Identification of Human T Cell Leukemia Virus Type 1 Tax Amino Acid Signals and Cellular Factors Involved in Secretion of the Viral Oncoprotein*‡§

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Human T cell leukemia virus type 1 (HTLV-1) is the etiologic agent of a number of pathologic abnormalities, including adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The viral oncoprotein Tax has been implicated in the pathogenesis of these diseases. Recently, cell-free Tax was detected in the cerebrospinal fluid of HAM/TSP patients, implying that extracellular Tax may be relevant to neurologic disease. Additionally, the presence of a nuclear export signal within Tax and its active secretion has been demonstrated in vitro. However, the mechanism of Tax secretion remains to be established. Studies reported herein elucidate the process of Tax secretion and identify domains of Tax critical to its subcellular localization and secretion. Tax was shown to interact with a number of cellular secretory pathway proteins in both the model cell line BHK (baby hamster kidney)-21 and an HTLV-1-infected T cell line, C8166, physiologically relevant to HTLV-1-induced disease. Silencing of selected components of the secretory pathway affected Tax secretion, further confirming regulated secretion of Tax. Additionally, mutations in two putative secretory signals within Tax DHE and YTNI resulted in aberrant subcellular localization of Tax and significantly altered protein secretion. Together, these studies demonstrate that Tax secretion is a regulated event facilitated by its interactions with proteins of the cellular secretory pathway and the presence of secretory signals within the carboxyl-terminal domain of the protein.

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of an array of pathologic abnormalities, including the leukoproliferative disease, adult T cell leukemia (ATL), and the neurologic disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (reviewed in Ref. 1). Among the proteins encoded by the viral genome, Tax has been identified as a key player in HTLV-1-induced pathogenesis. Functioning as a transcriptional trans-activator, Tax is known to aberrantly regulate cellular genes, prompting the genesis of ATL by interacting with a host of cellular transcription factors and signaling molecules to enhance or repress cellular gene expression (2–4). Tax affects the expression of several factors that may contribute to cellular transformation, including altered regulation of several cytokines and receptors involved in T cell growth and proliferation, such as granulocyte/macrophage colony-stimulating factor, TNF-α, IL-15, IL-2, and IL-2Rα (5–7). In addition, in vivo studies have demonstrated that transgenic mice expressing Tax develop a variety of cancers, including neurofibrosarcomas and large granular lymphocytic leukemias, depending on the promoter driving expression of the tax gene (8, 9).

Tax has been described as an intracellular protein localizing to the nucleus by virtue of an amino-terminal nuclear localization signal (10) and has also been shown to shuttle between the nucleus and cytoplasm (11), suggesting that cytoplasmic Tax serves additional roles (reviewed in Ref. 12). Supporting this hypothesis, we previously demonstrated that Tax contains a leucine-rich nuclear export signal between amino acids 188 and 202 that facilitates its export from the nucleus to the cytoplasm (13). Tax may localize to the cytoplasm as an intermediary step during the course of its release from HTLV-1-infected cells, as demonstrated by its presence in cytoplasmic secretory-like vesicles (14). Upon its release, secreted Tax may function as an extracellular cytokine.

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

§ The abbreviations used are: HTLV-1, human T cell leukemia virus type 1; ATL, adult T cell leukemia; HTLV-1-associated myelopathy/tropical spastic paraparesis; TNF, tumor necrosis factor; IL, interleukin; ER, endoplasmic reticulum; PM, plasma membrane; FTH6, FLAG-Tax-His6; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; LTR, long terminal repeat.

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3 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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Mechanisms of HTLV-1 Tax Secretion

Many pathologic abnormalities associated with HAM/TSP have been attributed to extracellular Tax. Important to the genesis of neurologic disease, cell-free Tax has recently been detected in the cerebrospinal fluid of HAM/TSP patients (15), and in vitro studies have demonstrated that extracellular Tax has detrimental effects on central nervous system-resident cell populations. Specifically, extracellular Tax causes the induction of TNF-α in human neuronal cells (16) and has been demonstrated to induce the production of TNF-α, IL-1β, and IL-6 in adult human microglial cells and peripheral blood macrophages (17). Local production of these factors could facilitate the maintenance of an inflammatory state and possibly contribute to the process of demyelination observed during the course of disease progression. For example, HAM/TSP has been characterized by a persistent and highly active immune response dominated by a large population of Tax-specific CD8+ cytotoxic T lymphocytes present in both the peripheral blood and the cerebrospinal fluid, with a number of studies suggesting their relevance to disease pathogenesis (18–23). These observations have suggested that Tax is available for immune recognition by antigen-presenting cells. Consistent with this avenue of thought, we have demonstrated that extracellular Tax can induce the expression of several genes involved in activation and maturation of dendritic cells, the most potent antigen-presenting cells (24). We have also demonstrated that Tax alters dendritic cell function and induces T cell proliferation in a mixed lymphocyte culture (25). Additionally, these studies have confirmed that cell-free Tax can be taken up by antigen-presenting cells and presented to T cells. It has also been suggested that extracellular Tax serves pathogenic functions in the central nervous system (26). However, the process by which Tax exits the cell and the mechanism of Tax secretion have not been fully delineated.

Studies have demonstrated that Tax can be released from HTLV-1-transformed cells by a mechanism other than cell death (27, 28). Based on these observations, we have investigated the active secretion of Tax in vitro. In this regard, we previously determined that Tax contains a leucine-rich nuclear export signal (13) and is secreted from Tax-transfected mammalian cells using a model cell line for secretion studies (baby hamster kidney, BHK-21), and a human kidney epithelial cell line (14). Moreover, an increase in Tax secretion was observed following treatment of cells with the secretagogues phorbol 12-myristate 13-acetate and ionomycin, indicating that a significant amount of Tax was present in the regulated secretory pathway (14). Furthermore, a Tax mutant lacking the carboxyl-terminal 139 amino acids displayed a reduced ability to be secreted,4 suggesting that critical amino acid motifs in the carboxyl-terminal domain of Tax may direct the protein to the cellular secretory pathway, since Tax does not contain a leader sequence typical of many secreted proteins (14).

Studies reported herein provide information concerning the transport of Tax from the cytoplasm to the extracellular environment as well as the identification of amino acid signals participating in Tax secretion. Using the BHK-21 model cell line, we have demonstrated the interaction of Tax with a number of proteins comprising the cellular secretory pathway, including those that facilitate transport from the nucleus to the cytoplasm, from the endoplasmic reticulum to the Golgi, and from the post-Golgi to the plasma membrane. These observations were reproducible in C8166 cells, an HTLV-1-infected T cell line serving as a physiologically relevant cell system to study Tax secretion. Comparison of the Tax amino acid sequence with amino acid signals known to target proteins of the cellular secretory pathway yielded the identification of a number of potential motifs that may be involved in targeting Tax for secretion. In agreement with previous studies, Tax localized to the nucleus and in a punctate distribution throughout the cell extending to the plasma membrane. However, mutations in the putative secretory signal DHE as well as a four-amino acid dihydrophobic tyrosine-based motif (YTNI) altered the subcellular distribution of Tax, resulting in an abundant amount of Tax localized to areas immediately surrounding the nucleus. Although Tax mutants retain the ability to trans-activate the HTLV-1 LTR, implying that aberrant localization to the ER or Golgi is not due to gross misfolding of Tax, a comparison of parental Tax versus YTNI and DHE double mutants revealed significant differences in percentage of total Tax secreted. These studies suggest that the putative secretory signals YTNI and DHE are involved in facilitating the egress of Tax through the secretory pathway en route to Tax secretion.

