation of IκB kinases. Indeed, a portion of Tax1 was found to reside in the Golgi and to recruit the IKK complex to this subcellular location (35–37). In the present study, we show that the Tax1 protein accumulates in the Golgi-associated lipid rafts in directing translocation of IKK to the microdomains by primarily targeting IKKγ. This process is crucial for Tax1 activation of NF-κB in both T cells and non-lymphoid cells. In contrast, Tax2 activates NF-κB in a manner independent of the lipid raft recruitment of IKK as seen in Tax2-immortalized T cells. These differential modes of IKK activation by the Tax proteins may have implications on the pathogenesis of T cell leukemia.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Antibodies, and Reagents**—Human T cell lines including MT1, MT2, MT4, SupT1, and Jurkat were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum plus antibiotics at 37 °C, 5% CO₂. SupT1 and Jurkat E6-1 cell lines were obtained from ATCC (Manassas, VA), MT1 and MT4 were kindly provided by Drs. Atsushi Koito and Takeo Ohsugi (Center for AIDS Research and Institute of Resource Development and Analysis, Kumamoto University, Japan). MT2 cells were obtained from Dr. Douglas Richman (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health). Antibodies for IκBα, Tak1, Hsp90, and HA epitope were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IKKα, IKKβ, IKKγ, and serine-phosphorylated IκBα were purchased from IMGENEX (San Diego, CA). Anti-LAT was from Upstate Biotechnology (Charlottesville, VA). Anti-β-actin, anti-FLAG M2 monoclonal antibodies, horseradish peroxidase-conjugated chrola toxin β subunit, methyl-β-cyclodextrin (MβCD), protease, and phosphatase inhibitor mixtures were obtained from Sigma. The proteasome inhibitor MG-132 was purchased from Calbiochem, and anti-Tax1 antibody was acquired from the AIDS Research and Reference Reagent Program.

**Mammalian Expression Plasmids, DNA Transfection, and GST Pulldown**—The expression plasmids for GST-tagged IKKα, IKKβ, and IKKγ as well as HA-tagged Tax1 were described previously (38). To generate Tax1 mutants, PCR-based site-directed mutagenesis was performed to construct the fragments with mutations at the positions illustrated in Fig. 3A. The fragments of Tax1 mutants were inserted into expression vector pCEF with a C-terminal HA or GST tag and verified with DNA sequencing. Tax2 was constructed in the same vector as Tax1. To construct lipid raft-targeted IKKγ, a myristoylation signal from Lck was attached to the full-length of IKKγ to generate the myristoylated IKKγ fusion fragment (Myr-IKKγ), which was inserted into the lentivirus vector. GFP-GaIT (galactosyltransferase) was purchased from Addgene Inc. (MA). To construct RFP-tagged caveolin-1, the full-length of the caveolin-1 (CAV1) cDNA was amplified from a human cDNA library, and C-terminal tag the with the RFP (monomeric red fluorescence protein) fragment. The CAV1-RFP fusion fragment was inserted into vector pLCEF8 and CAV1 was sequence-validated.

To examine the interaction of IKKγ with Tax1 and its mutants, the FLAG-IKKγ expression plasmid was co-transfected with GST-tagged wild type Tax1, the Tax1 mutants, or IKKβ into HEK cells using SuperFect transfection reagent (Qiagen, Alencia, CA). 24 h post-transfection, the cells were lysed in the Buffer A containing 1% Triton X-100, 40 mM Tris-Cl (pH 7.5), 150 mM NaCl, 2 mM MgCl₂, 0.5 mM dithiothreitol and protease inhibitor mixture at 15 °C for 30 min. Glutathione-Sepharose beads were added into the soluble supernatants and incubation was at room temperature for 2 h. The beads were then washed three times with the lysis buffer and subjected for SDS-PAGE plus Western blot analysis using anti-FLAG M2 monoclonal antibody to detect FLAG-IKKγ. To evaluate the interaction of endogenously expressed Tak1 with IκB kinases, GST-tagged IKKα, IKKβ, or IKKγ was transfected into HEK cells in the presence of either the empty vector or Tax1-HA. GST pulldown assay and immunoblot analysis were performed as described above.

