Human T-cell Leukemia Virus-I Tax Oncoprotein Functionally Targets a Subnuclear Complex Involved in Cellular DNA Damage-Response*

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The virally encoded oncoprotein Tax has been implicated in HTLV-1-mediated cellular transformation. The exact mechanism by which this protein contributes to the oncogenic process is not known. However, it has been hypothesized that Tax induces genomic instability via repression of cellular DNA repair. We examined the effect of de novo Tax expression upon the cell cycle, because appropriate activation of cell cycle checkpoints is essential to a robust damage-repair response. Upon induction of Tax expression, Jurkat T-cells displayed a pronounced accumulation in G2/M that was reversible by caffeine. We examined the G2-specific checkpoint signaling response in these cells and found activation of the ATM/chk2-mediated pathway, whereas the ATR/chk1-mediated response was unaffected. Immunoprecipitation with anti-chk2 antibody results in co-precipitation of Tax demonstrating a direct interaction of Tax with a chk2-containing complex. We also show that Tax targets a discrete nuclear site and co-localizes with chk2 and not chk1. This nuclear site, previously identified as Tax Speckled Structures (TSS), also contains the early damage response factor 53BP1. The recruitment of 53BP1 to TSS is dependent upon ATM signaling and requires expression of Tax. Specifically, Tax expression induces redistribution of diffuse nuclear 53BP1 to the TSS foci. Taken together these data suggest that the TSS describe a unique nuclear site involved in DNA damage recognition, repair response, and cell cycle checkpoint activation. We suggest that association of Tax with this multifunctional subnuclear site results in disruption of a subset of the site-specific activities and contributes to cellular genomic instability.

The human T-cell leukemia virus, type 1 (HTLV-1) viral protein Tax activates transcription of its cognate promoter contained in the 5′ long terminal repeat (1, 2). In addition, Tax transactivates a variety of cellular promoters and indeed displays pleiotropic functionality in the context of the host cell (3, 4). Tax also has oncogenic properties, which are not well understood mechanistically but may involve some of the activities described above. Increasing evidence now strongly suggests that Tax induces genomic instability in target cells (5–11). Thus, one compelling model for HTLV-1-mediated transformation involves increased genetic mutational impact resulting from tax-induced cellular genomic instability (12).

In support of this hypothesis, HTLV-1-transformed cells have reduced genomic integrity and harbor random chromosomal abnormalities (13, 14). Furthermore, HTLV-1-infected cells display increased mutational events, an observation also noted for Tax-expressing cells (5, 6, 15). Although Tax has been shown to contact DNA (16), it is unlikely that this viral protein directly causes DNA damage as a result of physical contact but rather induces the accumulation of damage by impairing the cellular DNA damage repair response. A variety of possible mechanisms have been proposed to explain Tax-induced damage accumulation. These include direct interaction with repair-response components such as the mitotic checkpoint protein hsMAD1 (7), transcriptional repression or activation of repair components such as h-pol (17) and PCNA (18, 19), respectively, or interference with the activity of tumor suppressors such as p53 (20–24). Although the focus has been upon mechanisms directly involved in the repair process, it is equally possible that Tax acts early to impair damage recognition and appropriate cell cycle response.

Essential to maintaining a robust DNA damage response is appropriate cell cycle phasing and accurate checkpoint activation (25, 26). To preserve the integrity of the genome, cells have evolved various sophisticated pathways to sense replication errors and overcome DNA damage (25, 27). Delay of cell cycle progression at specific checkpoints provides the time necessary to prevent propagation and fixation of damaged DNA (27). To accomplish this task it is essential that effective communication between cell cycle, cell division, damage repair, and cell death be established. One of the earliest events following DNA damage recognition is checkpoint activation (28, 29). Indeed these two processes are necessarily intimately linked. The Chk2 tumor-suppressor protein is a member of a series of checkpoint protein kinases that are activated upon sensing of replication defect and/or DNA damage (29, 31–33). ATM-dependent activation of Chk2 requires Nbs1 (34), and, because Nbs1 is a component of the multifunctional protein complex containing Mre11 and Rad50, involved in damage recognition and repair processing of DNA lesions (35), this interaction represents a link between chk2-regulated checkpoint activation and DNA repair. In addition, Chk2 directly phosphorylates p53 and is implicated in its stabilization (29), thus influencing repair and upon repair failure cell death. As data accumulates on the function of chk2, it is clear that the protein occupies a
central role in maintaining genomic integrity during genotoxic stress and/or cellular replication.