EXPERIMENTAL PROCEDURES

Cell Culture—BHK-21 cells (CCL-10; ATCC, Manassas, VA) were grown in Eagle’s minimum essential medium (ATCC). The HTLV-1-infected T cell line C8166 provided by Dr. Shao-Cong Sun (Pennsylvania State University College of Medicine) and the CD4+ T cell line Jurkat (clone E6-1; catalog number TIB-152; ATCC) were cultured in RPMI 1640 (Mediatech, Herndon, VA). All growth media were supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) with penicillin (100 units/ml) and streptomycin (100 µg/ml) (Mediatech Inc.). Cells were maintained at 37 °C in 5% CO2 at 90% relative humidity.

Tax Fusion Protein Constructs and Plasmid Purification—HTLV-1 Tax constructs utilized in fluorescence microscopy analyses were constructed by cloning full-length Tax cDNA sequence into yellow or green fluorescent protein constructs (pEYFP-N1 and pEGFP-N1, respectively) (Clontech, Palo Alto, CA), yielding constructs encoding fusion proteins in which YFP or GFP were fused to the carboxyl terminus of Tax as described (13). Mutations in putative secretory signals of Tax were made with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Specifically, Tax-YFP was converted to Tax-ATNI-YFP (312YTNI(315 to 312ATNI(315), Tax-YTNA-YFP (312YTNA(315 to 312YTNA(315), Tax-ATNA-YFP (312YTNI(315 to 312ATNA(315), Tax-AA-YFP (319LL(320 to 319AA(320), Tax-AHAYFP (330DHE(332 to 330AHA(332), and Tax-ΔV-YFP (deletion of Val153). The Tax-His6 protein expression vector (pTax-His6) was generously provided by Dr. Chou-Zen Giam (Uniformed

4 T. Alefantis and B. Wigdahl, unpublished observations.
Mechanisms of HTLV-1 Tax Secretion

For quantitative analysis, parental Tax and mutant Tax sequences were cloned into pRES-EGFP (Clontech) following PCR with Tax-specific primers that added FLAG (DDDK) or His<sub>6</sub> tags as described (14). Combination mutations in Tax that were constructed include Tax-AHA-ATNI (330DHE332 to 330AHA332 and 312YTN1315 to 312ATN1315), Tax-AHA-ATNA (330DHE332 to 330AHA332 and 312YTN1315 to 312ATN1315), and Tax-AHA-AA (330DHE332 to 330AHA332 and 312YTN1315 to 312ATN1315). GST-tagged Tax was generated by cloning the Tax coding sequence into pGEX-2T (Amersham Biosciences) between the BamHI and EcoRI sites. All constructs were confirmed by sequencing using a Beckman Coulter CEQ 8800 sequencer located in the DNA Sequencing Laboratory of the Center for Molecular and Functional Genomics in the Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine. The sequence was analyzed by MegAlign under the cluster-W method using Lasergene software (DNASTAR, Madison, WI).

Purification of Tax-His<sub>6</sub> and FLAG-Tax-His<sub>6</sub> Proteins—Tax protein was expressed in Escherichia coli HB101 by the pTax-His<sub>6</sub> plasmid and purified using the His-bind purification system (Novagen, Madison, WI) as described (13, 29). FLAG-Tax-His<sub>6</sub> (FTH<sub>6</sub>) was constructed by inserting the FLAG coding sequence at the 5′ end of the Tax-His<sub>6</sub> gene in the pTax-His<sub>6</sub> expression vector as described (14). Purified protein was subjected to SDS-PAGE and was stained using the Silver Stain Plus reagent (Bio-Rad). The concentration of FTH<sub>6</sub> protein was quantitated using densitometry. The activity of the protein was determined by electrophoretic mobility shift assay with respect to enhancement of cAMP-response element-binding protein (kindly provided by Dr. Jennifer Nyborg, Colorado State University, Ft. Collins, CO) binding to the 21-bp HTLV-1 promoter proximal repeat of Tax-responsive element 1 (30).

Cellular Secretory Pathway Protein Array—BHK-21 cells were plated at a concentration of 3 × 10<sup>6</sup> cells/plate (100 mm) and were transfectioned using 24 µl of Lipofectamine 2000 (Invitrogen) and 9.6 µg of FTH<sub>6</sub> DNA. The DiscoverLight™ protein array kit for use with His-tagged proteins was utilized to examine the interaction of Tax with cellular cytoplasmic proteins (Pierce). Antibodies to cellular proteins (actin (sc-1615; Sigma), rabbit IgG (sc-2045; Santa Cruz Biotechnology), calregulin (sc-1615; Santa Cruz Biotechnology), CRM-1 (N-19; Santa Cruz Biotechnology), SCAMP1, SCAMP2, SNAP23, and COPII) were manually spotted (1 µl) on a nitrocellulose membrane grid at predetermined positions. Arrays were blocked for 1 h and incubated for 2 h with either whole cell lysate (1.5 mg/ml) from untransfected BHK-21 cells or lysate from BHK-21 cells transfected with FTH<sub>6</sub> expression constructs, followed by incubation for 2 h with purified Tax-His<sub>6</sub> protein (2 µg/ml); this step was not necessary following membrane incubation with lysate from FTH<sub>6</sub>-transfected cells. After washing, the arrays were probed for 1 h with horseradish peroxidase-conjugated anti-His<sub>6</sub> monoclonal antibody (ab1187; Abcam), developed using the Western Lightning kit (PerkinElmer Life Sciences), and radiography and densitometry were performed on two separate experimental determinates.