**Lentivirus Vector and Transduction**—The full-length fragment of enhanced green fluorescence protein was fused with IKKβ and IKKγ to generate GFP-IKKβ and GFP-IKKγ fusion genes, which were constructed in the lentivirus vector pLCEF8, a modified vector of pLNL3.7 (39) in which human elongation factor 1α promoter replaced U6 promoter. The Tax1-GFP fusion fragment was also constructed in pLCEF8. The lentivirus production and transduction in T cell lines were performed as described previously (38), and ~10 multiplicity of infection was used for transduction of T cells. Transduction efficiency was verified with fluorescence imaging and immunoblot.

**Generation of Tax2 Immortalized T Cell Lines**—Primary CD4+ T lymphocytes from healthy donors were isolated with Dynal beads conjugated with anti-CD4 antibody (Invitrogen). The CD4+ cells were activated with phytohemagglutinin (1 μg/ml) and recombinant interleukin-2 (100 units/ml) for 5 to 7 days prior to transduction with the lentivirus expressing Tax2-GFP fusion protein. Following one month of in vitro culture of the transduced cells in the media supplemented with interleukin-2 (50 units/ml), virtually all of the viable cells were green fluorescence positive and the cells were maintained in culture for more than 6 months.

**Western Blot Analysis**—Cells were collected and lysed in lysis buffer B containing 40 mM Tris-Cl (pH 7.6), 1% Triton X-100,
HTLV-1 Tax-directed Lipid Raft Translocation of IκB Kinases

1% deoxycholate, 150 mM NaCl plus protease and phosphatase inhibitor mixtures at 4 °C for 30 min. Equal amounts of cellular proteins were analyzed by SDS-PAGE, followed by immunoblot. Anti-β-actin blot was used for the protein loading control.

In Vitro Kinase Assay and NF-κB Reporter Assay—In vitro kinase assay to detect the activity of IκB kinases and the NF-κB reporter assay were performed as previously reported (38).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from various T cell lines with or without TNFα stimulation (10 ng/ml for 10 min at 37 °C), using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The sequence of the oligonucleotide corresponding to the κB element from the interleukin-2Rα gene was 5′-gatCGGCAGGG-GAAATCTCCCTTCTCTC-3′. The underlined sequence is the κB cis-element. The oligonucleotide was 5′-end labeled with biotin (Integrated DNA Technologies, Coralville, IA) and annealed to Chemiluminescent EMSA Kit (Pierce). In brief, the κB binding activity to the κB element was examined by EMSA using the LightShift Chemiluminescent EMSA Kit (Pierce). In brief, 5 μg of the nuclear extracts were preincubated in a 20-μl total volume containing 100 mM Tris (pH 7.5), 500 mM KCl, 10 mM dithiothreitol, 200 mM EDTA (pH 8.0), 50% glycerol, and 1 μg of polydeoxyinosinic-deoxyctydilic acid and 2 μl of biotin-labeled probe (20 fmol) for 20 min at room temperature. The reactions were mixed with 5 μl of 5× loading buffer and run on a 6% non-denaturing polyacrylamide gel in 0.5× TBE (1× TBE: 89 mM Tris borate, 2 mM EDTA, pH 8.3) for 90 min at 100 V on ice, and transferred to nylon membranes (Amersham Biosciences) at 380 mA for 1 h in 0.5× TBE on ice. The membrane was optimally UV light cross-linked. Biotin-labeled DNA was detected by streptavidin-horseradish peroxidase, and followed by chemiluminescence.

