Human T cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T cell leukemia and HTLV-I-associated myelopathy/tropical spastic paraparesis. The HTLV-I protein Tax is well known as a transcriptional transactivator and inducer of cellular transformation. However, it is also known that extracellular Tax induces the production and release of cytokines, such as tumor necrosis factor-α and interleukin-6, which have adverse effects on cells of the central nervous system. The cellular process by which Tax exits the cell into the extracellular environment is currently unknown. In most cell types, Tax has been shown to localize primarily to the nucleus. However, Tax has also been found to accumulate in the cytoplasm. The results contained herein begin to characterize the process of Tax secretion from the cell. Specifically, cytoplasmic Tax was demonstrated to localize to organelles associated with the cellular secretory process including the endoplasmic reticulum and Golgi complex. Additionally, it was demonstrated that full-length Tax was secreted from both baby hamster kidney cells and a human kidney tumor cell line, suggesting that Tax enters the secretory pathway in a leaderless manner. Tax secretion was partially inhibited by brefeldin A, suggesting that Tax migrated from the endoplasmic reticulum to the Golgi complex. In addition, combined treatment of Tax-transfected BHK-21 cells with phorbol myristate acetate and ionomycin resulted in a small increase in the amount of Tax secreted, suggesting that a fraction of cytoplasmic Tax was present in the regulated secretory pathway. These studies begin to provide a link between Tax localization to the cytoplasm, the detection of Tax in the extracellular environment, its possible role as an extracellular effector molecule, and a potential role in neurodegenerative disease associated with HTLV-I infection.

Human T cell leukemia virus type I (HTLV-I), a retrovirus, is the etiologic agent of adult T cell leukemia (ATL) and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The HTLV-I transactivating oncoprotein Tax plays an integral role in productive viral replication and disease progression. Tax has been studied with respect to its interaction with a number of cellular signaling pathways and transcription factor families, including activating transcription factor/cAMP-response element-binding protein and NF-κB (1–5). Specifically, Tax enhances cAMP-response element-binding protein binding within the HTLV-I long terminal repeat, which in turn enhances transcription of viral mRNA (1). With respect to the NF-κB pathway, cytoplasmic Tax acts by binding directly to the IKK-γ subunit of the IKK complex. This association induces the phosphorylation and degradation of IκBα, the inhibitor of NF-κB, thereby allowing the NF-κB complex to migrate to the nucleus and enhance gene expression (6–8).

The pathogenesis of both ATL and HAM/TSP is coupled, at least in part, to the biological activity of Tax (9–13). Tax has been demonstrated to be a key player in the malignant transformation of HTLV-I-infected T cells. In addition, several of the pathogenic processes associated with HAM/TSP have been postulated to occur as a result of the extracellular activity of Tax. First, in some individuals with HAM/TSP, Tax has been shown to promote hyperstimulation of the immune system. Specifically, these individuals harbor an extremely large number of Tax-reactive CD8+ T cells that reside in the cerebrospinal fluid (14). Second, in some HTLV-I-infected individuals, antibodies directed against Tax also cross-react with the neuronal protein heterogeneous nuclear ribonucleoprotein-A1, implicating the process of molecular mimicry in the progression of HAM/TSP (15, 16). Finally, several studies have examined the effects of extracellular Tax on cells of the central nervous system (CNS). For example, microglial cells were previously shown to release tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in response to extracellular Tax (17). Additionally, neuronal cells were shown to release TNF-α in response to extracellular Tax (18). Each of these cytokines has been demonstrated to induce severe abnormalities in the CNS including, but not limited to, dysfunction of oligodendrocytes leading to demyelination of neurons, a pathology that is also observed in HAM/TSP patients.
There have been two cell types demonstrated to harbor HTLV-I proviral DNA in vitro and in vivo: the T lymphocyte and the astrocyte. Clearly, T lymphocytes represent the most commonly infected cell type and primary site of productive viral replication. Consequently, this cell type would represent an obvious source of extracellular Tax, since it infiltrates the CNS during the course of neurologic disease and has been associated with CNS lesions characteristic of HAM/TSP. Additionally, astrocytes have been demonstrated to harbor HTLV-I proviral DNA and exhibit an altered physiology after co-culture with HTLV-I-infected T cells (19–21). Thus, it is possible that both T lymphocytes and astrocytes may serve as a source of extracellular Tax in the CNS. In 1990, Lindholm et al. (22) addressed the possibility that Tax may be secreted from HTLV-I-infected T lymphocytes. Tax was detected in the extracellular environment after purification of medium derived from MT-2 cell cultures, an HTLV-I-infected CD4+ T lymphocyte cell line (22). The apparent release of Tax from MT-2 cells was most likely not the result of cell lysis or apoptosis, because the HTLV-I glycoprotein p24 could not be detected in the medium. Despite these interesting results, many questions still remain concerning the source of extracellular Tax and how the protein exits the cell.

