Dimerization and a Novel Tax Speckled Structure Localization Signal Are Required for Tax Nuclear Localization

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The human T-cell leukemia virus type 1 oncoprotein Tax has pleiotropic activities, a subset of which likely leads to immortalization of T cells. Tax is expressed and known to function in both the cell nucleus and the cytoplasm. Tax has defined nuclear localization (NLS) and nuclear export signals that enable shuttling between the two compartments. In this study, we identified a novel region in Tax that targets the protein to discrete nuclear foci that we have previously termed Tax speckled structures (TSS). We demonstrated that the identified region is both necessary and sufficient for directing proteins to TSS. This novel TSS localization signal (TSLS), spanning amino acids 50 to 75, is separable from and adjacent to the NLS of Tax. Coexpression of a Tax NLS mutant and a Tax TSLS mutant rescued the nuclear entry and subnuclear TSS targeting of both proteins, demonstrating that these signals are independent domains. Our analysis also revealed that Tax proteins deficient for dimerization fail to localize to the nucleus. Consequently, when we restored dimerization via induction of a heterologous “dimerizer” domain, nuclear localization was rescued. Thus, we defined additional domains in Tax specific for nuclear localization and subnuclear targeting. Our results reveal a more complex network for regulation of Tax subcellular localization and subsequent function.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia as well as subneoplastic disease states, the most prevalent being HTLV-1-associated myelopathy/tropical spastic paraparesis (22, 44, 47, 63, 68). Tax is a potent transcriptional transactivator of both viral and cellular genes and can physically associate with a number of cellular proteins, thus altering their functions (10). Expression of Tax results in the dysregulation of critical cell functions, including cell cycle regulation, proliferation, DNA damage recognition and repair, and apoptosis. Furthermore, although the pathogenesis of adult T-cell leukemia is not completely understood, the transformation of T cells infected with HTLV-1 can be logically attributed to genomic instability resulting from the pleiotropic activities of the viral protein Tax (30, 41).

Of particular note for this study, Tax has documented nuclear and cytoplasmic functions; thus, the regulation of Tax localization to subcellular compartments is a critical aspect of overall Tax function (11, 23, 52). Tax contains a nuclear localization signal (NLS) sequence in the N-terminal region of the protein that is necessary and sufficient for nuclear localization (23, 53, 55). We previously showed that Tax “shuttles” between nuclear and cytoplasmic subcellular compartments and identified a consensus nuclear export signal (NES) sequence defined by amino acids 190 to 203 (11). Subsequently, a fine-mapping mutational analysis of this region by Alefantis et al. (1) clearly demonstrated a functional NES at this site. These signals presumably mediate interactions between Tax and karyopherins in the nuclear pore complex to allow Tax to translocate through the nuclear membrane (58, 64). The specific importins and exportins involved in Tax transport have yet to be identified, but recent studies indicate that Tax can both import and export from the nucleus without the aid of carrier proteins or energy and can interact directly with the FG nucleoporins within the nuclear pore complex (67). Both the NLS and the NES of Tax are atypical. The NLS is large, involving the first 48 amino acids of Tax, and lacks the basic residues that define classical NLS (23, 53, 55). The NES of Tax contains a classic leucine-rich region between amino acids 188 and 202 that is capable of mediating export of Tax via the CRM-1 pathway but displays notably slower kinetics (1, 11). This NES is believed to be masked in the native Tax protein, and there is strong evidence that ubiquitylation serves to “unmask” the NES, in response to DNA damage, for instance (21). In fact, “nuclear” Tax is predominately sumoylated whereas “cytoplasmic” Tax appears to be primarily ubiquitylated (37).

