p53 plays a pivotal role in transmitting signals from many forms of genotoxic stress to genes and factors that control the cell cycle and apoptosis. We have previously shown that the human T-lymphotropic virus type I Tax protein can inhibit p53 function. Recently we reported that Tax inhibits p53 function in Jurkat cells and mouse embryo fibroblasts through a mechanism involving the nuclear factor κB pathway and correlates with phosphorylation on serines 15 and 392 of p53. However, several groups have also observed a mechanism that correlates with p300 binding of Tax. To address this controversy and to determine the mechanism by which Tax inhibits p53 function, we examined the activation functions of Tax required for p53 inhibition. In HeLa and H1299 cells the cAMP-response element-binding protein/activating transcription factor activation function is essential, as demonstrated by the Tax mutants M47 and K88A. In addition, expression of exogenous p300 in H1299 cells allows full recovery of p53 transactivation in the presence of Tax. Consistent with p300 being a limiting factor in H1299, Saos-2, and HeLa cells, we found that the level of endogenous p300 is relatively low in these cells compared with Jurkat cells or the human T-lymphotropic virus type I-infected C81 and MT2 cells. Thus our data suggest that Tax utilizes distinct mechanisms to inhibit p53 function that are cell type-dependent.

The human T-cell lymphotropic virus type-I (HTLV-I) is the etiologic agent of an aggressive and fatal disease termed adult T-cell leukemia (1–4). The principal target for HTLV-I infection in the lymphoid system is mature CD4+ CD45RO+ T lymphocytes, although other cell types of lymphoid origin have been infected by HTLV-I in vitro, including CD8+ T cells, B cells, and macrophages (5–7). The mechanism of leukemogenesis or neoplastic cell growth in adult T-cell leukemia remains unclear. Several groups have, however, established that the viral transcriptional activator protein, Tax, plays a critical role in cellular transformation (5–7). Tax not only activates expression of viral genes via the viral long terminal repeat (LTR) but has been reported to affect the expression or activity of a number of cellular genes. These genes encode proteins involved in cell growth and cell death including proto-oncogenes, growth factors and their receptors, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (5–8). Tax has been shown to cause tumors in transgenic mice (9, 10), to cooperate with the ras oncogene in transformation of rodent fibroblasts (11), and to immortalize human lymphocytes when expressed in either a herpesvirus or retrovirus vector (12, 13). More recently, it has been shown that the ability of Tax to activate the NF-κB pathway is critical for T-cell immortalization and factor-independent growth (13, 14).

The tumor suppressor p53 plays a pivotal role in regulating cellular transformation. This is underscored by the fact that the p53 gene is mutated in about 60% of all human cancers. Interestingly, the p53 protein is functionally inactive in the majority of adult T-cell leukemia patients although wild-type in sequence (15–18). It has further been demonstrated that expression of the Tax protein alone was sufficient for inhibition of p53 transactivation function (16, 19). Our laboratory has previously shown that in HTLV-I-transformed cells, the p53 protein is tetrameric and binds DNA in a sequence-specific manner. Moreover, p53 function is not inhibited by direct interaction with MDM2 or Tax (20). Rather p53 transactivation function appeared to be blocked due to constitutive phosphorylation on serines 15 and 392 (20).

More recently, using Tax mutants M22 (21), M47 (21), and V89A (22), we found that in lymphocytes, Tax activation of NF-κB is critical for Tax-mediated p53 inhibition (23). Consistent with these studies, blocking NF-κB activation using the dominant negative IκBα mutant (IκBαS32A,S36A (24, 25)) abologates the ability of Tax to inhibit p53 function (23). We do not see a correlation between HTLV-I LTR activation or p300/CBP binding and Tax-mediated p53 inhibition. Moreover, in Jurkat cells expression of exogenous p300 fails to rescue p53 activity in the presence of Tax. These results argue against the recent model proposed by Kuida et al., Suzuki et al., and Van Orden et al. (26–28) that suggests that the interaction of Tax with the coactivator p300/CBP is responsible for sequestering the coactivator from p53 and thus attenuating its transcriptional function.