Recent studies have uncovered discrete cellular compartmentalization as a supporting mechanism for linking and coordinating events that directly follow recognition of DNA lesions. Support for the existence of specific nuclear sites associated with the repair process has arisen from several studies showing that, following the occurrence of DNA damage, key DNA repair proteins; ATM, 53BP1, H2AX, Nbs1, Mre11, and Rad50 form DNA damage-induced nuclear foci (36–38). A repair complex containing BRCA1 forms specific nuclear structures and possesses dynamic nuclear localization following DNA damage (39, 40). Nbs1, a protein frequently found associated with both cell cycle regulatory and DNA repair proteins, is essential for the formation of radiation-induced nuclear foci, probably at the site of DNA breaks, together with Mre11 and HsRad50 proteins (41, 42). Although direct evidence of co-localization of repair components and cell cycle checkpoint components has not been demonstrated, such events would allow for coordination between damage recognition, repair, and cell cycle checkpoint activation. Indeed, it has recently been shown that mice lacking the damage recognition component H2AX were also impaired for DNA repair and checkpoint activation and had dramatically reduced genomic integrity (38, 43). Consequently, disruption of this coordinating mechanism will have profound effects on the maintenance of cellular genomic integrity.

There is increasing evidence that Tax expression results in disruption of normal cell cycle timing, suggesting that cell cycle responses to DNA damage may be impaired (11, 44–47). Mechanistically, Tax alters cell cycle progression by direct interaction/sequestration of inhibitors of kinase (INKs) or transcriptional activation of cellular proteins that support cell cycle progression. Tax was shown to bind to p16INK4a, which binds to and inhibits the cyclin-dependent kinases CDK4 and CDK6, resulting in suppression of G1 phase progression. The binding of Tax to p16INK4a results in decreased levels of the p16INK4a–CDK4 complex, with subsequent activation of CDK4 kinase (46, 48, 49). In addition, Tax also directly interacts with components of the cyclin D-CDK complex resulting in increased mitogenic activity (50). Tax expression leads to activation of the endogenous p21-waf1 promoter and alters complex formation to predominately cyclin A/cdk2 (51, 52). Absence of cell cycle pattern in response to Tax expression may lead to impaired repair response capacity. We recently showed that Tax-expressing cells fail to arrest at G1 in response to UV treatment (44). Thus, defective nucleotide excision repair observed in these cells may result from a loss of nucleotide excision repair-mediated cell cycle checkpoint; this implies that inappropriate cell phase may contribute to ineffective repair response. In the studies described here, we have examined the cell cycle and checkpoint responses of *de novo* Tax expressing cells to gain insight into the early cellular events that may contribute to genomic instability.

**EXPERIMENTAL PROCEDURES**

**Cells, Plasmids, and Antibodies—**Jurkat and JX9-9, human T lymphocytes cells, were grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum and 1% penicillin-streptomycin. 293T17 and HeLa cells were grown in Iscove's media (Invitrogen) containing 10% fetal calf serum and 1% penicillin-streptomycin. JX9-9 cells derived from Jurkat express Tax under the control of the metallothionein-inducible promoter activated by addition of cadmium chloride (CdCl2). The Tax expression plasmid pHFX is under the control of the HTLV-1 long terminal repeat. The Tax expression plasmid pLEX is driven by the CMV IE promoter. The Tax mutant expression plasmid pLEXA2–58 is deleted for amino acids 2–58. Plasmids pCMV-chk1 and pCMV-HAchik2, and anti-chk2 polyclonal rabbit sera were gifts from Steven Elledge (Baylor College, Houston, TX). The plasmids used for retroviral transduction are as previously described (53). pMD.G is used for the production of the envelope protein G of vesicular stomatitis virus. pCMV8.v2.2, the packaging construct, is used for the production of human immunodeficiency virus gag, pol, and regions of env. The delivery construct pHRTaxiGFP was made by inserting the tax-IREGFP cassette into the XhoI and BglII site of pHRMCV and produces "packagable" viral RNA. The anti-Tax mouse monoclonal was obtained from the AIDS Reference Reagent Program. The anti-Tax polyclonal rabbit antibody sera were raised against Tax peptide 104–124 (Biosource, Inc). Anti-Chk1, anti-Chk2, anti-Cdce25c, anti-Cyclin B1, and anti-GFP were purchased from Santa Cruz Biotechnologies. Anti-α-tubulin mouse monoclonal antibody was purchased from Sigma. Anti-53BP1 mouse monoclonal antibody and anti-P-Chk2 rabbit polyclonal antibody were a gift from Junjie Chen (Mayo Clinic, Rochester, MN). The Tax rabbit polyclonal antibody was purchased from Oncogene. The FITC- and rhodamine-conjugated secondary antibodies were purchased from ICN Biomedicals, Inc.