While in the cytoplasm, Tax activates transcription via the NF-κB pathway (61) by directly binding to the IκB kinase γ/NF-κB essential modulator, leading to the phosphorylation and degradation of IκB and the release of NF-κB (38, 43, 62). In the nucleus, Tax interacts with cellular transcription factors to activate or repress transcription of cellular and viral genes via the ATF/CREB (42), AP-1 (19), and SRF (29) pathways (reviewed in reference 33). The list of nuclear proteins that bind to Tax and are functionally modulated is growing and includes APC (39), CBP (7, 48), Cdk4 (25), Chk1 (45), Chk2 (26), DNA protein kinase (15), MAD1 (32), c-Myc (50), and retinoblastoma protein (34). While the NLS and NES provide one level of regulation for Tax localization, it is likely that there are other mechanisms at work regulating Tax subcellular and subnuclear localization and associated functions. In addition to interaction with other proteins, Tax is also capable of dimerization/oligomerization, and studies indicate that optimal transcriptional trans-activation by Tax requires that it be in a dimer/oligomeric form (5, 23, 31, 65).
In previous studies, we and others have demonstrated that Tax enters the nucleus and is directed to discrete nuclear foci that we termed Tax speckled structures (TSS) (6, 52). TSS coincide with interchromatin granules and consist of multiprotein complexes that partially overlap with subnuclear regions involved in splicing and transcription. We have also shown that Tax recruits cellular proteins involved in the DNA damage recognition and repair response into the TSS as well (15, 24, 26). The formation of TSS and the colocalization of splicing-some component 35 (SC35), DNA protein kinase, Chk2, and 53BP1 within the TSS places Tax near cellular machinery for transcription, splicing, DNA damage response, and checkpoint activation (26). This may explain how Tax is able to affect multiple cellular functions simultaneously (41). Although the NLS for Tax has been previously described, the domain in Tax that dictates TSS localization has not yet been defined.

In this study, we identified the region in Tax containing the sequence that targets the protein into TSS. We fine mapped the TSS localization signal (TSLS) to a region containing amino acids 50 to 75. This sequence lies adjacent to but is completely separable from the Tax NLS. We also demonstrated that dimerization of Tax is required for nuclear localization. We showed that deletion of the dimerization domain of Tax prevents nuclear accumulation and results in the cytoplasmic expression of the protein. The induction of dimerization in a Tax dimerization mutant restored nuclear localization. These results elucidate two novel mechanisms in the regulation of Tax subcellular localization and the subsequent function.

MATERIALS AND METHODS

Plasmids. Generation of the pSTaxGFP construct has been described previously (16). STaxGFP mutants were created with PCR-based deletion mutagenesis, using a QuickChange XL mutagenesis kit (Stratagene). The primer sequences used for cloning and mutagenesis are available upon request. pSNL-TaxGFP constructs were created by inserting the simian virus 40 (SV40) T antigen NLS in frame between the S tag and the tax-gfp fusion, using a PCR-based ExCite mutagenesis kit (Stratagene). pSTax-1 and pSTax-2 were generated by PCR-based mutagenesis with a Phusion site-directed mutagenesis kit (Finzymes, Inc.), using pSTaxGFP and pSNL-TaxGFP as the templates. A Myc peptide-tagged Tax construct (pMycTax) was a kind gift from Ralph Grassmann. A pMycTax(L30-52) construct was created by site-directed mutagenesis of pSTMycTax, using a QuickChange XL mutagenesis kit (Stratagene). The inducible dimerization construct pSTax(Δ100-150)-Fv-GFP was created by amplifying the Fv domain of pCγ2-Fv1E (Ariad Pharmaceuticals) and inserting it into the Clal site within pSTMycΔ99-150GFP.

Cell culture and transient transfection. HEK 293 cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in air in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen). Transfections were performed by the standard calcium phosphate precipitation method (11). The cells were washed 16 h posttransfection and incubated at 37°C until harvest. Cells were harvested 48 h posttransfection following a single wash with 1× phosphate-buffered saline (PBS).

Transcriptional trans-activation assays. HEK 293 cells were transfected with Tax-expressing plasmids and either HTLV-luciferase or the NF-κB-luciferase reporter, using the calcium phosphate method. Cells were lysed in 400 μl of reporter/lysis buffer (Promega), and lysates were immediately frozen at −80°C. Samples were allowed to thaw on ice, collected, and quantified, using a Bradford assay (Bio-Rad). A total of 1 μg of protein from each sample was applied to 100 μl of luciferase substrate (Promega), and a luciferase assay was immediately read in a Turner BD 20/20 luminometer. Transcriptional activation was analyzed and expressed as the level of activation over that of the reporter alone (level of activation = 1).