In this report, we have investigated the secretion of Tax utilizing a cell biology system that has been previously used to examine the general properties of protein secretion. To this end, the baby hamster kidney (BHK-21) cell line was utilized for several reasons, including its previous use in numerous studies examining the biological process of protein secretion, its high transfection efficiency, and its ease of use in microscopic analysis. In addition to this, 293T cells were selected to study Tax secretion because of their human origin and because they were of kidney origin and therefore physiologically consistent with the BHK-21 cell line. Furthermore, the 293T cell line has previously shown utility in protein secretion studies and other microscopic analyses and is also transfected with high efficiency in many experimental systems.

In the experimental analysis contained herein, Tax was demonstrated to co-localize with several cytoplasmic organelles associated with exocytosis including the endoplasmic reticulum (ER) and the Golgi complex (GC), suggesting that Tax may migrate through these two organelles during the course of secretion. Second, time lapse video microscopy demonstrated that a large fraction of Tax localized to the cytoplasm and moved in a manner consistent with that of microtubule-associated proteins or secretory vesicles. Third, Tax was purified from the medium of Tax-transfected BHK-21 and 293T cells, strongly suggesting that Tax was secreted from these cell types. Fourth, Tax was shown to be secreted as a full-length protein, suggesting that it entered the cellular secretory pathway in a leaderless and uncleaved manner, a result similar to several other secreted proteins, including HIV-1 Tat, IL-1β, and basic fibroblast growth factor (23, 24). Finally, a small increase in Tax secretion was detected after combined treatment of cells with the secretagogues phorbol myristate acetate (PMA) and ionomycin, indicating that at least a fraction of Tax was present in the regulated secretory pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**BHK-21 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA). All growth media were supplemented with 10% fetal bovine serum, antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/ml each), l-glutamine (0.3 mg/ml), and sodium bicarbonate (0.05%). Cell lines were maintained at 37 °C in 5% CO2 at 90% relative humidity.

**Secretion of the HTLV-I Oncoprotein Tax**

To facilitate the immunoprecipitation and quantitative estimation of secreted Tax, FLAG and His tags were added to the Tax. The FTH4 protein was purified by Western immunoblot analysis.

**Construction of FLAG-Tax-His6 (FTH6) Plasmid and Purification of Mutant Protein**—To facilitate the immunoprecipitation and quantitative estimation of secreted Tax, FLAG and His tags were added to the Tax. The FTH4 protein was purified by Western immunoblot analysis.

**Cell Culture**—BHK-21 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA). All growth media were supplemented with 10% fetal bovine serum, antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/ml each), l-glutamine (0.3 mg/ml), and sodium bicarbonate (0.05%). Cell lines were maintained at 37 °C in 5% CO2 at 90% relative humidity.

**Construction of Plasmids Encoding Fusion Proteins and Purification of Recombinant DNA**—Full-length Tax was cloned into pEYPF-N1 (Clontech, Palo Alto, CA) using PCR and Tax-specific primers, yielding a fusion protein in which yellow fluorescent protein (YFP) was fused to the carboxyl terminus of Tax. Tax and Tax mutant protein coding sequences were cloned into pRc/RS-EGFP (Clontech) using PCR and the Tax-specific primers that also encoded FLAG (DDDDK) or His6 (HHHHHH) tags. Mutation of FLAG-Tax-His6 to FLAG-Tax-COPII-His6 (330DHE332 to 330AHA332) was performed using site-directed mutagenesis (Stratagene, La Jolla, CA). Plasmid DNA used for screening and automated sequencing was isolated using the Concert miniprep system as described by the manufacturer (Invitrogen). The nucleotide sequence of all plasmid constructs was confirmed by automated sequencing and subsequent bioinformatics analysis using Lasergene software (DNASTAR, Madison, WI). Plasmid DNA used for transient transfections was isolated using the HiSpeed Plasmid Midi DNA purification system as described by the manufacturer (Qiagen, Valencia, CA).

**Construction of FLAG-Tax-His6 (FTH6) Plasmid and Purification of Mutant Protein**—To facilitate the immunoprecipitation and quantitative estimation of secreted Tax, FLAG and His tags were added to the Tax. The FTH4 protein was purified by Western immunoblot analysis.