In an attempt to address the discrepancies that exist in the mechanism of Tax-mediated p53 inhibition, we investigated whether there was a cell type dependence for the mechanism of Tax-mediated inhibition. Unlike what was observed in Jurkat T cells, Tax-mediated p53 inhibition in the p53 null fibroblast cell lines, HeLa, Saos-2, and H1299, was dependent on CREB/ATF activation. In addition, using p53 site-specific mutants, we
found that the CREB/ATF-dependent p53 inhibition did not correlate with phosphorylation on Ser-15 or Ser-392. Interestingly in H1299 cells, expression of exogenous p300 but not PCAF allows recovery of p53 activity in the presence of Tax. Of importance, endogenous levels of p300 in HeLa, Saos-2, and H1299 cells are relatively low as compared with Jurkat cells and the HTLV-I-transformed cells C81 and MT2.

Our results are consistent with a model in which Tax utilizes two distinct mechanisms for inhibiting p53 function. In lymphocytes, the in vivo target for HTLV-I, Tax-mediated p53 inhibition is NF-κB-dependent and correlates with p53 phosphorylation on Ser-15 and Ser-392. In contrast, in cells where p300 is limiting, Tax-mediated p53 inhibition is CREB/ATF-dependent and does not correlate with p53 phosphorylation. Whereas exogenous expression of p300 in the nonlymphoid cells can restore p53 transactivation function, Tax binding to p300 is not sufficient to explain the defect of M47 in p53 inhibition, indicating a more complex mechanism for Tax-mediated p53 inhibition in these cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfections—Jurkat and HTLV-I-transformed MT2 and C81 cells were maintained at 2 × 10⁶ to 2 × 10⁸ cells/ml in RPMI media supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. Transfection of Jurkat cells was performed as described previously (20). HeLa, Saos-2, and H1299 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. Transfections of adherent cells were performed using Effectene (Qiagen) as described by the manufacturer.

Plasmid Constructs and Antibodies—The luciferase reporters driven by the HTLV-I LTR (HTLV-I-Luc) or the p53-responsive elements (PG13-Luc) and p53 expression constructs (wild type, S9A, S15A, S392A, and S15A, S392A) were described previously (20, 23). The wild-type Tax (pTax) and Tax mutants M22 and M47 were kindly provided by Dr. W. Greene (University of California, San Francisco, CA). The Tax-K88A mutant was kindly provided by Dr. Y. Nakatani (NIH, Bethesda, MD). The p300 and PCAF cDNA expression constructs were kindly provided by Dr. W. Greene (University of California, San Francisco, CA). The luciferase reporter driven by the HTLV-I LTR (HTLV-I-Luc) or the p53-responsive elements (PG13-Luc) and p53 expression constructs (wild type, S9A, S15A, S392A, and S15A, S392A) were described previously (20, 23).

RESULTS

The Mechanism of Tax-mediated p53 Inhibition Is Cell Line-dependent—Several mutations in Tax have been described that selectively abrogate the ability of the Tax to activate transcription through the CREB/ATF and NF-κB transcriptional activation pathways (21, 22, 29). To compare the functions of Tax required for p53 inactivation in Jurkat cells, the well characterized Tax mutants M22, M47, and K88A (21, 22) were used. Consistent with our previous report (23), transient transfection of Jurkat T cells with Tax-wild type, -M47, and -K88A inhibits p53 transactivation (Fig. 1A). Expression of the NF-κB-deficient Tax mutant M22 did not inhibit p53 transactiva-