Global Antibody Microarray Analyses—A recently developed antibody microarray (XPRESS Profiler725; Sigma) targeting the global expression of a broad range of cellular proteins known to be involved in a variety of different biological pathways was used to examine the expression of these genes in a HTLV-1-transformed T cell line (C8166). This antibody array contains 725 antibodies spotted in duplicate on nitrocellulose-coated glass slides. HTLV-1-transformed C8166 cells plated at 5 × 10<sup>6</sup> cells/plate (100 mm) were left untransfected or transfected with 18 µl of FuGENE 6 transfection reagent (Roche Applied Science) and 6 µg of FTH<sub>6</sub> DNA. Nontransfected Jurkat T cells were used as a comparative negative control. Cells were harvested for protein extraction 24 h post-transfection, and equal amounts of protein extracts (>1 mg/ml) were labeled using Cy3 or Cy5 nonreactive reactive dyes (Amersham Biosciences) as described by the manufacturer (Sigma). Labeled samples with a dye/protein molar ratio >2 were applied to the antibody microarray in Array Incubation Buffer (Sigma) and incubated for 45 min protected from light with gentle shaking. The array was then washed three times with 5 ml of Washing Buffer (Sigma), air-dried completely, and scanned using a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). Image analysis was performed with GenePix Pro 5.0 software that provides a tool to obtain the position and content of each spot on the array. Data were mined to include proteins of the cellular secretory pathway as well as known Tax-interacting proteins.

GST Pull-down Assays—To confirm the interactions between Tax and selected proteins from the antibody array, a MagneGST™ pull-down system was utilized (Promega, Madison, WI). Full-length SCAMP1, SCAMP2, SNAP23, and COPII were cloned in expression vectors designed to construct prey proteins. The primer sequences used for PCR were as follows: SCAMP1, Forward 5′-CACCATGTCCGCTTTCGACA-3′, Reverse 5′-AGAGGCAGCCAGAAAGACG-3′; SCAMP2, Forward 5′-CACCATGTCGATTTCCACGTCATG-3′, Reverse 5′-GCGGAGAAAAAGTACAGG-3′; SNAP23, Forward 5′-CACCATGTGTTAGTGTGTTTCATCCTG-3′, Reverse 5′-TGGTGAACACCTGTCTTACAAACT-3′; COPII, Forward 5′-CACCAACGGGCGTGAAGAC-3′, Reverse 5′-GATGTGGACTCCGTAAGCC-3′. Following PCR amplification from a human cDNA template, plasmids were generated by cloning into pcDNA3.1/D/V5-His-TOPO (Invitrogen). Constructs were confirmed by sequencing in both directions as described above. To verify the binding of Tax to IKK-γ, the MGC full-length IKK-γ clone (Clone ID 606257; Invitrogen) was inserted into the pcDNA3.1/D/V5-His-TOPO vector. Prey proteins (IKK-γ, SCAMP1, SCAMP2, SNAP23, and COPII) were translated in vitro with the TNT™ quick coupled transcription/translation system (Promega).
Mechanisms of HTLV-1 Tax Secretion

Tax-GST and Rex-GST bait proteins and GST control vector were expressed in the DH5α-T1R strain of E. coli (Invitrogen). Bacterial cultures (1 ml) was lysed by freeze-thaw treatment and gentle mixing (30 min) in lysis buffer. Cell lysates were incubated with GSH-linked magnetic particles at room temperature with gentle mixing to allow GST fusion bait protein to be immobilized on the particles. The Tax-GST, Rex-GST, or GST alone bound particles were magnetically removed from the lystate, washed, and incubated with in vitro translation reaction (20 μl) for 1 h at room temperature. Particles were magnetically separated from the TNT reaction and washed six times, and the bound protein was eluted by incubating the particles in Laemmli Sample Buffer (Bio-Rad). Eluted protein samples were analyzed by western immunoblotting. Briefly, samples were heat-denatured and loaded onto 12% Tris-HCl SDS-polyacrylamide gels (Bio-Rad), subjected to electrophoresis, and blotted onto nitrocellulose membranes (Pall Corp., Ann Arbor, MI). Blots were blocked and incubated with antibodies directed against full-length IKK-γ, α-SCAMP1, α-SCAMP2, α-SNAP23, or α-COPII followed by either anti-rabbit horseradish peroxidase (Santa Cruz Biotechnology) and developed using the Western Lightening kit (PerkinElmer Life Sciences). Assays were performed in duplicate and repeated three times.

Silencing of Potential Tax-interacting Proteins and Their Effect on Tax Secretion—The interaction of Tax with proteins of the secretory pathway was further confirmed using RNA interference and examining the subsequent effect on Tax secretion. Briefly, a SmartPool containing four RNA duplexes (19 bp with a 3′-UU overhang and 5′-phosphorylated antisense strand) specific to SCAMP1, SNAP23, or COPII was purchased from Dharmacon RNA Technologies (Lafayette, CO). C8166 cells (1 × 10⁶ cells/well) were transfected using DharmaFECT transfection reagent and 2 μM small interfering RNA against components of the secretory pathway. Western immunoblot analyses were performed to confirm silencing at the protein level at 48 h post-transfection. Cells were then transfected with FTH₆ DNA using FuGENE 6 (Roche Applied Science), and the effects of silencing were examined by a FLAG-based ELISA to detect Tax in the supernatants of transfected cells.

Detection of Tax Secretion by ELISA—Culture medium harvested post-transfection was loaded onto a 96-well plate precoated with anti-FLAG antibody (Sigma). Reference standards were made with serial dilutions of purified FTH₆ protein. The plate was incubated at 37 °C for 2 h, washed, and incubated with anti-His₆-horseradish peroxidase antibody (ab1187; Abcam) at a 1:1000 dilution at 4 °C for 2 h. Bound protein was detected using tetramethyl benzidine substrate as described by the manufacturer (Pierce) and measured using a Spectramax Plus microplate reader at 450 nm (Molecular Devices Corp., Sunnyvale, CA).

Fluorescence Microscopy—For analysis of the subcellular localization of parental and mutant Tax-GFP, cells were plated in 35-mm culture plates at a density of 2.5 × 10⁶ cells/plate. Transfections were performed using 2.4 μg of parental or mutant Tax-GFP and 6 μl of Lipofectamine 2000 (Invitrogen). Cells were incubated with 10 μg/ml Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) diluted in culture medium 20 min prior to viewing. For co-localization of Tax-YFP with cytoplasmic organelles, 2.5 × 10⁵ cells/plate were plated in 35-mm culture plates and transfected using 6 μl of Lipofectamine 2000 (Invitrogen), 2.4 μg of Tax-YFP or Tax mutant-YFP DNA, and 1.0 μg of cyan fluorescent protein constructs (pECFP-N1 (vector control), pECFP-Nuc (nucleus), pECFP-ER (endoplasmic reticulum), or pECFP-Golgi (Golgi apparatus); Clontech). Cells were visualized using an Olympus IX-81 automated microscope equipped with the appropriate filter cubes for visualizing enhanced yellow fluorescent protein and enhanced cyan fluorescent protein or visualizing enhanced green fluorescent protein and 4′,6-diamidino-2-phenylindole. Images were obtained with a Cook CCD Sensicam digital camera controlled by Slidebook software (Intelligent Imaging Innovations, Denver, CO). All components of the microscopy system were controlled using an Apple Macintosh G4 dual 1-Ghz processor computer (Apple, Cupertino, CA). Raw fluorescent images were deconvolved (no-neighbors method) using Slidebook.