Immunofluorescence confocal microscopy. HEK 293 cells were seeded at 1 × 106 cells/well on ethanol-washed coverslips in 6-well plates. The cells were subjected to transient transfection to express the desired proteins. After 48 h, the cells were fixed by the paraformaldehyde/methanol method as previously described (52). The primary antibodies used were anti-SC35 mouse monoclonal S4045 (Sigma), anti-Myc rabbit polyclonal ab9106 (Abcam), and anti-Tax rabbit polyclonal (52) at a 1:1,000 dilution. The secondary antibodies were species-appropriate Alexa Fluor 594-conjugated antibodies (Molecular Probes) at dilutions of 1:1,000. Nuclei were counterstained with To-Pro-3 iodide (Molecular Probes) diluted 1:1,000 in 3% bovine serum albumin-PBS. The cells were then mounted on glass slides, using Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories). Confocal fluorescent images were acquired on a Zeiss LSM 510 confocal microscope (Carl Zeiss), using argon (488 nm), HeNe1 (543 nm), and HeNe2 (633 nm) lasers, and were imaged with LSM Image Browser software.

STaxGFP purification. HEK 293 cells expressing the STaxGFP fusion protein were lysed in 400 μl of M-Per mammalian protein extraction reagent (Pierce) with protease inhibitor cocktail (Roche Applied Science) and immediately frozen at −80°C. The lysates were assayed for total protein concentration using a Bradford protein assay and were normalized using a bovine serum albumin standard curve. The protein solution was brought to a total volume of 500 μl with M-Per for each sample, and 150 μl of S-protein agarose beads (Novagen) were applied to the sample. S-tagged proteins were purified from lysates as described previously (16). Supernatants containing isolated proteins were loaded onto an 8%- to 12%-gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel and separated by electrophoresis, transferred to Immobilon-P membranes (Millipore) by semidry transfer, and subjected to immunoblot analysis.

Immunoblot analysis. Proteins separated by electrophoresis were transferred to Immobilon-P membranes using the semidry transfer method with 400 milliamps applied for 50 min. The membranes were then blocked for 1 h at room temperature in 1× Odyssey blocking buffer (Li-Cor Biosciences). Primary antibodies diluted in 1× Odyssey blocking buffer were applied to the membranes and allowed to interact at 4°C overnight on an orbital shaker. Membranes were washed four times for 5 min with PBS-1% Tween. Li-Cor Odyssey secondary antibodies were diluted to a concentration of 1:20,000 in 1× Odyssey blocking buffer containing 0.5% SDS and 0.5% Tween and then incubated for 1 h at room temperature on an orbital shaker while protected from light. The membranes were washed four times for 5 min with PBS-1% Tween and then stored in PBS and protected from light until analyzed. Blots were scanned and analyzed with a Li-Cor Odyssey scanner and software.

Dimerization assay. Full-length MycTax protein was coexpressed with STaxGFP or mutant Tax protein in HEK 293 cells. The Tax-expressing cells were subjected to lysis and subsequent S-bead purification as described above. The isolated proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis. STaxGFP and the corresponding proteins were detected with mouse monoclonal anti-green fluorescent protein (GFP) primary antibody (Santa Cruz) at a 1:1,000 dilution followed by Odyssey goat anti-mouse 488 (Li-Cor Biosciences) secondary antibody at a 1:1,000 dilution. Copurified MycTax was detected by polyclonal anti-Myc peptide primary antibody (Abcam) at a dilution of 1:1,000 followed by Odyssey goat anti-rabbit 680 (Li-Cor Biosciences) secondary antibody at a 1:1,000 dilution.

Complementation analysis. Full-length MycTax was coexpressed with “test” mutant proteins in the STaxGFP backbone. The resulting cellular localization of the coexpressed proteins was visualized with immunofluorescence confocal microscopy as previously described. MycTax and Tax mutant proteins were detected by staining with rabbit polyclonal anti-Myc peptide primary antibody (Abcam) at a dilution of 1:1,000 followed by goat anti-rabbit secondary antibody conjugated to Alexa Fluor 594 (Molecular Probes) at a 1:1,000 dilution.