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Fig. 1. Tax-mediated p53 inhibition is cell type-dependent. A, Jurkat, H1299, and HeLa cells were transiently transfected as described under "Experimental Procedures" with the PG13-Luc reporter construct in the presence (+) or absence (−) of p53 and either control vector (−), wild-type Tax (WT), the M22 Tax mutant, M47 Tax mutant, or K88A Tax mutant. 18–24 h after transfection, cells were harvested, and luciferase assays were performed on a Berthold luminometer. Luciferase activity is expressed as light units. The results are an average of at least three independent experiments. B, cellular extracts from transfected HeLa cells (lane 1, no p53/no Tax; lane 2, p53/no Tax; lane 3, p53/wild-type Tax; lane 4, p53/M22 Tax; lane 5, p53/M47 Tax; lane 6, p53/K88A Tax) were separated by electrophoresis on 4–20% Tris-glycine gels (Novex), transferred to a polyvinylidene fluoride membrane (Immobilon-P), and analyzed for p53, Tax, and β-tubulin (as a loading control) using DO-1 antibody (Oncogene Research Products), Tab172, and anti-β-tubulin (Roche Molecular Biochemicals), respectively. Similar results were obtained in mouse embryo fibroblasts, which contain an endogenous wild-type p53 (23). In contrast, in H1299 and HeLa cells (Fig. 1A), Tax-wild type
-M22 inhibited p53 transactivation, but the CREB activation-deficient mutants M47 and K88A did not. Similar results were observed in Saos-2 cells (data not shown). We next analyzed the level of p53 and Tax protein expression. In contrast to our published results in Jurkat cells (23), inhibition of p53 transactivation correlates with increased steady state levels of p53 protein in HeLa and H1299 cells (Figs. 1B and 2B). Importantly, as shown in Fig. 1B, the Tax constructs were expressed to similar levels and therefore do not account for the differences seen on p53 function. These results are consistent with the recent report by Mulloy et al. (30) in which the M47 Tax mutant failed to inhibit p53 function in U2OS, Calu-6, and HeLa/Tat cells.

**Tax-mediated p53 Inhibition in H1299 Cells Is Phosphorylation-independent**—We have previously reported that in Jurkat cells, Tax-mediated p53 inhibition is dependent on phosphorylation of serines 15 and 392 of p53 (23). Because the properties of Tax required of p53 inhibition differed in the different cell types, it is also possible that the phosphorylation-related inhibition of p53 is also different. Using p53 site-specific mutants, we examined whether phosphorylation of p53 in H1299 cells correlated with p53 inhibition. Jurkat and H1299 cells were transiently cotransfected with either wild-type p53 or the p53 mutant constructs S9A, S15A, S392A, and S15A,S392A in the absence or presence of Tax. As reported previously, in Jurkat cells Tax could efficiently inhibit wild-type p53 or the p53 mutants S15A or S392A (Fig. 2A). Tax did not inhibit the p53 mutant S15A,S392A (23). In contrast, in H1299 cells Tax inhibited p53 transactivation of all p53 constructs examined (Fig. 2B). These results suggest that p53 inhibition in H1299 cells is not dependent on phosphorylation of Ser-15 and Ser-392. Consistent with the results presented above (Fig. 1B), expression of Tax increased the steady state level of wild-type p53 as well as p53 site-specific mutant constructs (Fig. 2B). As seen above, we find that inhibition of p53 by Tax correlates with increased steady state levels of p53 (Fig. 2B).

**Restoration of p53 Transcriptional Activity by Expression of p300**—The p300 coactivator has been shown to play an important role in transcriptional activation for a growing list of DNA-binding transactivator proteins (31–33). Several groups have proposed that Tax-mediated p53 inhibition is due to p300 squelching by Tax (26–28, 34). Although we did not find that Tax/p300 interaction played an important role in Tax-mediated p53 inhibition in Jurkat cells (23), the K88A mutant, which fails to bind p300, failed to inhibit p53 function in H1299 cells (Fig. 1A). Therefore, we examined whether p300 (Fig. 3A) is involved in Tax-mediated p53 inhibition in these cells. H1299 cells were transiently cotransfected with wild-type p53 in the presence or absence of Tax and increasing amounts of p300.
Interestingly, expression of exogenous p300 allowed recovery of p53 transcriptional activity in the presence of Tax (Fig. 3A).