**Flow Cytometry—**To analyze the cell cycle profile, Jurkat and JX9-9 cells were seeded at a concentration of 106 cells/ml. CdCl2 was added to a final concentration of 20 μM for the induction of Tax. In the experiments designed to release cells from G1, caffeine was added to a final concentration of 2 μM at 24 h after the addition of CdCl2. Cells were washed once with PBS and spun for 10 min at 1000 rpm at 4 °C. The cells were fixed with the addition of 1 ml of ice-cold 70% ethanol and incubated at 4 °C overnight. The cells were centrifuged and with 2 ml of PBS and resuspended in 1 ml of propidium iodide solution (PBS, 50 μg/ml propidium iodide and 100 units/ml RNase A) and incubated for 30 min at room temperature. The cells were washed in PBS, resuspended in 0.5 ml of flow buffer (PBS, 1% fetal bovine serum, and 1% sodium azide), then DNA flow analysis was conducted on a BD Biosciences FACScan with MODFIT software.

**Western Blotting—**To analyze the expression profile of the proteins, cells were washed in PBS and lysed in M-Per protein lysis buffer (Pierce) containing the Complete Mini mixture (1 tablet in 10 ml of lysis buffer) of protease inhibitors (Roche Applied Science). The cell lysate was centrifuged, and the proteins were collected and quantified using the Bradford assay (Bio-Rad). A total of 50 μg of protein was electrophoresed through a 10% SDS-PAGE and transferred to polyvinylidene membranes (Immobilon, Millipore). The membranes were first incubated in blocking solution for 1 h at room temperature then incubated overnight at 4 °C in blocking solution containing the appropriate primary antibody: anti-Tax (1:500 dilution), anti-Chk1 (1:500 dilution), anti-Chk2 (1:500 dilution), anti-Cyclin B1 (1:250 dilution), anti-Phospho-Cdk2 (1:200), anti-Cdc25c (1:500), or anti-α-tubulin (1:1000). The membranes were washed once for 15 min and then four times for 5 min each in PBST (PBS plus 0.1% Tween 20). The blots were then incubated in blocking solution for 45 min at room temperature with the appropriate alkaline phosphatase-conjugated secondary antibody (Tropix) and visualized. The detection was performed using the Western Star Detection kit (Tropix).

**Immunoprecipitation—**To analyze potential physical interaction between Tax and Chk2, 2.5 × 106 cells were lysed in 1 ml of M-Per lysis buffer (Pierce) containing the Complete Mini mixture of protease inhibitors (Roche Applied Science) for 30 min at 4 °C. Immunoprecipitation was carried out by incubating whole cell lysate with the appropriate antibody overnight at 4 °C on a rotator. 100 μl of 30% slurry of protein A-Sepharose beads in lysis buffer was added to the mixture and incubated for 3 h at 4 °C. The immune complexes bound to beads were pelleted by centrifugation at 12,000 rpm for 5 min at 4 °C. The beads were washed four times with lysis buffer. The bound proteins were eluted in SDS gel sample buffer at 95 °C for 5 min, placed on ice for 1 min, and further centrifuged at 12,000 rpm for 2 min. 30 μl of supernatant was loaded, separated by a 10% SDS-PAGE, and transferred to Immobilon-P (Millipore). All subsequent steps are the same as described for Western blotting.

**Microscopy and Immunofluorescence—**To analyze potential co-localization between Tax, Chk1, Chk2, and 53BP1, cells were seeded onto coverslips grown to ~60% confluence and washed three times with PBS and fixed by incubation for 12 min in PBS-buffered 4% paraformaldehyde. The cells were then washed four times in PBS to remove excess formaldehyde solution and permeabilized by subsequent incubation for 2 min in 100% methanol. Immunostaining was performed using the primary antibodies indicated in the Cy3 and/or Alexa Fluor-488 (Molecular Probes) conjugated secondary antibodies. The detection was performed using the Western Star Detection kit (Tropix).
expression of Tax by addition of CdCl
2. FACS analysis 72 h later as described.

Experimental Procedures

Viral Transduction—We used the lentiviral transduction system as described previously (44, 53). The successful transduction will result in expression of Tax from capped message and expression of GFP from internal ribosomal entry site-initiated translation. The viral titer is a Zeiss LSM510 confocal microscope outfitted with Metamorph software.

RNA Interference Assay—siRNA duplexes were synthesized by Xeragon Oligonucleotides (Huntsville, AL). The sequence of the chk2-targeting oligonucleotides was: 5’-GACAACUGAGGACAAAGAAC and 5’-GUUCUUUGGCCUCAGGUCG. Cells were co-transfected with 2 μg of the chk2 siRNA duplex and Tax expression plasmid using Lipo
fectAMINE 2000 (Invitrogen, Carlsbad, CA). Cells were subjected to infection with G2 or M is shown for each treatment group.