Lipid Raft Fractionation by Optiprep Density Gradient Ultracentrifugation—Cells (4 × 10⁵ for T cells and 1 × 10⁶ cells for HEK cells) were lysed in 2 ml of extraction buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 plus protease inhibitor mixture). Lysates were sheared by 20 passages through a 22-gauge needle, incubated for 20 min on ice before mixing with the OptiPrep density gradient medium (Iodixanol solution, final concentration, 40% v/v; AXIS-SHIELD PoC AS, Oslo, Norway), and placed at the bottom of a 12-ml tube. By overlaying 4 ml of 30% and 4 ml of 5% of OptiPrep density gradient medium on the top, the tube was centrifuged in a SW41 rotor. 1 ml of each fraction from the top to bottom was collected and subjected to Western blot analysis. Depletion of plasma and intracellular membrane cholesterol by MβCD in T cells was performed by pretreatment of the cells (4 × 10⁷ cells/sample) with 10 mM MβCD for 45 min at 37 °C in Hanks’ balanced salt solution. Following this step, the cells were centrifuged to density gradient ultracentrifugation for lipid raft fractionation analysis.

Fluorescence Imaging—MT2 cells were transduced with GFP, Tax1-GFP, GFP-IKKβ, or GFP-IKKγ using the lentivirus-mediated gene delivery system. Lipid raft was labeled with the Alexa Fluor 594 Lipid Raft Labeling Kit (Molecular Probes, Eugene, OR). Cells were centrifuged and gently resuspended in chilled, complete culture media. Following centrifugation, the cell pellets were gently resuspended in 500 μl of Alexa 594-conjugated cholera toxin B (6 μg/ml) at 4 °C for 20 min. After this incubation, the cells were washed twice with chilled 1× phosphate-buffered saline and resuspended in 500 μl of the chilled anti-cholera toxin B antibody (100-fold dilution working solution) for 15 min at 4 °C. The cells were washed twice with chilled 1× phosphate-buffered saline, and transferred to poly-L-lysine-coated dishes and fixed in 4% paraformaldehyde for 15 min. The Golgi-lipid raft association of Tax1 was assessed by co-transfection of Tax1-GFP with the Golgi lipid raft marker caveolin-1 tagged with RFP. The cells were then incubated with 300 nm 4′,6-diamidino-2-phenylindole (Sigma) to stain the nuclei. Microscopy was performed using Leica TCS SP2 AOBS confocal microscope.

RESULTS

IκB Kinases Are Constitutively Present in the Lipid Rafts in HTLV-1-infected T Cells That Express Tax—Five T cell lines, including non-HTLV-1-infected T cells (Jurkat and SupT1) and HTLV-1-infected T cells (MT1, MT2, and MT4) were assessed for Tax1 expression. MT2 cells, but not other T cell lines, produced a detectable level of Tax1 (Fig. 1A). A hyperphosphorylation of IκBα, a substrate for activated IκB kinase, was detected in MT2 cells when the cells were pretreated with the proteasome inhibitor MG-132 (Fig. 1B). Accordingly, MT2 cells exhibited a hyperactivity of the κB DNA binding, and TNFα stimulation did not further increase the κB binding activity (Fig. 1C), suggesting that NF-κB was maximally activated in these cells even in the absence of extracellular stimuli. IκBα was weakly phosphorylated in MT1 cells with a detectable basal activity of NF-κB, and this activity was further enhanced upon TNFα treatment (Fig. 1, B and C). In non-HTLV-1-infected, Jurkat T cells, the phosphorylation of IκBα was not detected and the basal activity of NF-κB activity was not observed, whereas the NF-κB activity was induced by TNFα (Fig. 1, B and C). These results implicate that a constitutive NF-κB activation is present in HTLV-1-infected T cells, and the expression of Tax1 leads to induction of full-scale activation of IκB kinases.