We have recently shown that PCAF is an important factor in Tax-mediated activation of the HTLV-I LTR and is responsible for the defect in M47 activation of the viral LTR (35). Likewise, PCAF has been shown to acetylate p53, which then increases the DNA binding affinity of p53 (36, 37). Because the M47 Tax mutant failed to inhibit p53 function, we examined whether expression of exogenous PCAF could also restore p53 transcriptional activity. Unlike p300, exogenous PCAF did not fully restore p53 transactivation in the presence of Tax (Fig. 3B).

Western blot analysis shows that the transfected PCAF protein was expressed, as detected by the anti-flag antibody (Fig. 3B). The level of PCAF does not increase as the level of PCAF expression plasmid is increased, which seems that the level of cellular PCAF may be tightly regulated. The transfected PCAF was active, because, consistent with previous reports (35), exogenous PCAF was capable of cooperating with Tax on the HTLV-I LTR (Fig. 3C). Based on these overexpression assays, it appears that, unlike p300, PCAF is not limiting in these cells. In addition, the level of endogenous PCAF appears to be similar for all cell types (data not shown). The lack of p53 inhibition by the M47 Tax mutant clearly shows, however, an important role for PCAF in Tax-mediated p53 inhibition in H1299 cells.

Endogenous p300 Levels—Because we observed a differential dependence of p300 for Tax-mediated p53 inhibition, we examined the level of endogenous p300 from nuclear extracts in Jurkat, H1299, HeLa, Saos-2, and HTLV-I-infected MT2 and C81 cells (Fig. 4). A significant difference in the level of endogenous p300 can be seen. The level of p300 is relatively high in lymphocytes as compared with the other cell lines. It is interesting to note that in lymphocytic cells where there are higher levels of p300 (Fig. 4, lanes 1 and 3–5), Tax inhibits p53 function due to p300 squelching but is dependent on NF-κB activation and correlates with p53 phosphorylation on serines 15 and 392 (23). In contrast, in cell lines containing low levels of p300 (Fig. 4, lanes 1–3), Tax inhibits p53 function through a CREB/ATF-dependent pathway, in part because of squelching of the coactivator p300. It is important to point out, however, that p300 binding does not sufficiently explain Tax-mediated p53 inhibition in these cells because the M47 Tax mutant that binds p300 (38) cannot inhibit p53 function. This suggests that an M47 function of Tax, perhaps the ability of PCAF to stabilize the CREB-Tax-p300 complex or CREB/ATF activation, is required for p53 inhibition.

DISCUSSION

The tumor suppressor p53 has been termed the guardian of the genome for its critical role in guarding cells against transformation. p53 is a short-lived, latent sequence-specific transcription factor that is activated and stabilized in response to a wide range of cellular stress including DNA damage and oncogenic activation (39–41). Once activated, p53 acts as a master switch to integrate the cellular stress signals with coordinated gene expression that leads to either cell cycle arrest or apopto-
sis. However, the precise mechanisms by which p53 responds to different external stimuli are not fully understood.