Luciferase Assays—Cells were plated at a density of 8 × 10⁴ cells/well in 24-well plates 24 h prior to transfection. Transfections were performed using 1.5 μl of Lipofectamine 2000 (Invitrogen), 36 ng of pU3R-luciferase, 8.5 ng of pRL-TK, and 286 ng of either pUC18 or each of the Tax-GFP constructs. Cells were harvested, washed once with cold phosphate-buffered saline, and lysed with radioimmune precipitation buffer (NaCl (150 mM), Igepal (1%), deoxycholate (0.5%), and Tris (50 mM, pH 8.0)). Cell extracts were assayed for luciferase and Renilla using a Fluoroskan Ascent FL (Thermo Labsystems, Beverly, MA) as described for the dual luciferase assay system (Promega).

Detection of Tax Secretion—BHK-21 cells were plated at a density of 2.5 × 10⁵ cells/well in 6-well plates 24 h prior to transfection and transfected using 4 μl of Lipofectamine 2000 (Invitrogen) with 1.6 μg of either control FTH₆ vector or each of the mutants. At 24 h post-transfection, new medium containing the protease inhibitors aprotinin and leupeptin (1 μg/ml each; Sigma and EMD Biosciences, San Diego, CA) was added. After an additional 24 h, the medium was collected and subjected to centrifugation (1200 × g for 5 min) to clear any cell debris. The supernatant was separated into two fractions. One fraction (100 μl) was utilized for the detection of lactate dehydrogenase using the CytoTox 96 nonradioactive cytotoxicity assay as described by the manufacturer (Pierce). The other fraction (1 ml) was concentrated to a volume of 200 μl using a nanosep 10K liquid concentrator (Pall Life Sciences, East Hills, NY). Remaining adherent cells were washed once with cold phosphate-buffered saline (2 ml) and lysed at 4 °C for 20 min using 600 μl of M-PER extraction reagent supplemented with HALT protease inhibitor (Pierce). Wells were scraped, and cell debris was removed by centrifugation (16,000 × g for 10 min at 4 °C). Cell lysates and supernatants were analyzed for Tax secretion by ELISA as previously described.

Statistical Analyses—All statistical analyses were performed using JMP version 3.0 (SAS Institute, Cary, NC). Briefly, each set of data was imported into JMP, and an analysis of variance was performed. The statistical significance of each comparison was determined using Student’s t test with a p value of ≤0.05.
RESULTS

Tax Interacts with Protein Components of the Cellular Secretory Pathway—If Tax is targeted for secretion, it probably interacts with a variety of cellular proteins that may facilitate the transport of Tax from the nucleus to the PM. To determine whether Tax interacts with proteins that may facilitate its egress from the nucleus and through the secretory pathway, antibody array experiments focusing specifically on the secretory pathway proteins were designed to permit the detection of cellular Tax-binding proteins with both purified Tax and endogenous Tax. The antibody array contained 16 antibodies directed against protein components of the cellular secretory pathway, including those that facilitate nuclear to cytoplasmic transport, ER to Golgi movement, and post-Golgi transport to the PM. The array membrane was incubated with either whole cell extracts derived from BHK-21 cells that had been transfected with a plasmid encoding Tax-His<sub>6</sub> or with nontransfected cell extracts followed by incubation with purified Tax-His<sub>6</sub> protein to identify Tax-interacting proteins (Fig. 1A). Comparing in vitro binding of purified Tax-His<sub>6</sub> protein with transfected Tax enabled the comparison between in vitro and in situ interactions ofTax with secretory pathway proteins. The antibody arrays identified the same set of cellular proteins along the secretory pathway with which Tax interacts utilizing purified Tax and Tax-containing cell extracts. Importantly, the identified putative Tax-interacting proteins have previously been shown to be involved in mediating nuclear to cytoplasmic export (calreticulin (a5), RanBP2 (b4), and NTF p97 (c1)), ER to Golgi transport (βCOP (c2) and COPII (c3)), and movement from the Golgi to the plasma membrane (SNAP23 (b3), SCAMP1 (b5), SCAMP2 (b6)). Tax did not directly bind to negative controls rabbit-IgG (a1) or mouse IgG (a2) or to actin (a3), IKK-γ (a4), calregulin (a6, a7), CRM1 (b1, b2), the nucleoporins Nup50 (b7) or Nup62 (b8), tubulin (c4), or kinesin (c5) (Fig. 1B). Although Tax is known to bind IKK-γ (31–33) the inability of Tax to bind to IKK-γ in this assay can be attributed to the fact that the antibody clone for IKK-γ (sc-8256) that was spotted on the array recognizes the COOH terminus of the protein, a domain that is known to bind to Tax (33) and was consequently not recognized in the antibody array analysis. Densitometric quantitation confirmed the qualitative observations and revealed a general trend of more binding in the array incubated with purified Tax-His<sub>6</sub> protein (Fig. 1C), reflecting the fact that less Tax was probably present in transfected cells as compared with the amount of purified Tax-His<sub>6</sub> protein used in the in vitro binding assay.

Analyzing Tax-interacting Proteins in HTLV-1-infected T Cells by Panorama Antibody Microarray—Once it was confirmed that Tax exhibited binding with the components of the cellular secretory pathway in BHK-21 cells, we proceeded to confirm if these interactions could be recapitulated in a relevant cell line model of HTLV-1 infection. To this end, we utilized HTLV-1-transformed C8166 cells, an HTLV-1-infected CD4<sup>+</sup> T cell line established from cord blood lymphocytes co-cultured with leukemic cells from patients with adult T cell leukemia (34). These cells are non-virus-producing and have been previously used to examine the expression of Tax in the supernatant as well as nuclear and cytoplasmic fractions (28). The expression of Tax was first confirmed in the lysate and supernatant of C8166 cells by Western immunoblot analyses using a murine monoclonal antibody against Tax (TAB 170; 1:50) (Fig. 2A). Protein extracts from HTLV-1-transformed C8166 cells transfected with FTH<sub>6</sub> DNA or Jurkat cells (negative control)
Mechanisms of HTLV-1 Tax Secretion

A

Purified Tax
C8166 cells
Lysate
Supernatant

40 kDa

B

| Protein | C8166 vs Jurkat | C8166+FTH6 vs Jurkat |
|---------|----------------|-----------------------|
| Calmodulin | 1.7 (0.015) | 1.4 (0.492) |
| Calreticulin | 2.4 (0.324) | 4.1 (0.320) |
| Calretinin | 1.7 (0.026) | 2.1 (0.689) |
| βCOP | 3.7 (0.06) | 4.9 (0.495) |
| Exportin T | 3.0 (0.115) | 3.6 (0.154) |
| IkBα | 3.7 (0.037) | 6.1 (0.437) |
| IKKγ | 3.4 (0.088) | 5.1 (0.085) |
| Importinα1 | 1.3 (0.091) | 1.4 (0.088) |
| Importin 5 7 | 1.9 (0.107) | 1.4 (0.014) |
| NF-κB | 3.7 (0.051) | 3.5 (1.016) |
| SNAP23 | 4.1 (0.634) | 5.2 (0.191) |
| SNAP25 | 2.7 (0.067) | 3.7 (0.331) |
| SNAP29 | 3.0 (0.361) | 4.3 (0.438) |