To determine whether the activation of IκB kinase by Tax1 requires a process that involves lipid raft translocation of IKK, density gradient ultracentrifugation was applied for lipid raft fractionation analysis. As shown in Fig. 1D, portions of three subunits of the IKK complex, including IKKα, IKKβ, and IKKγ, were constantly present in the lipid raft fractions in MT2 cells, corresponding to lipid raft biomarkers LAT and GM1 (fractions 4 and 5), whereas ERK1 exclusively resided in the soluble fractions. In contrast, in the HTLV-1-infected, non-Tax1 expressing cell line MT1, IKKs constantly remained in the cytoplasmic, soluble fractions.
We further tested another non-Tax1 expressing, HTLV-1-infected T cell line, TL-Om1, and found that IκB kinase, not ERK1, was found exclusively found in the lipid raft fraction (Fig. 3B). Indeed, disruption of lipid rafts was significantly reduced following MβCD inhibition of NF-κB (Fig. 2D). These results suggest that the lipid raft-translocated IκB kinases are in activated forms in Tax1-expressing T cells and that Tax1 is likely to be the causative factor to promote lipid raft translocation of IκB kinases.

**Tax1 Directs Lipid Raft Translocation of IκB Correlating with Its Ability to Activate IκK**—To evaluate structural recruitments of Tax1 in activating IκB kinases, we constructed several mutants of Tax1. M22 contains two amino acid substitutions at amino acids 130–131 and abrogates the ability of Tax to activate IκK but retains a full capacity to induce viral gene transcription through HTLV-1 LTR. As depicted in Fig. 3A, two additional mutants of Tax1, ΔGG and ΔPXXP, with deletions at amino acids 33–34 and 73–79, respectively, were generated, as these two amino acid sequences are highly conserved among the Tax proteins from HTLV-1 and HTLV-2. We assessed the ability of wild type (WT) Tax1 and Tax1 mutants in inducing the activity of IκKβ and found that the WT Tax1, but not any of the Tax1 mutants, stimulated the kinase activity of IκKβ (Fig. 3B). Accordingly, Tax1, but not any of the mutants induced NF-κB driven β-galactosidase activity (Fig. 3C). Similar to the WT Tax1, M22 maintained the ability to activate HTLV-1 LTR, whereas the ΔGG and ΔPXXP mutants lost such ability (data not shown). Because the ΔGG and ΔPXXP mutants abolished the abilities in inducing the activities of NF-κB and CREB that transactivates HTLV-1 LTR, it is likely that these two mutants may be improperly folded.

Tax1 activation of IκB kinases does not require lymphocyte-specific factors. To determine whether the lipid raft translocation of the IκK complex correlates with the ability of Tax1 to activate IκK, we transfected WT Tax1 and its mutants into a non-lymphoid cell line, HEK. The WT Tax1 induced the lipid raft translocation of IκKα, IκKβ, and IκKγ, corresponding to the GM1 fraction, whereas ERK1 remained in the soluble fractions (Fig. 3D, fraction 5). In contrast, all three Tax1 mutants lost the ability to drive the translocation of IκB kinases to lipid rafts (Fig. 3, E–G). More-
over, the IKK-associated chaperone protein Hsp90, an essential modulator of the IKK complex, and Tak1, an upstream kinase of IKK, were also able to translocate into the lipid raft fractions in Tax1-expressing HEK cells, but not in cells transfected with the Tax1 mutants (Fig. 3, H and I). In HEK cells transfected with Grb2 or in parental HEK cells, the subunits of IKK remained in the soluble fractions (data not shown). Together, these results suggest that Tax1 is the molecular determinant that directs lipid raft translocation of IKK, correlating with its ability to activate IκB kinases.