Induced dimerization assay. STaxGFP was transiently transfected in HEK 293 cells as described above. Forty-eight hours after transfection, the cells were treated with AP20187 from an Argent homodimerization kit (Li-Cor) secondary antibody at a 1:20,000 dilution. Copurified MycTax was detected by polyclonal anti-Myc peptide primary antibody (Abcam) at a dilution of 1:1,000 followed by Odyssey goat anti-rabbit 680 (Li-Cor Biosciences) secondary antibody at a 1:1,000 dilution. The membranes were washed four times for 5 min with PBS-1% Tween and then stored in PBS and protected from light until analyzed. Blots were scanned and analyzed with a Li-Cor Odyssey scanner and software.

RESULTS

Deletion-scanning mutational analysis of Tax. Previous structure/function studies of Tax involving mutational analysis involved amino acid substitution or refined scanning-linker approaches so as to preserve the transcriptional activity of the
protein (53, 56). In this study, we constructed a series of consecutive 25- to 50-amino-acid deletions that covered the length of the Tax protein as depicted in Fig. 1A. Our experimental objective in generating these larger deletions was to alter cellular localization. The parental construct, which we have previously described (16), provided an N-terminal S tag for protein purification and a C-terminal GFP fusion to facilitate localization studies and displays activity comparable to that of the wild-type protein. Shown in an immunoblot analysis of whole-cell lysates from 293 T cells transfected with the appropriate plasmids for expression of the indicated proteins. Rabbit polyclonal anti-GFP antibody was used to evaluate protein expression. (C) Transcriptional activity of Tax mutants on the HTLV-1-LTR-Luc and NF-κB-Luc promoter/reporter constructs. HEK 293 cells were transiently transfected with the indicated reporter construct and either STaxGFP or the indicated deletion mutant, and lysates were analyzed for activity. The reported level of activation is relative to that of the control in which the indicated reporter construct was cotransfected with empty vector expression plasmid.
wild-type Tax. When examined for protein expression levels via Western analysis, the deletion mutants generated comparable amounts of protein (Fig. 1B). We next analyzed each of the mutants for transcriptional trans-activation activity and subcellular localization. Nearly all of the deletion mutants displayed no transcriptional trans-activation for either an HTLV-1-responsive or an NF-kB-responsive promoter/reporter (Fig. 1C), consistent with previous observations that mutations within Tax ablate transcriptional activation (56). The one exception was the Δ323–353 mutant, which deletes the C-terminal portion of Tax downstream of the sequences mutated in the classic M47 mutant.

Confocal microscopy studies revealed that the mutants displayed one of three phenotypes: localization to discrete TSS foci, diffuse nuclear localization, or cytoplasmic localization (Fig. 2). One mutant protein with the Tax NLS deleted in part, the Δ1–29 mutant, showed cytoplasmic expression with weak nuclear expression in TSS foci, while the second NLS mutant, the Δ30–52 mutant, was completely confined to the cytoplasm. Our results confirm previous studies that define the Tax NLS function but disrupts TSS localization. In order to more fully characterize this putative TSLS, we designed a construct containing the first 75 amino acids of Tax fused to an N-terminal S tag and a C-terminal GFP. This construct, which contains the Tax NLS, was able to target GFP into TSS in a fashion that was indistinguishable from that of wild-type Tax (Fig. 5A and C). In comparison, a construct containing only the S tag and GFP was expressed diffusely throughout the cell, with no targeting to nuclear foci (Fig. 5B).

The NLS for Tax is contained in the first 50 amino acids (23, 53, 55), and deletion of this region ablates nuclear localization. In order to determine if the TSLS is distinct from the NLS, we inserted an exogenous NLS from the SV40 T in frame and N-terminally to Tax-1-75GFP. This insertion did not interfere with the targeting of S-NLS-Tax1-75GFP to TSS (Fig. 5D). We then effectively deleted the first 49 amino acids of Tax by creating S-NLS-Tax50-75GFP and observed that this construct was able to target the GFP fusion into the TSS (Fig. 5E). This result established that the TSLS is outside of the Tax NLS and resides within amino acids 50 to 75 of Tax.