FIGURE 2. Analysis of Tax-interacting proteins in the HTLV-1-transformed C8166 T cell line: A relevant model of HTLV-1 infection. A, C8166, an HTLV-1-transformed T cell line, was first examined for the expression of Tax in cell extracts as well as extracellular fluid by Western immunoblotting using a murine monoclonal antibody against Tax (TAB 170; 1:50) followed by secondary antibody staining using Protein G-horseradish peroxidase (1:10,000). Western immunoblot analyses detected the presence of Tax (40 kDa) in the lysate as well as supernatant of HTLV-1-transformed C8166 T cells, indicating active secretion of Tax. B, analyses of Tax-interacting proteins in HTLV-1-transformed C8166 T cells using antibody microarray analyses. Cell lysates (1 mg/ml) obtained from HTLV-1-transformed C8166 T cells or uninfected Jurkat T cells (negative control) were labeled with Cy5 and Cy3 monoreactive dyes, respectively, and applied to an antibody array (Sigma) spotted with 725 antibodies specific to proteins involved in a variety of biologic processes. The array was washed, dried, and scanned using a GenePix 4000B microarray scanner. Following appropriate image analyses and data normalization, -fold changes over the negative control of proteins known to be involved in the secretion process were calculated. To determine that the effects were specific to Tax, HTLV-1-transformed C8166 T cells transfected with FTH6 DNA were used in the antibody microarray analyses.

were subjected to microarray analyses using a very recently developed antibody microarray (XPRESS Profiler725; Sigma) designed to analyze the expression of 725 cellular proteins relevant to a variety of biologic processes. The earlier manually spotted array was highly focused on the secretory pathway proteins to first test if Tax binds with these proteins. However, since Tax may interact with many more proteins relevant to its subcellular localization and secretion, we have selected this array to provide an expanded view of the Tax-interacting proteins in the HTLV-1-transformed C8166 cell line model. The microarray analyses revealed significant -fold changes in the expression of proteins of the secretory pathway in both C8166 cells compared with Jurkat T cells, which were used as a negative control cell line (Fig. 2B). To determine whether the effects were specific to Tax, C8166 cells were also transfected with the FTH6 plasmid and similarly analyzed using the antibody microarray. Results in both cases demonstrated significant changes in the expression of proteins in HTLV-1-transformed C8166 T cell line compared with the uninfected Jurkat T cell line. A number of interesting proteins relevant to the secretion process have been identified (Fig. 2B). These included calmodulin, calreticulin (previously demonstrated to interact with Tax in the process of nuclear export and secretion (35)), βCOP, SNAP, SNAP23, SNAP25, and SNAP29. Additionally, a significant increase in the expression of proteins within the NF-κB complex, IkBα and IKKα, known to be phosphorylated by Tax upon its interaction with IKK-γ (31–33), was observed in Tax-expressing cells compared with the negative control. The other Tax-interacting proteins relevant to different biological processes are not included in this paper in order to maintain the primary focus of the study. Overall, the antibody microarray analyses using C8166 cells thus confirmed the interaction of Tax with proteins of the secretory pathway as well as serving to demonstrate that the observations made in our model cell line (BHK-21) used by many others to secretory pathway proteins can be recapitulated in the context of T cells, the primary target cell population during HTLV-1 infection and subsequently transformation.

Confirmation of Potential Tax-interacting Secretory Pathway Proteins by GST Pull-down Assays and Gene Silencing—To confirm the validity of the experimental results obtained utilizing the antibody arrays, GST pull-down assays were performed with selected Tax-interacting proteins identified in the Panorama antibody microarray analyses. The interactions of Tax with SCAMP1, SCAMP2, SNAP23, and COPII were specifically examined due to the specific roles these proteins play within the secretory pathway. IKK-γ, a known Tax-binding protein, was included as a positive control for the assay. The interaction of Rex (which is not believed to be secreted) with components of the secretory pathway was examined as an appropriate negative control. IKK-γ and proteins of the secretory pathway were synthesized in an in vitro transcription-translation reaction and incubated with GST-Tax or GST-Rex bait proteins. The magnetic GSH-linked particles provided a system in which GST-Tax or GST-Rex bait proteins could affinity-purify the prey proteins from solution. The presence of IKK-γ (48 kDa), SCAMP1 (31 kDa), SCAMP2 (38 kDa), SNAP23 (27 kDa), and COPII (85 kDa) was readily detectable in the eluted fraction where GST-Tax was present as opposed to GST alone (Fig. 3A) or GST-Rex (data not shown), as detected by Western immunoblot analysis. Particles bound to GST alone (negative control) yielded faint, if any, bands due to experimental background. These observations confirmed results obtained from the antibody array, suggesting that Tax interacts with these components of the cellular secretory pathway.

Gene silencing using small interfering RNA was used to demonstrate the effect of blocking the components of the secretory pathway on Tax secretion in a relevant cell model of HTLV-1
The Carboxyl-terminal Domain of HTLV-1 Tax Contains Putative Secretory Signals That Direct Its Subcellular Distribution—Previous observations have indicated that a Tax construct lacking the carboxyl-terminal 139 amino acids displayed a reduced capability to direct Tax secretion from BHK-21 cells.³ Therefore, it was hypothesized that this domain contained amino acid signals that direct the protein to the secretory pathway. Tyrosine-based sorting signals that conform to the YXXØ motif have been implicated in directing protein localization to several intracellular compartments, including the trans-Golgi network (36). A dileucine motif has been suggested to facilitate inclusion of proteins into vesicles that move along the secretory pathway (37). Finally, the DXXE motif has been shown to be critical to the process of concentrating cargo molecules and enhancing their rate of exit from the ER in COPII vesicles (38, 39). Analysis of the amino acid sequence of Tax revealed the presence of several putative secretory signals conforming to previously characterized secretory signal motifs within the carboxyl-terminal domain of the protein, including 312TYNI315, 319LL320, 330DHE332, and Val353 (36). To determine whether the putative secretory signals were responsible for directing Tax to the secretory pathway, site-directed mutagenesis was employed to mutate specific residues in the putative secretory signals in a Tax-GFP construct as described (14). A description of the mutants synthesized is shown in Fig. 4A. If the putative secretory signals are indeed involved in targeting Tax to the PM for secretion, mutation of these signals should alter the subcellular distribution of Tax, resulting in less Tax extending to the PM. Tax-GFP was found to be localized in both nucleus and cytoplasm of BHK-21 cells, consistent with a previous report in other cell types (11). Within the nucleus, Tax was found in the characteristic interchromosomal granules and splicosomal speckles, known as the Tax speckled structure (40). While in the cytoplasm, Tax-GFP was distributed in a punctate manner, extending through the cell body up to the plasma membrane. The Tax mutants ATNI, YTNA, ATNA, and AHA displayed dramatically altered subcellular localization compared with parental Tax-GFP. These mutants primarily displayed a nuclear or perinuclear distribution, with the majority of Tax localized within the nucleus or to areas immediately surrounding the nucleus. However, Tax mutants AA and ΔV displayed localization similar to that of parental Tax-GFP, indicating that in isolation, these signals are not critical for directing the proper subcellular distribution of Tax (Fig. 4B). Furthermore, the percentage of cells with aberrantly localized Tax dramatically increased with the mutants ATNI, YTNA, ATNA, and AHA, with ~35% of transfected cells displaying perinuclear accumulation, whereas AA and ΔV did not show any difference from parental Tax (Fig. 4C). Similar observations were made with HeLa cervical carcinoma and U87-MG astrocytic cell lines (data not shown), demonstrating that altered localization was not cell type-specific. Despite the altered localization of selected Tax mutants in the cytoplasmic compartment, mutated Tax localized to the nucleus was still found in the characteristic interchromosomal granules and splicosomal speckles, known as the Tax speckled structure, as previously reported with parental Tax (40).