**Tax1 Is a Virally Derived, Lipid Raft Modulator**—To investigate the possibility that Tax1 is associated with lipid rafts, we evaluated the lipid raft presence of Tax1. As shown in Fig. 4A, a portion of the Tax1 protein was constitutively present in the lipid raft fractions in HEK cells transfected with HA-tagged Tax1 (upper panel). The Tax1 mutants, M22, ΔGG, and ΔPXXP, were also found in this structure, whereas the control protein, HA-tagged Grb2, resided in the soluble fractions (Fig. 4A). To rule out the possibility of HA tag interference, we tested GFP-tagged Tax1 and found that Tax1-GFP was distributed predominantly in the lipid raft fraction (Fig. 4B, fraction 5). Accordingly, in Tax1-GFP transfected HEK cells, the subunits of IKK were shifted to the lipid raft fractions (Fig. 4B). Moreover, fraction 5 of the Tax1-GFP-transfected HEK cells exhibited a peak absorbance at $A_{600\text{ nm}}$, a peak of protein concentration and the alkaline phosphatase activity (Fig. 4, C–E, respectively), supporting the notion that fraction 5 is the lipid raft fraction in which Tax1 and the

![Figure 2](image2.png)

**FIGURE 2.** Disruption of lipid rafts suppresses NF-κB activity in Tax1-expressing T cells. A, lipid raft fractionation analysis of MT2 cells pretreated with MβCD. B, phosphorylation status of IKKα in MT2 and MT1 cells in the fractions (lipid raft fractionation) as detected by anti-phospho-IKKα immunoblot. C, NF-κB binding activity in nuclear extracts of Jurkat cells stimulated with or without TNFα, which were pretreated with or without MβCD as indicated in the figure.

![Figure 3](image3.png)

**FIGURE 3.** Tax1 directs lipid raft translocation of IKK. A, schematic structure of Tax1 and the positions of the mutations. B, in vitro kinase assay for the activity of IKKβ in HEK cells transfected with wild type Tax1 and its mutants. KA, kinase assay. C, NF-κB β-galactosidase reporter assay in HEK cells transfected with wild type Tax1 and Tax1 mutants. Lipid raft fractionation analysis of IKKα, IKKβ, IKKγ, and ERK1 in HEK cells transfected with wild type Tax1 (D), M22 (E), ΔGG (F), and ΔPXXP (G). Distribution patterns of Hsp90 (H) and Tak1 (I) in lipid raft fractions in HEK cells transfected with wild type Tax1 and Tax1 mutants.
IKK kinases are associated. In contrast, in GFP-transfected HEK cells, the GFP protein and IKKs resided exclusively in the soluble fractions (Fig. 4F). Furthermore, in HTLV-1-transformed MT2 T cells, Tax1 was expressed in two distinct forms, a major form of the Env-Tax fusion protein (p68) and a minor form of the full-length of Tax1 (p40) (40). Only p40 Tax1 was expressed in SLB-1 cells (40). As shown in Fig. 4G, significant portions of both p68 and p40 forms of the Tax1 proteins in MT2 and SLB-1 cells were found in the lipid raft fractions, supporting the notion that Tax1 is able to associate with lipid rafts in lymphoid and non-lymphoid cells.

To further evaluate the lipid raft presence of Tax1, we applied the confocal fluorescence imaging technique. To avoid potential nonspecific immunostaining from primary antibodies, we expressed GFP-tagged fusion proteins including Tax1-GFP, GFP-IKKβ, and GFP-IKKγ as well as the control protein GFP into MT2 cells using the lentivirus transduction method. These cells were co-stained with cholera toxin B labeled with red fluorescence dye, which binds specifically to the sphingolipid-enriched microdomains. A yellow cast is generated when green and red fluorescence signals overlap. As shown in Fig. 5A, GFP-tagged Tax1, IKKβ, or IKKγ was mostly expressed in the cytoplasm in MT2 cells and significant portions of these proteins overlapped with GM1, emitting a yellow cast in the merged pictures. GFP alone did not show apparent co-localization with GM1 as the merged picture emitted separate green and red fluorescence signals. These results indicate that Tax1 and IKK kinases co-localize in the lipid rafts. Next, as it was reported that the cytoplasmic Tax1 is localized in the Golgi, we examined the subcellular localization of Tax1 in HEK cells. We co-transfected HEK cells with the Golgi marker protein galactosyltransferase tagged with GFP (GFP-GalT) and the Golgi-lipid raft marker protein caveolin-1 (CAV1) tagged with RFP (CAV1-RFP). Two-color confocal imaging analysis showed that GFP-GalT and CAV1-RFP co-localized to form perinuclear clusters, a typical pattern of the Golgi (Fig. 5B, upper panel). Furthermore, Tax1-GFP and CAV1-RFP also co-localized in the perinuclear Golgi clusters, emitting a yellow cast (Fig. 5B, lower panel). Lipid rafts are synthesized in the Golgi and can be recycled to the plasma membrane. Taken together, these results indicate that Tax1 targets the Golgi-lipid raft microdomains in recruiting IKK to these structures for activation.

