The EMBO Journal Vol.19 No.5 pp.956–963, 2000

TIP30 has an intrinsic kinase activity required for up-regulation of a subset of apoptotic genes

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CC3 is a metastasis suppressor that inhibits metastasis of the variant small cell lung carcinoma (v-SCLC) by predisposing cells to apoptosis. The same protein was also reported as a cellular cofactor, TIP30, which stimulates HIV-1 Tat-activated transcription by interacting with both Tat and RNA polymerase II. We report here that TIP30/CC3 is a novel serine/threonine kinase. It phosphorylates the heptapeptide repeats of the C-terminal domain (CTD) of the largest RNA polymerase II subunit in a Tat-dependent manner. Amino acid substitutions in the putative ATP binding motif that abolish the TIP30 kinase activity also inhibit the ability of TIP30 to enhance Tat-activated transcription or to sensitize NIH 3T3 and v-SCLC cells to apoptosis. Furthermore, ectopic expression of TIP30/CC3 in v-SCLC cells induces expression of a number of genes that include the apoptosis-related genes Bad and Siva, as well as metastasis suppressor NM23-H2. These data demonstrate a molecular mechanism for TIP30/CC3 function and suggest a novel pathway for regulating apoptosis.

Keywords: apoptosis/metastasis suppressor/SCLC/Tat/transcription

Introduction

Metastasis is a complex process that involves differential expression of a number of genes whose products act as positive or negative regulators of metastasis in malignant tumor cells (Steeg, 1989). Recently, the CC3 gene was identified by differential display analysis of mRNAs from the highly metastatic human small cell lung carcinoma (SCLC) versus less metastatic SCLC cell lines (Shtivelman, 1997). Introduction of the CC3 gene into a variant SCLC (v-SCLC) line that does not express the CC3 gene results in significant suppression of its metastatic potential. This suppression appears to result, at least in part, in a propensity of the cells to undergo apoptosis. Therefore, CC3 was proposed to function as a metastasis suppressor that links the control of apoptosis to metastasis. The anti-tumor activity of CC3 was further confirmed by the finding that intravenous delivery of the CC3 gene to melanoma-bearing mice via a cationic liposome–DNA complex significantly reduced pulmonary and extrapulmonary metastases (Liu *et al*., 1999). Nevertheless, the molecular mechanism by which the CC3 gene mediates apoptosis has remained largely unknown (Shtivelman, 1997).

We have recently identified a transcription cofactor, called TIP30, that binds to the human immunodeficiency virus (HIV) Tat protein (Xiao *et al.*, 1998). TIP30 is essential for Tat-mediated transcription *in vitro* and potentiates Tat-activated transcription in transient transfection assays. Other cellular factors that include elongation factor P-TEFb (Mancebo *et al*., 1997; Zhu *et al*., 1997) and coactivators CBP/p300 (Hottiger and Nabel, 1998; Marzio *et al*., 1998) and PCAF (Benkirane *et al*., 1998) have also been found to interact with Tat and to potentiate transcription from the HIV-1 long terminal repeat (LTR). Ectopic expression of either the CycT1 component of P-TEFb or the histone acetyltransferase PCAF increases Tat-mediated transcription from the HIV-1 LTR in rodent cells (Benkirane *et al*., 1998; Wei *et al*., 1998). Although CycT1 appears to be a human-specific cofactor for Tat, as indicated by the inability of the mouse homologue to support Tat-mediated transcription (Bieniasz *et al*., 1998; Garber *et al*., 1998), P-TEFb appears to have a general role in the regulation of transcription elongation (Marshall and Price, 1995; Peng *et al.*, 1998). In contrast, TIP30 appears not to be required either for basal level transcription or for activated transcription by GAL4-SP1 and GAL4-VP16, suggesting that it is a gene-specific cofactor (Xiao *et al*., 1998). While it is evident that TIP30 can be usurped by the viral Tat protein to enhance transcription elongation from the HIV-1 LTR, cellular genes that are targeted by TIP30 remain to be identified. Significantly, however, the finding that TIP30 is identical to CC3 indicates that TIP30 might facilitate suppression of metastasis by regulating the transcription of genes involved in apoptosis.

In this study, we have employed *in vivo* analyses to delineate further the molecular mechanisms whereby TIP30 potentiates Tat-mediated transcription and apoptosis. We demonstrate that TIP30 contains an intrinsic kinase activity that is capable of phosphorylating the C-terminal domain (CTD) of the largest subunit of RNA polymerase II and, further, that phosphorylation of the CTD by TIP30 is enhanced specifically by Tat. We further show that the kinase activity of TIP30 is required not only for Tat-mediated transcription from the HIV-1 LTR, but also for predisposing cells to apoptotic signals. By using a cDNA microarray analysis, we have identified a number of TIP30-responsive genes that are involved in the regulation of apoptosis, metastasis and DNA repair.