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Butler, PA) and incubated at 37 °C. Some cells were treated with either the microtubule polymerization inhibitor nocodazole (50 μM, 90 min; Sigma) or the kinesin inhibitor monastrol (100 μM, 4 h; Sigma) just prior to insertion into the live cell chamber system. The live cell chamber was then attached to the Olympus IX-81 microscope system as outlined above. Time lapse images (60) were obtained at intervals of 3 s as guided by the Slidebook software system.

Detection of Native Tax and FTH6 in Cell Culture Medium—BHK-21 cells (1 × 10⁶) were seeded in a 100-mm culture dish and transfected (in triplicate) with pCMV4, pCMV-Tax, or pCMV-FTH6 using Lipofectamine 2000 as described under "Transient Transfections." After transfection (24 h), fresh medium containing protease inhibitors apro tinin and leupeptin (1.0 μg/ml each; Sigma) was added. After an additional 24 h, the medium was collected and subjected to centrifugation (600 × g, 5 min) to pellet cell debris. Cells remaining in culture were washed once with ice-cold PBS (1.0 ml) and lysed at 4 °C with shaking for 10 min. First, M-PER extraction reagent supplemented with HALT protease inhibitor (Pierce). Both cell lysate and supernatant were concentrated using a nanosep 10K liquid concentrator (PerkinElmer Life Sciences, Ann Arbor, MI). Total protein in the supernatant was precipitated by the addition of ice cold 10% trichloroacetic acid for 20 min on ice. The precipitate was collected by centrifugation at 14,000 rpm for 15 min at 4 °C. The protein pellet was washed twice with 100% acetone (10-min intervals on ice) and resuspended in M-PER extraction reagent supplemented with HALT protease inhibitor (Pierce). Cell lysates were diluted 1:2 with sample buffer. All of the samples were denatured at 95 °C for 5 min and loaded onto a 12% Tris-HCl SDS-polyacrylamide gel (Bio-Rad). Cell lysates were detected using a 1:10,000 dilution (PBST plus 1% bovine serum albumin) of anti-Tax monoclonal antibody (TAB 170), a generous gift from Dr. Fatah Kashanchi (George Washington University, Washing- ton, D.C.). After primary antibody incubation, blots were washed as before and incubated with a 1:10,000 dilution (PBST plus 1% bovine serum albumin) of Protein G-peroxidase (Sigma) for 1 h. After the final wash, blots were developed using Western Lightening (PerkinElmer Life Sciences) and exposed to x-ray film.

Purification of Secreted FTH6—Six plates (35 mm) of BHK-21 cells were transfected with each plasmid construct used (pIREs-EGFP and FTH6) as described under "Transient Transfections." After transfection, medium in each well was replaced with new medium containing the protease inhibitors aprotinin and leupeptin (1.0 μg/ml; Sigma). After 24 h, the medium (12 ml/sample) was collected and subjected to centrifugation (600 × g, 1 min) to pellet all cell debris. Medium was then transferred to a 15-ml conical tube, and 250 μl of anti-FGAR-sagaroose beads (Sigma) was added. Samples were then placed on an end-over-end rotor overnight at 4 °C. After incubation, samples were subjected to centrifugation (400 × g, 5 min). The medium was removed. Arosearose beads were washed three times (500 μl) with 1× wash buffer (Sigma), resuspended in 50 μl of 2× SDS loading buffer (Sigma), and heated at 95 °C for 5 min. The presence of FTH6 was assayed using Western immunoblot analysis as described above using either anti-GFP antibody ab-290 (Abcam Ltd.), the M2 anti-FLAG antibody (Sigma), or the anti-His6-horseradish peroxidase antibody (Sigma) as primary antibody and anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences).

Quantification of Tax Secretion by ELISA—BHK-21 cells were transfected with FTH6, as described above, and 24 h post-transfection, new medium containing the protease inhibitors aprotinin and leupeptin (1 μg/ml; Sigma) was added. After an additional 24 h, the medium was collected and subjected to centrifugation (16,000 × g, 1 min) to pellet all cell debris. The supernatant was separated into two fractions. One fraction (100 μl) was utilized for the detection of lactate dehydrogenase (LDH) using the CytoTox96 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, Ann Arbor, MI). Cells remaining in the culture well were washed once with ice-cold PBS (1 ml) and lysed on ice for 10 min using 200 μl of M-PER extraction reagent supplemented with HALT protease inhibitor (Pierce). Cell debris was pelleted by centrifugation (16,000 × g, 10 min). Cell extract and corresponding concentrated medium were loaded onto a 96-well plate precoated with anti-FLAG antibody (Sigma). Purified FTH6 protein was also loaded onto a 96-well plate as a series of quantification reference standards (1000, 100, 10, and 1 ng/well). The plate was incubated at 37 °C for 2 h, at which time each well was washed three times with 300 μl of PBS with 1% Tween (PBST). A solution of PBST and anti-His6-horseradish peroxidase antibody (ab1167, 200 μl, 1-1000 dilution; Abcam), was then added to each well and incubated at 4 °C for 2 h. Each well was then washed three times with 300 μl of PBST (1%). Detection of bound anti-Tax-horseradish peroxidase was detected using tetramethyl benzidine substrate as described by the manufacturer (Pierce) and measured using a microplate reader (450 nm). This procedure was repeated for a total of three times with each cell type.