**Complementation analyses of NLS and TSLS mutants.** We then hypothesized that if the Tax NLS and TSLS are independent sequences, then coexpression of the two mutant proteins should result in dimerization and rescue of Tax localization to TSS. We therefore coexpressed the MycTaxΔ30–52 mutant with the Δ53–99 mutant to test whether the mutants could rescue nuclear localization and focus formation. The MycTaxΔ30–52 mutant failed to localize to the nucleus, and the Δ53–99 mutant failed to form TSS, as expected (Fig. 6A). However, coexpression of the MycTaxΔ30–52 and Δ53–99 mutants resulted in normal nuclear expression and TSS formation (Fig. 6A). To confirm that the foci formed by the coexpression of the mutants were TSS, we expressed the GFP fusion version of each mutant either alone or in combination (Fig. 6B) and observed colocalization of each with SC35. As was the case for the previous experiment, expression of either the Δ30–52 or Δ53–99 mutant resulted in Tax protein that failed to localize to the nucleus or to form TSS, respectively. Coexpression of the Δ30–52 and Δ53–99 mutants resulted in nuclear localization and the formation of TSS that overlap with the expression of SC35. Complementation by these two different domain mutants clearly demonstrated that the Tax NLS and TSLS are separate and sufficient for directing specific subcellular localization.
Full-length Tax rescues nuclear localization of Tax midregion mutants. Previous studies have established that Tax forms dimers and that optimal transcriptional trans-activation by Tax requires Tax dimerization (5, 31). The described dimerization domain spans approximately 150 amino acids in the midregion of Tax. Our observation that Tax mutants with deletions in this region are expressed in the cytoplasm suggests a possible role for dimerization in nuclear localization. Three of our four midregion mutants of Tax that were nuclear excluded contained deletions within the Tax dimerization domain. This large domain reportedly contains three subdomains, I, II, and III, that are involved in Tax dimerization, but the interdepen-
In our original mutational analysis, we created constructs that were each missing one of these subdomains of the Tax dimerization region. The $\Delta 100-150$ mutant is missing the first subdomain, the $\Delta 151-202$ mutant is missing the second subdomain, and the $\Delta 203-254$ mutant is missing the third subdomain. In addition, the double mutant, the $\Delta 100-150 \Delta 203-254$ mutant, is missing the first and third dimerization subdomains. As shown in Fig. 7, immunofluorescence confocal microscopy studies revealed that coexpression of full-length Tax was able to partially restore nuclear localization of those mutants missing only one dimerization subdomain (Fig. 7A to D) but not of the mutant missing two dimerization subdomains (Fig. 7E).

**FIG. 3.** Colocalization of Tax deletion mutants with TSS. Shown are confocal microscopy images of fixed HEK 293 cells transiently expressing "nuclear" Tax mutants. Tax proteins (green) were observed via fluorescence of the GFP fusion. SC35 (red) was detected with mouse anti-SC35 primary antibody (1:5,000) and goat anti-mouse Alexa Fluor 594-conjugated secondary antibody (1:1,000). The nuclei were counterstained with To-Pro-3 iodide (1:1,000). The overlap (white) was displayed by merging the two images.
Mutual complementation by coexpression of different single-subdomain mutants revealed that none of the mutants could be rescued for nuclear localization (data not shown). These results suggest that the midregion Tax mutants are impaired for dimerization and that presentation of a dimer interface is a prerequisite to nuclear localization.