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**FIGURE 3.** Confirmation of Tax interaction with select components of the secretory pathway by GST pull-down assays and gene silencing. A, GST-based pull-down of IKK-γ, SCAMP1, SCAMP2, SNAP23, and COPII (prey proteins) using Tax-GST as bait protein followed by Western immunoblotting confirmed the interaction of Tax identified with the antibody array. Tax-containing plasmids are represented as pGEX-Tax, and the negative control plasmid expressing GST alone is represented as pGEX-2T. The experiment was performed in duplicate and repeated twice. B, proteins most relevant to Tax secretion, including SCAMP1, SNAP23, and COPII, were silenced by using specific small interfering RNAs, and the effect of silencing on Tax secretion was examined using a FLAG-based ELISA. Results demonstrated a decrease in the amount (ng) of Tax secreted from HTLV-1-transformed C8166 T cells.

infection. HTLV-1-transformed C8166 cells were silenced using a SmartPool containing four RNA duplexes against SCAMP1, SNAP23, and COPII. Western immunoblot analyses using antibodies specific to SCAMP1, SNAP23, and COPII were performed to confirm the silencing of these proteins (data not shown), and cells were subsequently transfected with FTH₆ DNA. The effect of silencing on Tax secretion was examined using a FLAG-based ELISA designed to detect secreted Tax in the supernatants of transfected cells. Results demonstrated that silencing of the secretory pathway components had a significant effect on Tax secretion, with reduced amounts of FTH₆ protein (2.5-fold) being detected in the supernatants of HTLV-1-transformed C8166 cells (Fig. 3B) following silencing of SCAMP1, SNAP23, and COPII.

| Prey Protein | Bait Protein | kDa |
|-------------|-------------|-----|
| SCAMP1      | pGEX-Tax    | 31  |
| SCAMP2      | pGEX-Tax    | 38  |
| SNAP23      | pGEX-Tax    | 27  |
| COPII       | pGEX-Tax    | 85  |
| IKK-γ       | pGEX-Tax    | 48  |

**FIGURE 3.** Confirmation of Tax interaction with select components of the secretory pathway by GST pull-down assays and gene silencing. A, GST-based pull-down of IKK-γ, SCAMP1, SCAMP2, SNAP23, and COPII (prey proteins) using Tax-GST as bait protein followed by Western immunoblotting confirmed the interaction of Tax identified with the antibody array. Tax-containing plasmids are represented as pGEX-Tax, and the negative control plasmid expressing GST alone is represented as pGEX-2T. The experiment was performed in duplicate and repeated twice. B, proteins most relevant to Tax secretion, including SCAMP1, SNAP23, and COPII, were silenced by using specific small interfering RNAs, and the effect of silencing on Tax secretion was examined using a FLAG-based ELISA. Results demonstrated a decrease in the amount (ng) of Tax secreted from HTLV-1-transformed C8166 T cells.
Perinuclear Accumulation Is the Result of Tax Retained in the ER and Golgi—The perinuclear accumulation of Tax-GFP observed following mutation of the putative secretory signals YTNI and DHE suggested that these Tax-GFP mutants may fail to progress through the secretory pathway. Several cytoplasmic organelles are critical with respect to transporting secreted proteins from their site of synthesis to the PM. Proteins targeted for secretion move along the secretory pathway in a highly organized and directional manner, beginning with targeting to the ER, movement from the ER to the Golgi, and vesicular transport from the Golgi to the PM, where secretion occurs (41, 42). We have previously shown that in BHK-21 cells, a proportion of cytoplasmic Tax localized to the ER and Golgi with additional cytoplasmic Tax present in a punctate distribution extending to the PM (14). Therefore, to examine the subcellular distribution of parental and mutated Tax with respect to the location of cytoplasmic organelles involved in mediating protein secretion, parental Tax-YFP and mutated Tax-YFP constructs were transiently transfected into BHK-21 cells in conjunction with commercially available plasmids encoding the nucleus (CFP-Nuc), ER (CFP-ER), and Golgi (CFP-Golgi). Consistent with previous observation, parental Tax-YFP was localized in part to each of these organelles, nucleus, ER, and Golgi, whereas additional Tax was present in areas beyond these organelles extending to the PM (Fig. 5A). In contrast, Tax-ATNA-YFP and Tax-AHA-YFP localized primarily within the nucleus or to the areas immediately surrounding the nucleus alongside the ER and Golgi, but no cytoplasmic Tax was present in areas other than these organelles (Fig. 5, B and C). The same localization was observed following transfection of Tax-ATNI-YFP and Tax-YTNI-YFP (data not shown). These experimental observations suggest that although Tax secretory signal mutants could localize to cytoplasmic organelles involved in mediating protein secretion, complete egress through the secretory pathway was probably inhibited. The absence of punctate Tax within the cytoplasm extending to the PM indicated that the Tax mutants were most likely unable to assemble in post-Golgi vesicles for distribution to the PM.