Treatment of Cells with Brefeldin A or a Combination of PMA and Ionomycin—Cells were transfected with each plasmid construct used (pIRES-EGFP and FTH6) as described under "Transient Transfections." After transfection (24 h), the medium was removed and replaced with 2 ml of medium containing either brefeldin A (10 μg/ml) or a combination of PMA (100 nM; Sigma) and ionomycin (1 μM; Sigma). Cells were incubated for 4 h, after which time 1 ml of medium was removed and replaced with 1 ml of new medium containing either brefeldin A or a combination of PMA and ionomycin. After initial medium replacement (24 h), the medium was removed and subjected to centrifugation at (400 × g). Medium and cells were then prepared as outlined under "ELISA Detection of Tax." Results

Detection of Apoptosis and Necrosis of Transfected BHK-21 Cells—Cells were plated in 6-well glass slides and either not transfected or transfected (pCMV-EGFP, pCMV-FTH6) as described under "Transient Transfections." Transfected cells incubated in medium containing 10 μM camptothecin (Sigma) for 4 h were used as the positive control for the experiments designed to detect apoptosis. Transfected cells incubated in medium containing 50 μM ebselen (Sigma) for 6 h were used as the positive control for experiments to detect necrosis. Reactions with only ethanol and MeSO were utilized to control for each solvent used with ebselen and MeSO. Transfected cells (24 h) treated with the stated length of time for each positive control, apoptosis and necrosis were detected using the Vybrant apoptosis detection kit 7 as described by the manufacturer (Molecular Probes, Inc., Eugene, OR). Cells were always used as described under "Microscopic Analyses." RESULTS

Tax-YFP Co-localizes with Organelles Associated with Protein Secretion—Several studies have examined the intracellular localization of the HTLV-I Tax protein (19, 26–29). Most of the investigations, which examined Tax intracellular localization in HeLa and COS-7 cells and the HTLV-I-infected cell lines C8166-45 and MT2, have concluded that Tax resides mainly in the nucleus in the form of interchromatin granules and spli ceosomal speckles (30), sites of high rates of gene transcription. As a result, most studies of Tax function have focused on the role of nuclear Tax and its involvement in gene regulation. However, recent studies have demonstrated that there is a significant amount of Tax localized to the cytoplasm, an amount that is cell type-dependent. For example, recent studies utilizing HeLa cells and primary astrocytes or astrocytic cell lines infected with HTLV-I demonstrated that Tax accumulated to significant levels in the cytoplasm as well as the nucleus (19, 31–34). Furthermore, the localization of Tax in the cytoplasm of these cells was observed in both small and large punctate particles. These punctate structures suggested that Tax interacted with cytoplasmic proteins or localized to specific cytoplasmic organelles. In either case, many of the proteins involved in interfacing with cytoplasmic Tax have not been characterized in detail. Many studies have examined the adverse effects of extracellular Tax, including the stimulation of the production and release of cytokines. However, the pathway whereby Tax gains access to the extracellular environment has not been identified. Theoretically, Tax may exit the cell through at least three avenues, cell necrosis, cell apoptosis, or via the cellular secretory pathway. To begin to examine this hypothesis, we proceeded to determine whether cytoplasmic Tax was localized to cellular organelles associated with the secretory pathway, specifically the ER and GC. To allow for
proteins to halt secretory vesicle movement. Consequently, these methods were also utilized to track Tax-YFP in BHK-21 cells co-transfected with CFP-Nuc to demarcate the nucleus. A representative cell from a population of Tax-YFP-transfected cells is shown in Fig. 2A. Each cell transfected with Tax-YFP contained vesicle-like particles present within the cytoplasm. Time lapse photographic analysis of these particles indicated that many of the Tax-YFP-containing structures moved in a manner similar to microtubule-associated structures and other secretory proteins. The stop-and-go and change-of-direction motion of several Tax-YFP-containing cytoplasmic structures is shown in Fig. 2B. Additionally, many of the Tax-YFP particles migrated significant distances (4–10 μm) over the time period examined (Fig. 2B), another characteristic feature of microtubule-associated vesicles. To provide additional evidence that cytoplasmic Tax-YFP may be associated with the secretory pathway, Tax-YFP-transfected cells were incubated with either nocodazole or monastrol, two compounds known to inhibit movement of secretory vesicles. Specifically, nocodazole inhibits microtubule polymerization, whereas monastrol inhibits the microtubule-associated motor protein kinesin. In both treated cell cultures (Fig. 2, C and E), movement of the cytoplasmically localized Tax-YFP (Fig. 2, D and F) was abrogated or severely retarded. These results provided further evidence that at least some fraction of the cytoplasmic Tax-YFP particles were associated with the microtubule-mediated secretory pathway.