Tax1 Directs Lipid Raft Translocation of IKK by Targeting IKKγ—Because it has been shown that Tax1 primarily interacts with IKKγ to stimulate the kinase activity of the catalytic subunits of IKKs, we then investigated the structural requirements of Tax1 for the interaction with IKKγ. As shown in Fig. 6A, WT Tax1, but not any of the Tax1 mutants, co-precipitated with IKKγ. These results implicate that the interaction of Tax1 with IKKγ correlates with Tax1-induced IKK kinase activation and Tax1-directed lipid raft translocation of IKK. In addition, we found that Tax1 co-precipitates with IKKγ, but not with IKKα or IKKβ, regardless of the presence of Tax1 (Fig. 6B). Moreover, by expressing Tax1-GFP in the IKKγ-deficient Jurkat T cells, the depletion of IKKγ apparently impaired Tax1-mediated lipid raft translocation of IKKα and IKKβ (Fig. 6C). Therefore, these results validated the role of Tax1 as a viral lipid raft protein, which actively hijacks the IKK complex and Tax1 to the Golgi-associated lipid rafts through selective interaction with IKKγ.

To determine whether lipid raft targeting of IKKγ alone is sufficient to mediate lipid raft translocation of the catalytic

FIGURE 4. **Tax1 is a lipid raft protein.** A, lipid raft fractionation analysis of HA-tagged Tax1, the Tax1 mutants, and Grb2 in transfected HEK cells. B, lipid raft fractionation analysis of Tax1-GFP transfected HEK cells. C, protein concentration (D), and alkaline phosphatase activity (E) of each fraction in Tax1-GFP-transfected HEK cells. F, lipid raft fractionation analysis of GFP-transfected HEK cells. G, lipid raft fractionation analysis of Tax1 in MT2 and SLB-1 T cells.
HTLV-1 Tax-directed Lipid Raft Translocation of IκB Kinases

subunits of IκKs and to activate NF-κB, we generated myristoylated IκKγ (Myr-IκKγ). Expression of Myr-IκKγ in HEK cells resulted in exclusive distribution of Myr-IκKγ in the lipid raft fractions, yet the endogenous IκKα, IκKβ, and Hsp90 remained in the soluble fractions (Fig. 6D). In addition, Myr-IκKγ did not induce activation of NF-κB in transfected HEK cells (Fig. 6E), suggesting that lipid raft targeting of IκKγ alone is not sufficient for mediating lipid raft translocation and activating the catalytic IκKs.

Activation of NF-κB by Tax2 Is Independent of the Lipid Raft Recruitment of IκK—We tested the ability of Tax1 and Tax2 in inducing the activity of NF-κB in transfected HEK cells and found that both Tax proteins activated NF-κB efficiently (Fig. 7A). Tax1 and Tax2 interacted with IκKγ equally well in co-transfected HEK cells (Fig. 7B). The lipid raft fractionation analysis showed that Tax2 did not direct the lipid raft translocation of the IκK complex, although a portion of Tax2 was localized in the lipid raft fraction (Fig. 7C).