PBS-BSA (3% BSA in PBS) and left overnight at 4 °C. Excess primary antibody was removed with four washes of PBST (0.1% Tween 20 in PBS). The secondary antibodies (FITC-conjugated goat anti-mouse and/or rhodamine-conjugated goat anti-rabbit) were diluted 1:100 in PBS-BSA and incubated with the cells for 1 h at room temperature. Excess antibodies were removed with four washes of PBST, and cover-slips/cells were mounted in Vectashield medium (Vector Laboratories, Inc). The cells were viewed using either a Nikon ES300 fitted with the ORCA-ER and Improvision software (conventional microscopy) or with a Zeiss LSM510 confocal microscope outfitted with Metamorph software.

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fectAMINE 2000 (Invitrogen, Carlsbad, CA). Cells were subjected to FACS analysis 72 h later as described.

RESULTS

Tax Expression Induces Accumulation of Cells in G2 Phase—To elucidate the effects of Tax expression on cell cycle status, we induced Tax expression in the Jurkat-derived cell line JPX9 that expresses tax under a cadmium chloride (CdCl2)-inducible promoter. We analyzed the cell cycle status in the presence or absence of CdCl2 in both JPX9 and Jurkat cells. The percentage of cells accumulating in 4N following Tax expression at 24 h induction was ~2-fold over non-induced JPX9 cells (Fig. 1, compare A and B). Jurkat cells identically treated with CdCl2 showed no increase in the percentage of cells accumulated in 4N when compared with untreated Jurkat cells (Fig. 1, compare C and D). The relative level of Tax expression following 24-h induction is shown in Fig. 3A. Thus, de novo expression of Tax in Jurkat T-cells results in an accumulation of cells into a 4N ploidy state.

To determine whether the 4N stage results from G2 or M arrest signals, we observed the effects of caffeine addition on Tax-expressing JPX9 cells. Caffeine interferes with ATM signaling and abrogates G2 checkpoint activation. Because caffeine is unable to release M checkpoint, this is a convenient way to distinguish between G2 and M checkpoint activation in a 4N population. In this study JPX9 cells were incubated in the presence or absence of CdCl2 followed by addition of caffeine. The addition of CdCl2 alone to JPX9 Tax-expressing cells did not significantly alter the viability of cells (data not shown). We compared the percentage of cells in G2/M from uninduced JPX9, induced JPX9, uninduced JPX9 treated with caffeine and induced JPX9 treated with caffeine. As is shown in Fig. 2, the addition of caffeine to Tax-expressing cells reversed the 2-fold increase 4N accumulation. There was no effect of caffeine treatment on the uninduced cells. These results indicated that the observed cell cycle alteration is due to activation of G2 checkpoint signals. This finding provided the rationale for the following studies in which we analyzed in more detail the signaling cascade for G2 arrest following Tax expression.

Tax Expression Induces G2 Accumulation through Chk2 Activation—We examined key signaling events in an attempt to identify the molecular pathway activated in response to Tax expression leading to G2 accumulation. Proteins were extracted from JPX9 cells before Tax induction or after 12- and 24-h induction with CdCl2 and subjected to Western blot analysis. We found that optimal detection of Tax expression occurred after 24-h induction (Fig. 3A), although reduced levels of Tax can be detected at earlier times (data not shown). We also analyzed Chk1, Chk2, and p53 steady-state expression over the same time course, because they represent known targets for ATM/ATR following DNA damage and are directly involved in cell cycle checkpoint control. Because the G2 checkpoint can be activated via ATM/Chk1 and ATM/Chk2, we examined the status of these signal components using antibodies to chk1 and chk2. We found that, although the steady-state levels of Chk1

FIG. 1. Cell cycle analysis by FACS of Tax-expressing cells. The inducible Tax-expressing T-cell JPX9 and parental Jurkat cell lines were analyzed by FACS as described under “Experimental Procedures” following treatment with CdCl2. The histograms represent the distribution of cells through the cell cycle measured by flow cytometry and analyzed with ModFit. A, uninduced JPX9 cells. B, JPX9 cells induced to express Tax by addition of CdCl2. C, Jurkat T-cells in the absence of CdCl2. D, Jurkat T-cells after addition of CdCl2. The percentage of cells in G2 or M is shown for each treatment group.