**Full-length Tax Is Secreted from BHK-21 Cells**—The co-localization of Tax-YFP to the ER and GC and the association of Tax-YFP-containing particles in the cytoplasm with secretory vesicles suggested Tax movement through the cellular secretory pathway. To continue the investigation of Tax secretion, it was important to determine whether Tax is released from the cell into the extracellular environment. In order to effectively detect, immunoprecipitate, and quantify Tax in an efficient manner, two amino acid tags were fused to the protein. Specifically, a FLAG tag (DDDDK) was fused to the amino terminus, and a His₆ tag was fused to the carboxyl terminus of Tax (Fig. 3). To ensure, by visualization, that each Tax construct was effectively transfected into the target cell population, the FTH₆ construct was cloned into the pIREs-EGFP vector (pIREs-FTH₆) where the expression of GFP was promoted by an internal ribosome entry site (IRES). Thus, any green cell within a culture could also be expected to express FTH₆.

Numerous studies have demonstrated that even small changes in amino acid structure or the addition of long amino acid tags to Tax may significantly alter many functional properties of Tax, including its intracellular localization and/or its ability to activate cellular or viral gene expression (20). As a result, several functional properties of FTH₆ were examined. First, FTH₆ expression was examined using transfected BHK-21 cell lysates for SDS-PAGE analysis and Western immunoblotting (Fig. 3C). Tax was detected using antibodies against both the amino terminus FLAG tag and the carboxyl terminus His₆ tag to ensure that full-length Tax was being expressed. In addition, Western immunoblotting with an anti-GFP antibody demonstrated that GFP was expressed evenly throughout all transfected cultures. Furthermore, immunofluorescence microscopy was utilized to determine whether the FLAG and His₆ tags affected intracellular localization of Tax following transfection of BHK-21 cells (Fig. 3B). As shown, FTH₆ localized to both the nucleus and cytoplasm, consistent with the nucleocytoplasmic localization of Tax-YFP in BHK-21 cells (Fig. 1).

Confident that the additional FLAG and His₆ peptide tags did not affect Tax expression and intracellular localization, experimentation was performed to analyze the secretion of...
Secreted FTH₆ was immunoprecipitated from either the medium or the cellular lysate utilizing an anti-FLAG antibody and subsequently examined by Western immunoblot analysis using both anti-His₆ and anti-FLAG antibodies (Fig. 4, top). This method resulted in the purification and detection of a 43-kDa protein, the predicted size of FTH₆ protein, from both the lysate and medium, and the protein was detected using both anti-His₆ and anti-FLAG tag antibodies. This result is significant for two reasons. First, it is the most conclusive evidence to date that Tax can be detected in the medium of cells transfected with a recombinant plasmid encoding Tax. Second, since extracellular Tax was purified and detected utilizing amino acid tags found at both the amino and carboxyl terminus of the protein, Tax was probably released from the cell in a leaderless manner, a mechanism common to only a few proteins, such as IL-1β/H9252 and HIV-1 Tat. Finally, it was important to ensure that the FLAG and His₆ tags were not the causal reason for Tax secretion. To assess this possibility, proteins contained in the medium of Tax (pCMV-Tax) and FTH₆ transfected cells were precipitated and subjected to SDS-PAGE. Western blot analysis was then performed using an anti-Tax monoclonal antibody (TAB 170) (Fig. 4, bottom). This method resulted in the detection of both FTH₆ and native Tax, strong evidence that native Tax was secreted and that the addition of the FLAG and His₆ was not the basis for this observation.

Quantification of Tax Secretion by ELISA—Since we have demonstrated that secretion of FTH₆ behaves in the same manner as native Tax, FTH₆ was utilized for the quantitative analysis of secreted Tax. Using an ELISA-based system, FTH₆ secretion from both BHK-21 and 293T cells was demonstrated (Fig. 5). This system is advantageous over previous systems that have been described (22) in that it provides a simple, yet rapid and sensitive analysis of Tax secretion. In addition, this system has also allowed examination of Tax protein mutants that may be of specific relevance to Tax protein secretion. For example, examination of the Tax primary amino acid sequence determined that Tax contained a putative D/E signal (amino acids 300–302; Fig. 3). The D/E amino acid signal was previously reported to be important in binding to the COP91 complex, a complex important in the progression of secretory proteins from the ER to the GC, and concentration of these proteins into secretory vesicles (40). This report also demonstrated reduced secretion of the protein after the D/E signal was mutated to A/A. Thus, a Tax protein construct A/A, FLAG-Tax-COP91-His₆ (FTCOP91H₆; Fig. 3), containing a mutation in the D/E signal was designed for use in these studies.