Putative Tax Secretory Signal Mutants Retain the Ability to trans- Activate the HTLV-1 LTR—Evidence presented thus far has indicated that introducing point mutations into putative secretory signals YTNI and DHE within Tax-GFP/YFP alters the subcellular distribution of the protein. Presumably, since putative Tax-GFP/YFP secretory mutants do not localize beyond the ER and Golgi, there is probably a defect in Tax secretion. However, it is critical to determine whether the Tax-GFP/YFP mutants still retain functional activity, since it remains possible that the point mutations caused Tax-GFP/
YFP to become misfolded. If misfolded, Tax-GFP/YFP may accumulate in the ER, where it would eventually be degraded. However, this is unlikely due to the fact that a portion of the Tax-GFP/YFP mutants still localized to the nucleus, where the protein probably performed its function as a transcriptional trans-activator. Furthermore, since YTNI and DHE lie within or close to the Tax trans-activation domain between amino acids 284 and 325 (43, 44), mutating these signals may affect Tax-mediated HTLV-1 LTR activation (Fig. 6A). To determine the activity of Tax mutants as compared with parental Tax, BHK-21 cells were co-transfected with a plasmid encoding the HTLV-1 LTR, driving expression of firefly luciferase (pU3R-luciferase) along with either pCMV-Tax (positive control), pEGFP-N1 (negative control), parental Tax-GFP (positive control), or each of the Tax-GFP mutants. Parental Tax-GFP was able to activate expression from pU3R-luciferase to similar levels as does pCMV-Tax, indicating that the GFP tag itself did not alter the ability of Tax to function as a trans-activator. Similarly, Tax-GFP mutants retained LTR trans-activation capability. Each experiment was performed in duplicate, and data represent mean of the duplicated samples ±S.D.

FIGURE 5. Tax mutants localize to the ER and Golgi but not in the outermost areas as compared with the parental Tax. Tax-YFP, Tax-ATNA-YFP, Tax-AHA-YFP, and organelle-targeting CFP plasmids (nucleus, ER, and Golgi) were transiently transfected into BHK-21 cells. After a 24-h incubation, cells were viewed on an inverted fluorescent microscope. After image capture, each image was deconvolved using the no-neighbors method in Slidebook. Colors represent Tax-YFP (green), CFP constructs (blue), and regions of co-localization (phase-contrast image overlaid with blue). A portion of intracellular Tax localized to the cytoplasm, the ER, and the Golgi and extended in a punctate distribution to the plasma membrane (A). Point mutations in DHE (B) and YTNI (C) failed to localize in structures beyond these cytoplasmic organelles. Images represent ×40 magnification.

FIGURE 6. Tax-GFP secretory signal mutants retain functional activity. A, schematic representation of various Tax domains and the location of DHE/YTNI signals. B, BHK-21 cells were co-transfected with pU3R (HTLV-1 LTR)-luciferase, pRL-TK (control plasmid), and Tax-GFP (full-length protein) or Tax mutant and analyzed for the ability of Tax-GFP (parental or mutants) to trans-activate pU3R by Renilla lucinescence. The control pEGFP-N1 did not affect luciferase expression, whereas Tax-GFP activated luciferase expression to similar levels as does pCMV-Tax, indicating that the GFP tag did not affect the ability of Tax to function as a trans-activator. Similarly, Tax-GFP mutants retained LTR trans-activation capability. Each experiment was performed in duplicate, and data represent mean of the duplicated samples ±S.D.
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tory signals YTNI and DHE resulted in aberrant subcellular localization with cytoplasmic Tax concentrated in areas immediately surrounding the nucleus. Perinuclear accumulation appears to result from localization of Tax to the ER and Golgi, but not to areas beyond these organelles. This implies that mutant Tax is probably targeted for movement through the secretory pathway but fails to egress through the latter part of the pathway via inclusion into post-Golgi secretory vesicles. If this were the case, it is likely that the release of Tax secretory signal mutant protein into the extracellular environment would be decreased compared with the release of parental Tax. To investigate this hypothesis, the FTH6 detection system was utilized (14). Having previously demonstrated that the FLAG and His6 tags did not effect Tax expression or intracellular localization (14), mutations were generated within the putative secretory signals of the FLAG-Tax-His6 construct. A description of the constructs utilized in this study is illustrated in Fig. 7A. The expression of each construct was confirmed by Western immunoblot analyses using a murine monoclonal antibody against Tax (TAB 170; 1:50) and was found to be equal in lysates of BHK-21 cells transfected with each of the constructs (Fig. 7B).

To quantitate the secretion of Tax from BHK-21 and HTLV-1-transformed C8166 cells, a FLAG-based ELISA system was utilized as described (14). The relative amount of secreted Tax was converted to percentage of total Tax (present in lysate and medium) in each transfected culture once normalized for the total amount of Tax present in the lysate and the medium. As shown in Fig. 7B, compared with 42% secretion with parental Tax in BHK-21 cells, the YTNI mutation significantly decreased the secretion of Tax. Specifically, the percentage of secretion exhibited by ATNI and ATNA was 28 and 0%, respectively, as compared with parental Tax (42%). Mutation of DHE itself did not alter the overall secretion of Tax, but when combined with mutations in YTNI, the secretion of Tax was completely abrogated (Fig. 7B).

To confirm that the detection of extracellular Tax protein resulted from secretion as opposed to nonspecific release due to cell death, relative to parental Tax, release of lactate dehydrogenase was quantitated. Quantitation of lactate dehydrogenase release demonstrated no significant alterations between parental and mutant Tax constructs (Fig. 7C), indicating that Tax release was not the result of increased cell death. Similar results were obtained when Tax secretion was examined in the HTLV-1-transformed T cell line C8166 (data not shown), a relevant model of infection.

**DISCUSSION**

The vast majority of the cytotoxic T cell response observed during HAM/TSP is specific for the Tax immunodominant peptide (11–19), indicating that Tax is readily available for immune recognition. Several studies have provided evidence suggesting that progression of HAM/TSP involves an indirect mechanism involving the ability of Tax to function as an extracellular cytokine. Studies with two central nervous system-resident cell populations, neurons and microglia, demonstrated the release of the proinflammatory cytokine TNF-α in response to extracellular Tax (16, 17). Exposure of oligodendrocytes to TNF-α induces demyelination and inflammation. The immune response to Tax probably plays a key role in the initiation and maintenance of the inflammatory state characteristic of HAM/TSP. Therefore, understanding the mechanism of Tax release is crucial to defining the precise role of extracellular Tax as a mediator of immune dysfunction.
In addition to being an intracellular/nuclear protein, Tax has been shown to be present in HAM/TSP patients in a cell-free form (15). However, it is not clear whether the presence of extracellular Tax is a result of apoptosis or necrosis of HTLV-1-infected cells or if it has been secreted from infected cell populations. Consistent with the concept of Tax secretion, it has been previously demonstrated that full-length Tax can be secreted from HTLV-1-transformed lymphoid cells (27, 28, 45) and Tax-transfected BHK-21 cells in vitro (14). However, the intracellular events that direct Tax to the secretory pathway and the mechanisms that facilitate Tax secretion remain to be delineated. Since Tax is primarily a nuclear protein, it must be transported into the cytoplasm and directed to the secretory pathway.