FIG. 2. Caffeine reverses the Tax-induced 4N accumulation. Induced and uninduced JPX9 cells were incubated in the presence or absence of 2 μM caffeine and analyzed by FACS. The percentage of cells in G2/M were determined as described under “Experimental Procedures.” The addition of caffeine to uninduced JPX9 (JPX9+Caff) did not noticeably affect the percentage of cells in G2/M from JPX9 alone (JPX9). Conversely, Tax-expressing JPX9 cells (JPX9+CdCl2) showed reduced G2/M population following the addition of caffeine (JPX9+CdCl2+Caff). Data shown are the average from three independent experiments.
CDC2 and cyclin B1 within the same Tax-induction time course ing phosphatase Cdc25c and its target complexes containing chk2.

Activation of the ATM/chk2-dependent G2 cell cycle arrest cascade following Tax (Fig. 4). Specifically, we analyzed the expression of the inactivating phosphatase Cdc25c and its target complexes containing Cdc2 and cyclin B1 within the same Tax-induction time course described above. Consistent with a chk2 signal we observed an inactivation of Cdc25c, as shown by its reduced levels suggesting signal-mediated degradation, which coincides with induction of Tax expression (Fig. 4). When using a phospho-cdc2 (Tyr-15) antibody, we found that the phosphorylated form of Cdc2 was overexpressed after Tax induction. Phosphorylation of Cdc2 is an inactivating regulatory step preventing progression through mitosis. Dephosphorylated Cdc2 enhances its activity through binding with and subsequent stabilization of cyclin B1. In agreement with inactivation of cdc2, cyclin B1 also showed dramatically reduced steady-state levels following induction of Tax (Fig. 4A). These data clearly demonstrate the activation of the ATM/chk2-dependent G2 cell cycle arrest cascade following Tax expression.

We next examined the role of ATM signaling in Tax-mediated activation of Chk2. The cell lines GM00498B (ATM+/+) and GM05849D (ATM−/−) were transduced with HTLVTaxiGFP at a titer of 2.1 infectious viral units per cell. The resulting expression was confirmed by GFP to be >94%. Following transduction the cells were harvested and analyzed for steady-state levels of the active phosphorylated form of Chk2 using a T68 phosphor-specific antibody. In both ATM+/+ and ATM−/− cells Tax expression resulted in activation/phosphorylation of Chk2 (Fig. 4B). Thus, activation of Chk2 by Tax may be independent of ATM signals possibly involving a stabilization of P-Chk2. Such a scenario would be consistent with increased intensity of Chk2 observed when co-localized with Tax.

**Tax Co-immunoprecipitates with Chk2**—One possible mechanism for Tax activation of G2 arrest is via direct interaction with cellular complexes containing the Chk2 protein. To examine this possibility we performed an immunoprecipitation of Tax, Chk1, and Chk2 from HeLa cell lysates prepared either from mock-transfected cells or from cells transfected with tax, chk1, or chk2 or cells co-transfected with tax/chk1 or tax/chk2. Cells transfected with a tax-expressing plasmid demonstrated efficient expression and immunoprecipitation of Tax (Fig. 5A, compare lanes 1 and 2). Those cells transfected with either chk1 or chk2 alone failed to support co-immunoprecipitation of Tax when the lysates were immunoprecipitated with either anti-Chk1 or anti-Chk2, respectively (Fig. 5A, lanes 3 and 4). However, cells co-transfected with both tax- and chk2-expressing plasmids fully supported co-immunoprecipitation of Tax using either anti-Tax or anti-Chk2 (Fig. 5A, lanes 7 and 8) but not with control antibodies such as anti-GFP (Fig. 5A, lane 6). Consistent with a Chk2-specific interaction, co-transfection with tax- and chk1-expressing plasmids followed by immuno-
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Fig. 5. Tax co-immunoprecipitates with Chk2. A, co-immunoprecipitation of Tax and Chk2 from 293T cells. Non-denatured cell lysates from mock-transfected (lane 1), Tax-transfected (lane 2), Chk1-transfected (lane 3), Chk2-transfected (lane 4), Tax + Chk1-transfected (lane 5), or Tax-Chk2-transfected (lanes 6–8) cells were immunoprecipitated with anti-Tax, anti-Chk1, anti-Chk2, or anti-GFP antibodies as indicated in the left-hand column. The resulting precipitates were subjected to SDS-PAGE as described under “Experimental Procedures” and subjected to immunoblot analysis using anti-Tax antibody. The band corresponding to Tax is indicated. B, cell lysates from mock transfected (lane 1), Tax-transfected (lane 2), Chk2-transfected (lane 3), or co-transfected with Tax and Chk2 (lane 4) were subjected to immunoblot analysis as described under “Experimental Procedures” and probed with anti-Tax antibody. The band corresponding to Tax is indicated. C, cells were transfected with wild type Tax-expressing plasmid IEX (lanes 1, 4, and 5), wild type Tax-expressing plasmid HPX (lane 3), Tax mutant expressing plasmid IEX2–58 (lane 2) or mock transfect (lane 6). The resulting cell lysates were reacted with anti-Tax polyclonal (lane 1), anti-Chk2 (lanes 2, 3, 4, and 6), or anti-GFP (lane 5). The precipitates were then analyzed by Western with anti-Tax monoclonal. D, the same fractions used in C were analyzed for Chk2 expression. E, 293T cells were transfected with wild type Tax-expressing plasmid (lane 3) or mock transfected (lanes 1 and 2). The cell lysates were reacted with anti-Chk1 monoclonal (lanes 1 and 3) or anti-GFP (lane 2). The precipitates were analyzed by western using anti-Chk1 monoclonal.