To compare the amount of Tax released between experimental repetitions, the relative amount of Tax secreted was converted to percentage of total Tax secreted in each transfected culture. Surprisingly, a large percentage of Tax was released from BHK-21 cells into the medium, an average of 53.1% of all Tax detected in both the medium and cell lysate fractions (Fig. 5A). Additionally, 293T cells secreted 16.2% of Tax detected in both the medium and cell lysate. The difference in the percentage of...

**Fig. 2.** Time lapse analysis of Tax-YFP movement in BHK-21 cells. A, Tax-YFP was transiently transfected into BHK-21 cells, and the movement of Tax-YFP was observed using time lapse video microscopy. A, representative image from the population of Tax-YFP transfected cells. B, pictorial representation of the path of some Tax-YFP-containing vesicles within the cytoplasm corresponding to the image in A over the total capture time of 54 s. The green dots indicate the starting position of the vesicle, black dots represent the path traveled, and red dots indicate the end position of the vesicle at the end of video capture. Other cell populations were also treated with either nocodazole (C, 50 mM, 90 min), a microtubule polymerization inhibitor, or 100 μM monastrol (E, 100 mM, 4 h), an inhibitor of the microtubule-associated motor protein kinesin. Both treatments halted almost all movement of Tax-YFP in the cytoplasm (D and F), indicating that movement of the cytoplasmic punctate Tax-YFP structures, possibly secretory vesicles, is controlled in large part by microtubule polymerization.
Tax secreted by BHK-21 versus 293T cells may be the result of a factor(s) that would result in a general difference in the secretion output of either cell type and any of a number of cellular or species differences that could specifically alter the ability of Tax to enter the secretory pathway as a result of changes in cellular recognition of secretory signal sequences within Tax. Importantly, these results demonstrated that Tax secretion could be observed in cells of hamster and human origin. Purified FTH6 protein was utilized as both a positive control for detection of the chimeric protein and a protein concentration standard that could be used to calculate the specific amounts of FTH6 released in each culture. Utilizing this method, an average of 132.5 and 82.3 ng of Tax was detected in each 2 ml of BHK-21 and 293T cell culture medium, respectively. Surprisingly, the amount of FTCoPIIH6 mutant protein secreted from BHK-21 cells was only slightly below that detected with the parental protein. This result was somewhat unexpected, given that our hypothesis predicted that Tax interaction with the COPII machinery would be necessary for the migration of Tax to the GC. The results from 293T cell cultures were more consistent with the hypothesis suggesting that the putative DXE signal within Tax was more functional in this cell type. More recent reports have suggested that other amino acid signals surrounding the DXE motif are just as important, or more so, than that of the DXE motif itself for migration of proteins from the ER to the GC (41). Thus, study of the putative DXE signal within Tax must be examined in context with other signals at the carboxyl-terminal end of the protein.

**Tax Secretion Is Inhibited by Brefeldin A**—Brefeldin A is a chemical inhibitor of protein secretion that specifically blocks secretory vesicle migration from the ER to the GC. Thus, to provide additional evidence that Tax utilized the cellular secretory system, analysis of FTH6 secretion in BHK-21 cells was performed in the absence or presence of brefeldin A (10 μg/ml). After 24 h of treatment, the ELISA secretion assay was performed to determine the effects of brefeldin A. The results (Fig. 3) illustrated that Brefeldin A inhibited Tax secretion, confirming that Tax used the cellular secretory pathway. The experiment was repeated with different concentrations of brefeldin A to determine the optimal concentration for inhibition. The results showed a dose-dependent inhibition of Tax secretion, with complete inhibition observed at 20 μg/ml.