To further evaluate the differential effect of Tax1 and Tax2 in induction of the lipid raft translocation of IκK, we generated Tax2-immortalized, primary human CD4+ T cell lines. As shown in Fig. 7D, in Tax2-immortalized T cells, IκKs were mostly in the soluble fractions. Examination of two additional Tax2-immortalized T cells yielded similar results (data not shown). These results indicate that Tax1 and Tax2 exhibit differential modes of activation of IκB kinases.

Although both Tax1 and Tax2 can interact with IκKγ, it is likely these homologous viral proteins may differentially regulate the catalytic subunits of IκKs. To investigate this possibility, we first assessed the interactions of the Tax proteins with IκKα and IκKβ. In consistency with the previous reports, transient co-transfection of HA-tagged Tax and GST-tagged IκKs demonstrated that Tax1 co-precipitated with either IκKα or IκKβ (Fig. 7E). In contrast, at the identical conditions, Tax2 failed to co-precipitate with IκKα or IκKβ. Next, we generated IκKα-specific knockdown HEK cells (Fig. 7F). Expression of Tax1 in the IκKα-depleted HEK cells failed to activate NF-κB (data not shown), and abrogated the ability of Tax1 to promote lipid raft translocation of the IκK complex, although Tax1 was still able to translocate into the lipid rafts (Fig. 7G). Thus, these results suggest that the interaction of Tax1 and IκKγ is necessary but not sufficient to promote the translocation of IκK to lipid rafts. A preconditioning event of the catalytic subunits of IκKs such as IκKα by Tax1 is critical to execute the ability of Tax in hijacking IκKs to lipid rafts for persistent activation.

DISCUSSION

The present study illustrated a novel mechanism that the Tax protein derived from HTLV-1 actively recruits IκKs to the Golgi-associated lipid raft microdomains as one of the critical processes for the persistent activation of IκB kinases. The ability of Tax1 to drive the IκK complex to lipid rafts in both T cells and non-lymphoid cells implicates that such a process is independent of lymphocyte-specific factors such as Carma1, PKCθ, and Bcl10, which are otherwise critical in TCR-directed membrane lipid raft recruitment and activation of the IκB kinases. This process is dependent on the lipid raft targeting of Tax1 and the interaction of Tax1 with IκKγ, thereby promoting lipid raft translocation of IκB kinases.

The Tax protein of HTLV-1 exhibits both cytoplasmic and nuclear distribution patterns as it consists of nuclear import and export signal peptides, allowing Tax1 shuttling from the nucleus to the cytoplasm (41–43). These unique distribution patterns are in accordance with two major activities: activation of IκB kinases by the cytoplasmic Tax1 and transactivation of HTLV-1 LTR through induction of the activity of CREB by nuclear Tax1 (44). The percentage of Tax1 protein distributed in the cytoplasm and nucleus varies in HTLV-1-infected T cell lines. Predominant cytoplasmic distribution of Tax1 was found in MT2 cells (45). In this study, we utilized...
several approaches to assess the subcellular distribution of Tax1 in MT2 cells and non-lymphoid cells. Our results confirmed that Tax1 is mostly distributed in the cytoplasm in MT2 cells by confocal imaging analysis. One concern about the subcellular localization of Tax1 is that an active viral replication of HTLV-1 occurs in MT2 cells, which requires the nuclear Tax1. It is likely that the nuclear Tax, even at a low level, is sufficient to promote viral transcription. Alternatively, Tax1 may be rapidly exported from the nucleus to the cytoplasm. The presence of nuclear import and export signal peptides in Tax1 supports this notion, suggesting that Tax1 has a dynamic cellular distribution in HTLV-1-infected T cells.