**Dimerization subdomain Tax mutants are deficient for Tax binding.** To directly test whether the subdomain mutants were deficient for dimerization, we coexpressed MycTax with STaxGFP or a Tax mutant missing one or two dimerization subdomains, the Δ100–150 or Δ100–150 Δ203–254 mutants, respectively. We then assessed dimerization function by purification of STaxGFP, the Δ100–150 mutant, or the Δ100–150 Δ203–254 mutant, followed by immunoblot analysis for the presence of coprecipitated MycTax (Fig. 8A). Full-length STaxGFP was able to bind to MycTax, demonstrating dimerization between the two full-length proteins (Fig. 8A, lane 7). When the Δ100–150 mutant was tested, a measurable but reduced level of MycTax was detected (Fig. 8A, lane 6), consistent with weak dimerization with full-length Tax. However, when the Δ100–150 Δ203–254 mutant was examined, no evidence of dimerization with full-length Tax could be detected (Fig. 8A, lane 5). MycTax did not bind to the S beads alone (Fig. 8A, lane 8). We quantitated the dimerization by deter-
mining the ratio of MycTax that coprecipitated with STaxGFP or the STaxGFP-derived deletion mutant. When this analysis was performed, the single deletions between amino acids 100 to 289 resulted in similarly reduced dimerization compared to that of the wild-type protein (Fig. 8B). The double deletion that removes two dimerization subdomains resulted in a further reduction in binding. These results confirm that this region is required for dimerization and that deletion of the dimerization subdomains has an additive effect.

**Nuclear localization requires Tax dimerization activity.** We have shown that deletion of Tax dimerization subdomains impairs protein-protein interactions in a dimerization assay and results in cytoplasmic expression of Tax protein. Clearly, these results strongly support that dimerization is a prerequisite to nuclear localization of Tax. However, we wanted to determine if restoration of dimerization activity is sufficient to rescue nuclear localization of Tax. To accomplish this, we designed a Tax dimerization mutant protein that contains an inducible dimerization domain, STax1-75-Fv-GFP. The inducible dimerization cassette is based on the binding domain of the human protein FK506 binding protein (FKBP) and its ability to bind to the immunosuppressive drugs FK506 and rapamy-

![Image](http://jvi.asm.org/)
cin. Fv is a modified version of the FKBP binding domain containing a phenylalanine-to-valine substitution that increases the affinity of a rapamycin derivative, AP20187, for the Fv fusion protein by 1,000-fold over that for the wild-type protein. Thus, AP20187 is able to associate any two proteins containing the FKBP binding domain by inducing dimerization. The expression of STax(H9004100–150)-Fv-GFP in the absence of the chemical dimerizer AP20187 resulted in cytoplasmic localization of the protein. Upon the addition of increasing concentrations of the dimerizer, the Tax protein accumulated in the nucleus (Fig. 9A). The nuclear form of the protein dimer does not target TSS because the Fv region was inserted into the TSLS region using the unique ClaI site within the Tax open reading frame. Semiquantitative analyses of the percentages of Tax expression in the nucleus revealed dose-dependent responses of from less than 1% to more than 40% nuclear accumulation (Fig. 9B). This result clearly shows that dimerization function is required for Tax nuclear localization.

DISCUSSION

Compartmentalization is a well-established mechanism for regulating protein function (14, 60). Clearly, this critical relationship between cellular targeting and function goes beyond nuclear-versus-cytoplasmic accumulation. For instance, a formidable number of nuclear proteins target specific subnuclear domains, many of which denote specific cellular functions (13, 35, 36). It is also understood that since localization is dynamic (for instance, proteins may exhibit compartmental “shuttling”), the stoichiometry is critical to overall protein function and an ideal target for protein regulation. In the case of the HTLV-1 Tax protein, localization is influenced by the presence of both NLS and NES sequences, as well as by the action of posttranslational modifications such as sumoylation and ubiquitylation (1, 11, 21, 23, 37, 53, 55). Our results from the current study provide an additional regulatory sequence, the TSLS, which allows for accumulation of Tax within the subnuclear structure TSS. We have further shown that dimerization of Tax is required for overall nuclear accumulation of this important viral protein.