**Fig. 3.** Intracellular localization and expression analysis of plasmid constructs utilized in secretion analyses. A, pictorial representations of the plasmid constructs utilized in secretion analyses of Tax and Tax mutant proteins. Each construct contains an amino-terminal FLAG tag as well as a carboxyl-terminal His6 tag. Additionally, each construct was inserted into a plasmid with a GFP coding sequence expressed under control of an IRES. Tax-COPII contains a DXE to AXA mutation at amino acids 330–332. The DXE motif was previously described as a domain important in protein localization to the secretory pathway. B, the intracellular localization of secretion constructs was determined to ensure that the additional amino acid tags did not alter the normal intracellular distribution of Tax in BHK-21 cells. Secretion construct plasmids were transiently transfected into BHK-21 cells. Subsequent to the transfection procedure (24 h), cells were fixed and stained as outlined under “Experimental Procedures.” Images of cells representative of the entire population are shown. Red, anti-FLAG; blue, nucleus (DAPI). Cells were viewed on an inverted fluorescence microscope using a ×40 objective as described under “Experimental Procedures.” After image capture, each image was deconvolved using the no neighbors method within Slidebook (Intelligent Imaging Innovations). C, Western immunoblot analysis of constructs used in this study. Cell lysates of transfected BHK-21 cells were subjected to electrophoresis on a 10% Tris-HCl SDS-polyacrylamide gel and then blotted onto nitrocellulose. Constructs were detected using an anti-FLAG and anti-His6 polyclonal antibody. Expression of GFP protein was detected using a GFP polyclonal antibody. The dots represent the bands corresponding to construct names listed at the top of each lane.
demonstrate that the amount of detectable extracellular Tax was reduced by a small amount, suggesting that brefeldin A at least partially blocked Tax secretion. This result was not unexpected based on the co-localization of Tax with the GC.

**Tax Secretion Is Promoted after Incubation with PMA and Ionomycin—**
Release of proteins localized to the regulated secretory pathway is promoted by a variety of extracellular signals, also known as secretagogues. These signals vary in both their ability to induce secretion and the distinct regulated secretory pathway they induce. Two chemical compounds, PMA and ionomycin, have been shown to be effective secretagogues in a variety of cell types (42, 43). Therefore, studies were designed to determine whether combined treatment with PMA and ionomycin induced the secretion of FTH₆. After transfection with secretory plasmid constructs (24 h), cell culture medium was removed and replaced with medium containing PMA and ionomycin as well as protease inhibitors. After an additional 24 h, Tax was detected in both medium and cell lysate by Western blotting using anti-Tax monoclonal antibody (TAB 170). Both native Tax and FTH₆ were detected at the expected size by the Tax-specific monoclonal antibody. Each experiment was performed in triplicate and repeated at least twice.

Since PMA and ionomycin induced overall protein production, more Tax was released in the supernatant, whereas the overall ratio of Tax in the supernatant as compared with the cells was the same.

**Tax Secretion Is Not the Result of Increased Plasma Membrane Permeability—**
Tax has been associated with cellular dysfunction and apoptosis, and it has been generally thought that the primary source of extracellular Tax stemmed from a loss of cell membrane integrity instead of through the cellular secretory pathway. To determine whether the presence of Tax in the medium of Tax-transfected BHK-21 cells was the result of a loss in cell membrane integrity rather than release through the secretory pathway, two assays were performed. First, the LDH activity, a measurement of plasma membrane integrity loss, of each transfected cell culture was determined. Consequently, detection of LDH activity in Tax-transfected cells above that of mock-transfected cells would suggest that at least some extracellular Tax would be attributed to cell membrane integrity loss. Utilizing this method, it was determined that the level of LDH release in Tax-transfected cultures was 1.42-fold over that of mock-transfected cultures (Fig. 6A). However, this level was similar to mock and vector-only controls. Furthermore, the level of LDH release ob-
The role of extracellular Tax in the progression of HTLV-I-associated pathogenesis has not been a major focus of research in recent years. The primary reason for this may be the belief that Tax does not enter the extracellular environment to any biologically relevant level. However, several studies have reported that extracellular Tax may impact a number of cellular functions. For example, Tax has been shown to induce the production and release of TNF-α, a potent cellular cytokine that has been demonstrated to have effects on cells similar to those present in pathogenic lesions observed in HAM/TSP (17). Additionally, both neurons and microglia have been demon-

**DISCUSSION**

The role of extracellular Tax in the progression of HTLV-I-associated pathogenesis has not been a major focus of research in recent years. The primary reason for this may be the belief that Tax does not enter the extracellular environment to any biologically relevant level. However, several studies have reported that extracellular Tax may impact a number of cellular functions. For example, Tax has been shown to induce the production and release of TNF-α, a potent cellular cytokine that has been demonstrated to have effects on cells similar to those present in pathogenic lesions observed in HAM/TSP (17). Additionally, both neurons and microglia have been demon-