We have previously identified a leucine-rich nuclear export signal between amino acids 188 and 202 that targets Tax for nuclear export (13). Once in the cytoplasm, Tax probably proceeds through the cellular secretory pathway via interactions with cytoplasmic proteins. In order to identify Tax-interacting proteins, we have employed an antibody array designed to detect the proteins of cellular secretory pathway with which Tax directly interacts. Tax-interacting proteins that were identified in this study have been shown to mediate nuclear to cytoplasmic export (calreticulin, RanBP2, and NTF p97), ER to Golgi transport (βCOP and COPII), or Golgi to plasma membrane migration (SNAP23, SCAMP1, and SCAMP2). Calreticulin, in addition to functioning as an ER-resident protein involved in calcium binding, acts as a nuclear export protein, facilitating the exit of proteins from the nucleus to the cytoplasm (46, 47). We have previously observed that Tax binds to calreticulin (35), and the antibody array results further substantiate this interaction. Other Tax-interacting proteins also serve key roles; NTF p97 is a nuclear transport factor involved in mediating transport of proteins from the cytoplasm into the nucleus, whereas RanBP2, a component of the nuclear pore complex, binds Ran GTPase and helps stimulate GTPase activity to facilitate transport of macromolecules across the nuclear membrane (48–51). Coatamer proteins shuttle proteins through the secretory pathway; βCOP is implicated in the retrograde transport of proteins from the Golgi to the ER, whereas COPII facilitates movement of proteins from the ER to the Golgi (52–54). Soluble N-ethyl maleimide-sensitive factor attachment protein receptors facilitate post-Golgi membrane fusion during intracellular vesicular transport (55), and SNAP23, a target soluble N-ethyl maleimide-sensitive factor attachment protein receptor previously identified at the plasma membrane, is involved in exocytic membrane fusion in both polarized and nonpolarized cells (56–62). Interestingly, SNAP23 has been demonstrated to associate with SCAMP2 (63), another protein identified by the antibody array capable of interacting with Tax. SCAMP2 has also been found to interact with SCAMP1 (64), again supporting the results obtained by the antibody array. SCAMPs are proteins of post-Golgi compartments that reside in vesicles comprising cell surface recycling pathways, including secretion granules (65). Although the precise function of SCAMPs is unknown, it is postulated that SCAMP1 and SCAMP2 function together in a single protein complex (64). Results presented herein substantiate this hypothesis, since Tax was found to interact with both SCAMP1 and -2. Most of the proteins identified by the array were found to be significant for studying the mechanisms of Tax nucleocytoplasmic transport and/or secretion. In this study, the focus was to define the events that lead to Tax shuttling through the secretory machinery and eventual release from the cells. In this regard, GST pull-down assays further confirmed the interaction of Tax with selected components of the secretory pathway, namely SCAMP1, SCAMP2, SNAP23, and COPII by GST pull-down assay, suggesting that these cellular factors are critical mediators of Tax transport from ER to Golgi (COPII) and post-Golgi movement to the plasma membrane (SCAMP1, SCAMP2, and SNAP23). Future investigations will explore the functional consequences of such interactions.

Proteins trafficking through the secretory pathway use a vesicular transport mechanism in which proteins are concentrated into vesicles and are delivered to the endosome, lysosome, plasma membrane, or extracellular environment (37). This transport system requires selective sorting signals present in the proteins themselves, which are necessary for selection of cargo into vesicles, and also direct the retention along this pathway or diversion to peripheral organelles. One such signal of interest has been identified in the cytosolic sequence of proteins that exit the ER and is composed of a diacidic sorting signal, Asp-X-Glu (DHE). This signal has been found in the type-1 transmembrane protein, the vesicular stomatitis virus glycoprotein, which has been utilized extensively to define the basic operations of the secretory pathway (36). Previous studies have investigated a number of transmembrane glycoproteins and identified two families of post-Golgi sorting signals. First, YXXΦ contains a critical tyrosine residue, which is separated by two random amino acids (Φ) from a hydrophobic side chain (Φ), and has been shown to be responsible for targeting from the Golgi network. Second, a dileucine (LL) motif has been shown to also mediate vesicle formation at the Golgi and has been demonstrated to be involved in targeting proteins to outer organelles (66). Based on the observations that a Tax construct lacking the carboxyl-terminal 139 amino acids displayed reduced Tax secretion from BHK-21 cells, the carboxyl-terminal domain of Tax was examined for the presence of putative secretory signals, since Tax does not contain a leader sequence to direct its secretion. Analysis of the amino acid sequence of Tax confirmed the presence of a number of putative secretory signals, including 312YTNI315, 319LL320, 330DHE332, and Val353. Mutation of 312YTNI315 and 330DHE332, but not 319LL320 or Val353, resulted in aberrant subcellular localization of Tax, characterized by the cytoplasmic Tax accumulating in the perinuclear region. This localization was apparent in BHK-21, HeLa, and U87-MG astrocytic cells, demonstrating that aberrant localization of the Tax mutants was not cell type-specific. In addition, aberrant localization in astrocytes demonstrated that the putative secretory signals are probably functional in cells that are pathogenically relevant to the HTLV-1-induced disease state HAM/TSP (data not shown).

Further analyses of the subcellular localization of Tax and Tax mutants demonstrated that perinuclear Tax was the result of Tax accumulation in areas corresponding to the ER and Golgi. Very few, if any, Tax-containing mutations in the YTNI
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and DHE signals were found to be present in the cytoplasm in areas outside of the ER and Golgi. This suggested that Tax-containing mutations in putative secretory signals were probably targeted for entry into the cellular secretory pathway, but inclusion into post-Golgi secretory vesicles may have been inhibited. It is likely that amino acids surrounding the YTNI and DHE signals may be equally important to successful targeting of Tax for secretion. As previously demonstrated by Nishimura et al. (39), mutating the diacidic (DXX) motif in the vesicular stomatitis virus glycoprotein caused the protein to exit the ER ~10-fold more slowly but failed to completely block the exit of the protein. It is also possible that signals within the carboxy-terminal domain may function together rather than as individual signals (67). In this regard, the studies reported herein demonstrate that the ATNI and ATNA mutations played a significant role in Tax secretion, resulting in an inhibition of secretion. Furthermore, the combined mutation of YTNI and DHE also inhibited the secretion of Tax. These observations suggested that the YTNI and DHE signals participate in interactions required for the efficient coupling of Tax to the export machinery.

Given the consequences of extracellular Tax acting on cells within the central nervous system, understanding the mechanism of Tax release is crucial to defining the precise role of extracellular Tax in the participation of the initiation and/or maintenance of HAM/TSP. Taken together, the results presented herein begin to identify the cellular factors that facilitate Tax secretion and also identify secretory signals within Tax that direct the protein to the secretory pathway. Further studies will extend these observations in clinically relevant cell types under various pathological conditions as observed in disease states, such as stress induced by oxygen deprivation.

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