Although nuclear localization is delineated from cytoplasmic localization by a clear nuclear membrane, subnuclear localization is largely dictated by interactions with protein complexes present in the nucleus, with membership in these complexes defining the specific structure. All of the well-known subnuclear structures, represented by nuclear speckles (36), paraspeckles (36), Cajal bodies (36), gems (20), and ND10/PML bodies (4, 59), are dynamic structures that contain characteristic sets of nuclear proteins and protein complexes that reside in distinct subnuclear regions (46). Nearly all of these nuclear structures contain subpopulations of nuclear factors, but each is distinguished by the presence of a nuclear protein(s) unique to each structure (36). One specific class of
structures, called nuclear speckles, are interchromatin granule clusters that contain the pre-mRNA splicing machinery, including small nuclear ribonucleic proteins, non-small nuclear ribonucleic protein splicing factors, and spliceosome subunits such as SC35 (59). We previously observed that Tax localized to specific TSS, so named because of their overlap with nuclear speckles, colocalized with SC35, did not contain promyelocytic leukemia protein, and did not colocalize with nucleoli (3). However, TSS are distinct from nuclear speckles in that they contain various other nonsplicing cellular proteins. These proteins include DNA protein kinase, Chk2, 53BP1, and γH2AX, proteins involved in DNA damage recognition and repair (15, 24, 26). The colocalization of Tax with cellular machinery for transcription/splicing, DNA damage response, and checkpoint activation suggests that targeting to TSS is an important integrating event for the varied functions of Tax.

Subnuclear protein targeting to nuclear speckles appears to be mediated by at least two separable trafficking signals: one for nuclear import (the NLS) and one for mediating inclusion into speckles (12, 28, 49, 54). As might be expected, the identified domains are structurally variable and include examples such as ankyrin repeats in the IκBα, FF domains in CA150, or interaction with RNA and are believed to represent modalities for protein-protein interactions. In our study, we found that the TSLS that is required for localization to TSS was physically distinct from the NLS and could function independently to...
direct Tax to its specific intranuclear site. There are no remarkable features of the 25-amino-acid TSLS except that it is enriched for proline residues, a characteristic shared by many nuclear speckle proteins, but it does not contain an arginine-serine (RS) motif common to targeting signals of splicing speckle components (8, 9, 17, 18, 27). This may reflect the unique nuclear address of TSS and the ability of Tax to form novel protein complexes.

FIG. 8. Midregion Tax mutants are deficient for dimerization. (A) MycTax was coexpressed with STaxGFP or Tax mutants missing one (Δ100–150) or two (Δ100–150, Δ203–254) dimerization subdomains. Dimerization of STaxGFP and Tax mutants with MycTax was assayed by S-bead purification of protein followed by immunoblot analysis for coprecipitated MycTax. Starting levels of each protein are shown in the left panel (input). Analyses of the isolated Tax complexes are shown in the right panel (S-bead purification). Protein complexes were normalized to levels of GFP expressed by Tax fusions (lanes 5, 6, and 7). Corresponding levels of coprecipitated MycTax are indicated. (B) Quantitation of the dimerization assay. Following the isolation of the Tax complexes shown in the right panel of panel A above, the ratios of MycTax and the test proteins were determined for relative quantitation of dimerization ability. The bar graph shows the percentages of MycTax relative to those of the isolated STaxGFP or mutant proteins as indicated (ratio).
It is also important to point out that the 25-amino-acid sequence comprising the TSLS is sufficient for targeting to TSS when fused to either the native Tax NLS or a heterologous NLS. This implies that sumoylation of Tax is not a requirement for either nuclear localization or the formation of TSS. Likely, the role for sumoylation is in the context of the whole protein and may involve the removal of a block to entry or facilitate protein-protein interactions that enable nuclear accumulation. Such a model is reminiscent of that established for the tumor suppressor Wilms’ tumor gene (WT1) in which modification by SUMO proteins is separable from nuclear speckle localization (57). As with Tax, although sumoylation is required for nuclear entry of WT1, an independent sequence is sufficient for targeting to nuclear speckles in the absence of a SUMO protein target. It may also be that sumoylation serves to mask the function of the NES, as has been suggested previously (37).