precipitation with anti-Chk1 did not result in co-immunoprecipitation of Tax (Fig. 5A, lane 5). The steady-state expression levels of Tax were comparable in both single and co-transfection systems (Fig. 5B). We next addressed the specificity of the Tax-Chk2 interaction. This was accomplished by transient transfection of a Tax-expressing plasmid into 293T cells. The cells lysates were then reacted with anti-Chk2 to co-immunoprecipitate Tax (Fig. 5C, lanes 3 and 4). Thus, Tax co-precipitates with endogenous Chk2. Second, we tested a Tax mutant, unable to target to TSS (see Fig. 9 below), for the ability to co-precipitate with endogenous Chk2. The mutant protein was unable to interact with Chk2 in this assay (Fig. 5C, lane 5) and did not interact with exogenously expressed Chk2 (data not shown). The efficiency of Chk1 precipitation was examined, because one explanation for the failure of Tax to co-precipitate with Chk1 is inefficient immunoprecipitation of Chk1. As shown in Fig. 5E, Chk1 is efficiently immunoprecipitated with our Chk1 antibody. Thus, the failure of Tax to co-precipitate with Chk1 is not due to lack of expression or immunoprecipitation of either protein. These results demonstrate that Tax and Chk2 reside in the same subcellular complex and suggest a direct interaction as a mechanism for Tax-induced G2 checkpoint signaling.

Tax Protein Co-localizes with Chk2—To determine if Tax and Chk2 physically associate with or target to identical subcellular sites, we used indirect immunofluorescence microscopy and examined the subcellular distribution of Chk2 in Tax-expressing cells. HeLa cells were transiently transfected with a Tax-expressing plasmid to generate sub-optimal expression efficiency (∼25% of cells express Tax). Thus, Tax-expressing cells were seeded beside non-Tax-expressing cells. In the absence of Tax, the endogenous Chk2 showed a speckled nuclear distribution that was spatially unchanged by and overlapping with the Tax-containing speckles (Fig. 6). These Tax-containing speckles were previously described by our laboratory and termed Tax Speckled Structures (TSS) (54). Although the distribution of Chk2 remained unchanged, the intensity of the Chk2 staining noticeably increased. Thus, Chk2 forms complexes that are similar to TSS and pre-exist Tax expression. We then applied confocal microscopy to determine precise overlap of the Tax and Chk2. Tax-expressing cells were co-immunostained with anti-Chk2 and anti-Tax and subjected to dual probe indirect immunofluorescence and confocal imaging. Distinct nuclear foci containing both Tax and Chk2 were revealed (Fig. 7A, left three panels). Conversely, when Tax-expressing cells were co-immunostained for both Tax and Chk1, the individual nuclear foci were in close proximity but not directly overlapping (Fig. 7B, left three panels). We estimated overlapping areas of greater than 87% between Tax and Chk2 using Metamorph co-localization software (Fig. 7A, right panel). The Chk1 pattern was more diffuse compared with Tax, and the co-localization was determined to be <9% (Fig. 7B, right panel).

Tax Induces the Formation of Discrete 53BP1 Nuclear Foci—One of the earliest events following DNA damage is the targeting of 53BP1 to the site of damage. This relocation of 53BP1 is rapid and precedes chk2 signaling. Thus, analysis of the 53BP1 localization would provide insight into the location of subcellular sites of DNA damage relative to Chk2/Tax. Interestingly, 53BP1 appears as discrete nuclear foci and co-localizes with >88% with Tax protein in Tax-expressing HeLa cells (Fig. 8A). In the absence of Tax, 53BP1 exhibited a diffuse nuclear staining (data not shown). These data clearly indicate that Tax targets the same protein complex containing key
components of both DNA damage-recognition response and cell cycle checkpoint. If the 53BP1 relocalization to TSS is associated with a cellular DNA repair response, then this event would be dependent on initial ATM signaling. To establish the dependence of 53BP1 relocalization on ATM signals, we examined cells defective for the ATM gene for 53BP1 nuclear localization following Tax expression. We found that 53BP1 showed fewer nuclear foci and an overall diffuse pattern in ATM/H11002/H11002 cells (Fig. 8B), as compared with ATM-competent cells. Thus, the relocalization of 53BP1 is an ATM-dependent process as would be expected in a damage response event.