Our data demonstrated that Tax1 is a viral lipid raft protein as evidenced by lipid raft fractionation analysis and confocal imaging. A significant portion of the Tax1 protein is constitutively present in the lipid rafts. Through selective interaction with IKKγ, Tax1 hijacks the entire IKK complex to the lipid rafts. The Tax1 mutants that fail to interact with IKKγ are unable to bring the IKK complex to the microdomains. Depletion of IKKγ abrogates Tax1-directed translocation of IKKs to lipid rafts and activation of NF-κB. However, our data also show that lipid raft targeting of IKKγ alone is not sufficient for lipid raft translocation of the catalytic subunits of IKK and activation of NF-κB. Modification of IKKγ or a catalytic activity of IKKs prior to lipid raft translocation of IKKs may be critical. Nevertheless, these findings are in strong support of the process involved in Tax1-dependent, lipid raft translocation of IκB kinases. This scenario is necessary for induction of IκB kinase activation by Tax1. Aside from Tax1, the viral transactivator Tat of HIV-1 has been shown to induce the NF-κB activity in a manner highly dependent of the lipid raft protein, Lck (46, 47). In another scenario, the latent infection membrane protein 1 of the Epstein-Barr virus resides in lipid rafts and mediates engagement with tumor necrosis factor receptor-associated TRAF to activate NF-κB via the NF-κB-inducing kinase/IKKα-dependent pathway (48). Indeed, the lipid raft microdomains are critical mediators for supporting NF-κB activation induced by certain types of viral proteins and upon TCR activation.

The association of viral proteins with lipid rafts plays crucial roles in dysregulation of cell function and viral budding. For instance, HIV-1 Nef protein is a myristoylated protein that associates with T cell-specific Src kinase Lck within lipid rafts (49–54), which directs a signaling cascade to enhance viral infectivity (52). The lipid rafts are critical for viral budding of HIV-1 and HTLV-1 (55–58). Viral assemblies of these viruses occur within lipid rafts through interaction of the viral envelope, capsid, and host factors. It is therefore plausible that Tax1 may have a potential role in facilitating viral budding. This possibility is yet to be determined. Generation of a Tax mutant with defective lipid raft targeting but with preservation of CREB activation should be able to prove the point. Tax1 does not possess a defined lipid raft-targeting leader. However, it has been shown that certain cellular proteins that lack well defined lipid raft targeting signals, such as stress-induced Hsp70 (59), are capable of relocating to the microdomains. Our data confirmed the lipid raft presence of Tax1, and it is plausible that Tax1 targets to this structure directly with an undefined signal or through interaction with a cellular lipid raft protein. Although it is still not clear about the mechanism of activation of IκB kinases in
lipid rafts, the experimental data have indicated that the membrane translocation of IKK is crucial for activation of IKK upon engagement of TCR. Similar to the lipid raft recruitment of signaling molecules such as ZAP70 and phosphatidylinositol 3-kinase, translocation of IKK and its upstream kinase Tak1 to the lipid raft microdomain enriches these molecules in this structure, providing an optimal microenvironment necessary for the activation event. In contrast to the physiological activation of TCR in which IKK is transiently translocated to lipid rafts in response to antigen stimulation, Tax1 induces a persistent lipid raft presence of NF-κB, correlating with the constitutive activity of NF-κB.

Similar to Tax1, Tax2, a homologous Tax protein from non-pathogenic HTLV-2, interacts with IKK and activates NF-κB in a comparable potency to that of Tax1. Moreover, like Tax1, Tax2 can translocate to lipid rafts. In sharp contrast, Tax2 does not possess the ability to direct the lipid raft translocation of IKK in transfected HEK cells and in Tax2-immortalized primary T cells. Such differential effects of the Tax proteins may be due to their distinct abilities to modulate the catalytic subunits of IKKs, IKKα, and IKKβ. Tax1, but not Tax2, is able to interact strongly with both IKKα and IKKβ, a crucial step to precondition the IKK complex prior to their lipid raft translocation. Thus, it is clear that Tax1, which acts as a viral lipid raft modulator, holds a unique ability in hijacking 1κB kinases to lipid rafts, thereby contributing to the persistent activation of NF-κB and HTLV-1 oncogenesis.

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