**FIG. 5.** Quantitative determination of FLAG-Tax-His6 secretion from BHK-21 and 293T cells by ELISA. A, BHK-21 or 293T cells were transiently transfected with secretion constructs. After transfection (24 h), medium was removed and replaced with medium containing protease inhibitors. Subsequently (after 24 h), medium and cell lysates were utilized in the ELISA-based secretion assay as outlined under “Experimental Procedures.” Results are presented as the percentage of total Tax secreted into the medium. B, BHK-21 cells utilized in ELISA secretion assays were also exposed to either brefeldin A (BFA), a known inhibitor of the secretory process, or PMA and ionomycin, known inducers of some regulated secretory vesicles. BHK-21 cells were transiently transfected with FLAG-Tax-His6 or FLAG-Tax-COPII-His6. Tax-COPII contains a DXE mutation at amino acids 330–332. The DXE motif was previously described as a domain important in protein localization to the secretory pathway. After 24 h, cell medium was replaced with medium containing protease inhibitors, protease inhibitors plus brefeldin A, or protease inhibitors plus PMA and ionomycin. Cells were incubated for an additional 24 h, at which time the media and cell lysates were utilized in the ELISA-based secretion assay as outlined under “Experimental Procedures.” Results are represented as either a percentage of total FLAG-Tax-His6 released or as the average total amount (ng) of FLAG-Tax-His6 released per 2 ml of culture. Assays were repeated for a total of three times for each cell type.
strated to release TNF-α in response to extracellular Tax (17, 18). Each of these cell types coexists with oligodendrocytes, cells that form the protective myelin sheath surrounding CNS neurons. Upon exposure to TNF-α, oligodendrocytes enter a process of demyelination, an effect subsequently followed by neuronal defects and abnormalities similar to those observed in individuals with HAM/TSP. Thus, the study of the effects of extracellular Tax is likely to be of great importance with respect to understanding the pathogenesis of HTLV-I. As a result, studying the process by which Tax exits the cell and migrates into the extracellular environment will improve our understanding of the role of extracellular Tax in neurologic disease.

The results presented herein begin to solidify a link between HTLV-I-infected cells, the release of Tax into the extracellular environment, the reported effects extracellular Tax has on cells, and the progression of HAM/TSP. First, these results substantiate previous observations suggesting that Tax is released into the extracellular environment by a process other than apoptosis or lysis of the cell. This process may occur through a formal cellular secretory pathway. This was evident based on several results including the co-localization of Tax and myelin organelles associated with the cellular secretory pathway including, but not limited to, the ER and the GC. Second, the real time movement of Tax-containing punctate structures within the cytoplasm was congruent with the move-
ment of microtubule-associated secretory vesicles. Third, the Tax molecules released from these cells and detected using our system were full-length proteins, demonstrating that Tax enters the secretory pathway utilizing a leaderless system. Fourth, the initiation of Tax secretion using brefeldin A supports observations of Tax localization to both the ER and GC, since brefeldin A blocks migration of proteins from the ER to the GC. Fifth, the increase in Tax secretion observed after treatment with PMA and ionomycin indicated that at least some Tax resided in the regulated secretory pathway.

Two final results noted here will assist the study of Tax secretion as well as provide a source of Tax made from mammalian cells instead of the bacterial source now used by many investigators. First, an ELISA-based system for detection of full-length Tax released from the cell has been established. Second, a new method for the purification of full-length Tax made within the context of a eukaryotic system has been identified. Traditionally, biochemical analysis of Tax has utilized Tax purified from bacterial cell cultures. The system presented herein utilized mammalian cells for production of Tax protein and has several advantages over purification from bacterial cultures. For example, mammalian cells are able to add secondary modifications to proteins that are not achieved in a bacterial background.

 Whereas the CD4+ T lymphocyte is the primary cell targeted by HTLV-I, the role of other HTLV-I-infected cell types has become an area of great interest. For example, HTLV-I-infected astrocytes have been demonstrated to produce and release many cytokines that have dramatic effects on the immediate cellular environment (19–21, 34). These results are congruent with observations of spinal cord lesions in patients with late stage HAM/TSP. These lesions are the result of several consequences including, but not limited to, immune system interactions, molecular mimicry, and apoptosis. Additionally, it is possible that extracellular Tax is taken up by cells through a receptor-mediated process, similar to that suggested for HIV-1 Tat. If this is the case, extracellular Tax may be able to induce intracellular signaling pathways and gene transcription in preparation for infection by whole virus. A combination of cutting edge techniques including proteomics and mass spectrometry would now make such a study feasible. Thus, the release of cytokines and Tax, especially from HTLV-I-infected astrocytes, has tremendous effects on neighboring cells and the progression of HAM/TSP. With these pathogenic mechanisms in mind, the study of Tax secretion from infected astrocytes, CNS-infiltrating HTLV-I+ T cells, or a yet to be identified cell type become important in the elucidation of the exact mechanisms of HAM/TSP progression.

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