The p53 protein has been shown to be a target of several viral transforming proteins including SV-40 large T antigen, hepatitis B X protein, adenovirus E4orf6, cytomegalovirus IE2, and human papillomavirus E6 protein (42–46). Our laboratory and others have shown that the HTLV Tax protein inhibits p53 function. In this report we addressed the recent controversy over the mechanism by which Tax inhibits p53 function. Suzuki et al. (27), Van Orden et al. (28), Kaida et al. (26), and Ariumi et al. (34) suggest that the Tax-p300 interaction is responsible for p53 inhibition. These groups used wild-type Tax and the Tax mutant K88A in their analyses. The K88A mutant fails to bind p300/CBP and fails to activate the viral LTR. Importantly, we demonstrate here using additional Tax mutants M22 and M47 that in cells where p300 is limiting (H1299, HeLa, and Saos-2 cells), the CREB/ATF activation function of Tax is responsible for Tax-mediated p53 inhibition. Tax-mediated p53 inhibition does not appear to be due simply to squelching of p300 by Tax, because the Tax-M47 mutant can still bind p300 but fails to inhibit p3 function. These results are in agreement with those obtained by Mulloy et al. (30). One distinct difference in our report versus Mulloy et al. (30) is in Jurkat cells. Because we see a clear dependence on p300 levels in the Tax-mediated p53 inhibition pathway, one possible explanation for the differing results is in p300 levels in our Jurkat cells versus those used by Mulloy et al. (30). Differences in cell culture could directly effect the level of endogenous p300.

We have recently shown that PCAF is an important factor in Tax-mediated activation of the HTLV-I LTR and is responsible for the defect in M47 activation of the viral LTR (35). PCAF does not appear to be a limiting factor in our system, because we observe only a modest increase of p53 activity with exogenous expression of PCAF. In contrast, exogenous PCAF significantly enhanced viral LTR expression. At present the role of PCAF in Tax-mediated p53 inhibition is unclear. It is possible that PCAF could be involved in CREB-Tax-p300 promoter complex stability, which would be consistent with a p300-squelching model. Alternatively, although we consider it less likely, the Tax-CREB/ATF transcriptional activation function may be required to increase expression of cellular genes that inhibit p53 function. Further investigation is required to determine the exact mechanism.

Our studies clearly show that there is a cell type dependence on the mechanism used by Tax for p53 inhibition. Whereas the CREB/ATF pathway is clearly important for p53 inhibition in HeLa, H1299, and Saos-2 cells, the NF-B pathway is important for p53 inhibition in Jurkat cells as well as mouse embryo fibroblasts (23). In this report (Fig. 1) and in a recent publication (23), we demonstrated that in cells that have a high level of p300 Tax-mediated p53 inhibition involves phosphorylation on serines 15 and 392 by a processes that involves NF-B activation. This is not the first report of a cell type-specific dependence for Tax function. In recent reports by Courtois et al. (47) and Harhaj et al. (48), they found that in B cells the IKKγ component of the IKK complex is not necessary for NF-B activation, whereas in T cells and rat embryo fibroblasts it is essential. More recently, Ohtani et al. (49) have also shown a cell type-specific E2F activation that is also linked to the M22 mutant of Tax.

It is well known that the NF-B/Rel proteins play important roles in the regulation of lymphocyte activation and proliferation, and these proteins have been implicated in the development of a number of human leukemias and lymphomas (50). Consistent with our findings on p53 inhibition, several groups have demonstrated a clear link between NF-B activation by Tax and virus-induced cellular transformation (13, 14, 51, 52). Other groups have suggested that the CREB/ATF activation pathway is important for virus-induced transformation (53, 54) using Rat1, Rat2, or rat embryo fibroblast cells. Perhaps the link between p53 inhibition, p300 expression levels, and cell type-dependent pathways could explain the differences observed in these systems. It is also interesting to speculate that these two distinct mechanisms of Tax-mediated p53 inhibition may be reflected in HTLV-I-associated pathologies. HTLV-I infection has long been linked not only with lymphocytic transformation but also with inflammatory and neurodegenerative diseases (6, 7). Perhaps the CREB/ATF-mediated inhibition of p53 function by either viral infection or soluble Tax contributes to the pathologies seen in diseases such as tropical spastic paraparesis/HTLV-I-associated myelopathy. In contrast, we would argue that the NF-kB-mediated inhibition of p53 by Tax is linked to lymphocytic transformation. At present, more investigation is required to pinpoint the exact mechanism by which Tax inhibits p53 and the importance of p53 inhibition to disease outcome.

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