Our results also demonstrate that the dimerization of Tax is a necessary prerequisite for nuclear localization/accumulation. There are numerous examples of proteins that must dimerize or oligomerize prior to nuclear translocation, including the

![Induced dimerization of a dimerization-deficient Tax mutant restores nuclear localization.](image-url)
human cytomegalovirus protein ppUL44 and the cellular protein p53 (2, 66). Other proteins, such as those of the AP-1 family, including c-Jun, JunD, JunB, and c-Fos, enter the nucleus as monomers but require heterodimerization in order to remain in the nucleus (40). In our studies, we were able to link the inability of Tax mutants to dimerize with a failure to accumulate in the nucleus in spite of a competent NLS. In fact, the addition of a heterologous NLS failed to rescue this defect. The relationship between dimerization efficiency and nuclear accumulation is linear in that weakly dimerizing proteins were able to weakly accumulate in the nucleus. Specifically, we generated a deletion of two subdomains within the Tax dimerization domain as well as single-subdomain mutants. The single-subdomain Tax mutant retained a slight dimerization ability, whereas the Tax mutant in which the larger dimerization domain is removed showed no dimerization. The rescue of single-subdomain dimerization mutants by wild-type Tax protein supports a complementation phenotype, and our observation that none of the subdomain mutants could complement distal subdomain mutants implies a requirement for a dimer interface. Finally, we showed that induction of dimerization in a previously cytoplasm-restricted Tax dimerization subdomain mutant resulted in restored nuclear accumulation. Although our studies definitively link dimerization as a prerequisite to nuclear accumulation, additional studies are required to determine if this dimerization occurs in the cytoplasm as a prerequisite to nuclear entry or within the nucleus as a means of retaining the protein within its subnuclear address.

Previous studies on the dimerization of Tax have suggested that the N-terminal zinc finger domain (51) is important for Tax self association (5, 31). These findings were based on yeast two-hybrid assays where an N-terminal deletion of Tax failed to interact with wild-type Tax. In mammalian cells, as used in our system, deletion of the zinc finger region (Δ30-52) was rescued for nuclear localization by the coexpression of wild-type protein, demonstrating the ability to interact. Our functional complementation assay between the NLS mutant, the Δ30-52 mutant, which is missing the zinc finger domain, and the TSLS mutant, the Δ50-75 mutant, demonstrated that the zinc finger domain is not required for dimerization, since a complementation phenotype requires interaction. Likewise, the fusion of a heterologous NLS to the Δ30-52 mutant was able to restore nuclear localization and subsequent TSS accumulation, providing further evidence that the zinc finger domain is dispensable for Tax self association in our system.

Our results also allow for some inferences regarding the stoichiometry of Tax self association. The complementation assays between the NLS mutant and TSLS mutant suggest that at the minimum, a single copy of each, NLS or TSLS, is required for nuclear accumulation of Tax in TSS. Thus, Tax exists at least in a one-to-one dimer state. In addition, the inability of the dimerization subdomain mutants to complement distal subdomain mutants implies that at least two intact molecules of Tax are needed to form the required dimer interface. This finding is supported by findings in the induced dimerization assay. The design of the heterologous “dimerizer” domain is such that only dimers, and not nonpaired higher-order oligomers (for instance, trimers), are formed upon the addition of the chemical dimerizer. Although induced dimerization was able to increase the nuclear accumulation of the mutant protein, it did not result in a total restoration of nuclear accumulation to wild-type levels. This suggests the necessity of the presentation of specific dimer interfaces for normal Tax nuclear accumulation. Perhaps these specific interfaces mediate interaction with specific proteins. Interestingly, the ability of wild-type Tax to rescue dimerization subdomain mutants suggests the existence of a higher-order (>2) oligomeric structure. This conclusion derives from the reasoning that if dimer interfaces are required for nuclear accumulation and the mutants are incapable of forming interfaces, then wild-type Tax must provide them. In order for this to occur, the rescued Tax complex must contain at least 2× molar equivalents of the wild-type protein. Thus, it is likely that Tax is capable of forming a homotetrameric complex. Higher-ordered Tax oligomerization could provide for more-complex regulation of Tax functions. Oligomerization could generate new intermolecular interfaces to improve stability, control the accessibility and specificity of active sites, and increase the number of cellular binding partners for Tax. The complexity of such a model helps explain how a single protein displays such a wide range of activities.

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