Inactivation of Chk2 through RNA Suppression—The reported limitations of RNA interference by means of double-stranded RNAs have been largely overcome by using small interfering RNAs (siRNAs) that mimic intermediates in the RNA interference pathway (55). Recently, effective suppression of Chk2 was achieved using siRNA resulting in dramatic repression of Chk2 activity (56). To establish that Chk2 mediates the Tax-induced G2 accumulation, we used the same 21-bp siRNA duplex to repress Chk2 in our system. As previously shown, transfection of 293T cells with wild type Tax-expressing plasmid resulted in pronounced accumulation of cells in G2 (Fig. 9C). However, transfection of the same cells with either a mutant Tax-expressing plasmid or co-transfection of wild type Tax-expressing plasmid and Chk2 siRNA duplex failed to result in G2 accumulation (Fig. 9, B and D). Thus, suppression of Chk2 blocked the Tax-induced G2 "checkpoint."

DISCUSSION

We present evidence that Tax targets to discrete nuclear foci where the early damage-response protein 53BP1 and G2 checkpoint activator Chk2 were also found to co-localize. These nuclear foci are identical to subnuclear sites that we previously showed preferentially contain Tax and referred to as “Tax Speckled Structures” (TSS) (54). Specifically, in this earlier study we showed that the periphery of TSS overlap with so-called “transcription hot spots” and the core of TSS overlap with SC-35 and a previously unassigned function. Thus, Tax targeting of TSS domains places the protein in complex with or close proximity to subnuclear sites for transcription and potential coordination of DNA damage response with cell cycle checkpoint.

In these experiments we demonstrate a physical association of Tax involving protein-protein interaction with a Chk2-containing complex following de novo expression of Tax in the
target cell. Chk2, the mammalian homolog of Saccharomyces cerevisiae Rad53 and Schizosaccharomyces pombe Cds1, is a kinase whose activation by DNA damage prevents entry into mitosis. Chk2 kinase is activated by phosphorylation in an ATM-dependent manner (31, 57). Subsequently, Chk2 is capable of phosphorylating all members of the Cdc25 family. Coincident with this physical association of Tax with the nuclear speckles complex was activation of members of the ATM-Chk2-Cdc2 pathway and resulting G2 arrest. Interestingly, although the association of 53BP1 with this complex was dependent upon ATM, the activation of Chk2 by Tax was independent of ATM. Thus, it is likely that Tax exerts its activation of Chk2 signals downstream of ATM; a scenario consistent with a mechanism initiated via direct contact with the Chk2 complex. This could specifically involve stabilization of Chk2 oligomeric complexes, an event shown to facilitate autophosphorylation and neighboring Chk2 phosphorylation (58–61). The resulting impact upon the cell is accumulation in G2 in the absence of increased de novo DNA damage. Thus, it is possible that the resulting G2 accumulation occurs directly as a result of the association of Tax with the Chk2-containing complex or as a result of a Tax-induced dysfunction in the complex.

One model for coordinated regulation of cell cycle, genome integrity, and cell death provides that the individual factors are co-localized and/or may dynamically relocalize to a discrete functional subcellular compartment (62). This compartment would contain the activities associated with recognition of DNA damage, repair, checkpoint activation, and initiation of apoptosis. We also observed that, upon expression of Tax and subcellular localization of Tax to the Chk2 complex, the DNA damage response protein 53BP1 relocalized to the same Chk2-containing complex. Although there have been reports of interaction between chk2 and individual DNA repair components (63, 64), this would represent the first evidence that the repair response protein 53BP1 co-localizes with the checkpoint activator chk2. We also point out that the Chk2 localization was unchanged by Tax expression or 53BP1 relocalization events, although the fluorescent intensity and perhaps concentration of Chk2 increased with these events. Thus, Chk2 is a permanent occupant of the multifunctional nuclear foci, whereas 53BP1 appears to be induced to associate. Therefore, Tax likely targets to the Chk2-containing complex prior to subsequent 53BP1 relocalization events and thus may interfere with appropriate assembly of a competent damage-response complex.

Previous work by others and ourselves has shown that Tax-expressing cells show evidence of increased genomic instability, a result consistent with a Tax-mediated defect in efficient repair resulting in accumulated damaged DNA. Considering the central role of chk2 in both cell cycle and DNA repair response, and the convergence of checkpoint and repair proteins to a single complex, it is possible that functional disruption of Chk2-containing complexes results in defects in repair of a variety of DNA damage. The subnuclear site ascribed to both Tax and chk2 was the same site to which 53BP1 relocalized.
following Tax expression. Because 53BP1 is one of the earliest repair signals following damage recognition and coincident with H2AX relocalization, this result strongly suggests that the Chk2-containing complex targeted by Tax is proximal to sites of damage. Thus, this same subcellular site houses proteins actively involved in signaling damage repair. It may be that the association of Tax with this multifunctional complex results in an effective interference of damage-recognition and/or damage repair function. A second consideration is that cell populations, which normally occupy predominately G1 phase of the cell cycle, have more time to repair damage prior to the genetic fixation of damage associated with passage through M than do cells accumulated in G2. Thus, accumulation of cells in G2 alone may contribute to increased accumulation of unrepaired damage.

One final consideration is the role chk2 plays in relaying signals from ATM to p53. Recent findings have reinforced the notion that ATM enhances p53 accumulation by triggering the release of this protein from MDM2 (65, 66). One proposed mechanism for p53 stabilization involves Chk2, which relays ATM-dependent signals to p53 and many other downstream target proteins in ionizing radiation-damaged cells. Specifically, ATM activates Chk2 by phosphorylating an amino-terminal threonine residue (Thr-68) (67, 68), and hChk2, in turn, phosphorylates another amino-terminal Ser residue in p53 (29, 69, 70). Therefore, because Chk2 directly phosphorylates p53 and thus influences p53 activity, inappropriate activation of Chk2 could result in mis-regulation of p53, as has been reported following Tax expression (21), and initiate events favorable for accumulation of DNA damage. In fact we show an increased phosphorylation of Chk2 and p53 following Tax expression and have previously reported p53 nuclear stabilization as an early effect of Tax expression (44).

It is interesting to speculate that control of cell cycle is of obvious advantage to viruses. For example, human immunodeficiency virus type 1 (HIV-1) has been reported to cause G2 arrest in infected cells (71, 72). The HIV-1 Vpr protein promotes nuclear entry of viral nucleic acids in non-dividing cells, causing G2 cell cycle arrest and is involved in cellular differentiation and cell death. Control over cell cycle would allow for coordination of a variety of activities essential to virus life cycle, including latency/activation, replication, and viral entry/exit. HTLV-1 has been shown to be capable of influencing G1/S transition (11, 44, 73) and evidence for regulation of G2/M via hSAD1 (7) and now Chk2. Because HTLV-1 does not express the so-called accessory proteins encoded by HIV, such as Vpr, these may have been adopted by the Tax protein. It is also intriguing speculation to suggest that an inadvertent result of virus-mediated regulation of cell cycle could result in a negative impact on cellular genomic integrity. Thus, efforts by the virus in controlling cell cycle result in pathogenesis and disease development. Indeed it may be to the long term benefit of the virus to strike a compromise between the extremes of virus-centric and cell-centric activities thus moderating the overall cellular impact. In this respect it appears that subcellular localization of the viral protein Tax would be an effective means of regulating virus-cell interactions. We previously reported that Tax can shuttle between the nucleus and cytoplasm (30) and would favor a model ascribing control of overall Tax function via regulation of targeting to subnuclear sites. Uncovering the mechanism by which Tax targets to multiple subcellular sites would help explain the pleiotropic functionality of this protein. Indeed the targeting of Tax to the TSS nuclear foci may potentially address the need for a unifying functional strategy for Tax that provides benefit to the virus and provides a mechanism that satisfies several reported activities of tax.

In summary, we have described a Tax-induced accumulation of cells in G2 resulting from activation of the ATM/Chk2 signaling cascade. We further demonstrate that Tax targets to a Chk2-containing subnuclear complex and co-immunoprecipitates with Chk2. The early damage response factor 53BP1 alters its cellular redistribution from diffuse to nuclear speckles co-localized with Tax and Chk2. Thus, Tax targets to and alters the makeup of a nuclear complex containing proteins involved in both early recognition of DNA damage and checkpoints.

Collectively, our results support a model whereby the interaction between Tax, Chk2, 53BP1, and perhaps other DNA repair factors results in the rapid activation of Chk2 and accumulation of Tax-expressing cells at G2/M. Overall, these studies provide further insight into the role of compartmentalization in regulating cellular functions and as targets for viral infection.