Results

TIP30 displays an intrinsic kinase activity

To understand better the underlying mechanism by which TIP30 stimulates Tat-activated transcription and predis-

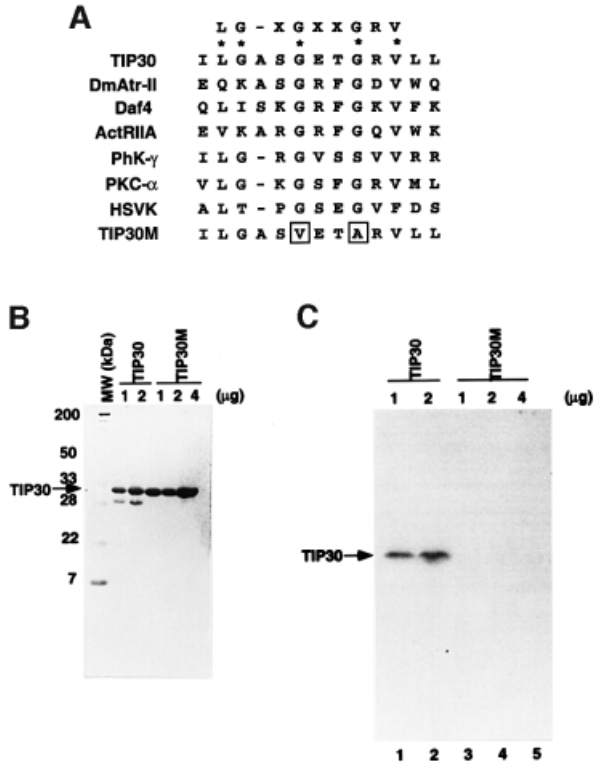

Fig. 1. TIP30 has an intrinsic kinase activity. (**A**) Alignment of the putative TIP30 ATP-binding motif with other kinases. The ATP-binding motif sequences of the known kinases were derived from the Protein Kinase Resource (www.sdsc.edu/kinases). DmAtr-II, *Drosophila melanogaster* activin type II receptor; Daf4, cell-surface receptor DAF-4 precursor; ActRIIA, activin type II A receptor; PhK-γ, phosphorylase kinase γ; PKC-α, protein kinase C α; HSVK, herpes simplex virus kinase. The amino acids that are often found in the serine/threonine kinases are listed in the first row. In the bottom row, the same region of TIP30M is shown and the substituted amino acids are boxed. (**B** and **C**) Bacterially expressed recombinant TIP30 is autophosphorylated. The recombinant proteins purified as described (Xiao *et al*., 1998) were incubated with [γ-^{32}P]ATP for 30 min, resolved on 12.5% SDS—PAGE and visualized with Coomassie Blue staining in (B) and autoradiography in (C). Arrows indicate the positions of TIP30 protein.

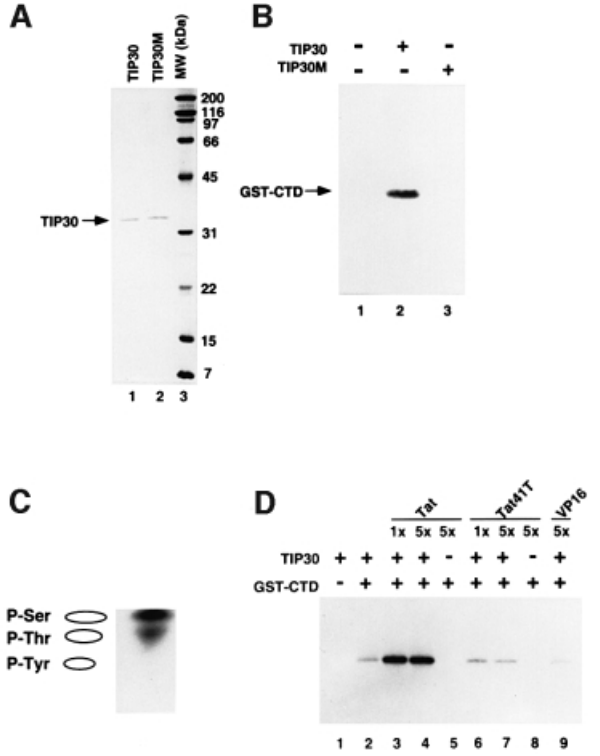

Fig. 2. TIP30 phosphorylates the CTD of RNA polymerase II. (**A**) TIP30 and TIP30M expressed with baculovirus expression system. Wild-type Flag-tagged TIP30 and mutant TIP30 were expressed in Sf9 cells and purified with M2–agarose beads. Aliquots of the proteins were resolved on SDS–PAGE and stained with Coomassie Blue. (**B**) *In vitro* phosphorylation of GST–CTD by TIP30. The purified proteins GST–CTD (lane 1) plus TIP30 (lane 2) or TIP30M (lane 3) were subjected to kinase assays followed by SDS—PAGE and autoradiography. (**C**) Phosphoamino acid analysis. [γ-^{32}P]ATP GST—CTD labeled with TIP30 was separated on SDS—PAGE and transferred into a nitrocellulose filter by electrophoresis. The labeled band was excised from the filter, hydrolyzed in 6 M HCl, and phosphoamino acids with unlabeled standard phosphorylated amino acids were separated by electrophoresis on a thin-layer cellulose plate in one dimension. The positions of ^{32}P-labeled amino acids were determined by autoradiography (right panel). The amino acid standards are visualized by ninhydrin staining (left panel). (**D**) Tat stimulates phosphorylation of the CTD by TIP30. Wild-type TIP30 was incubated with GST (lane 1) or GST–CTD (lane 2) and increasing concentrations of wild-type Tat (lanes 3 and 4) or mutant Tat (lanes 6 and 7) or GAL4-VP16 (lane 9). As controls, the kinase reactions contained GST–CTD and either wild-type Tat (lane 5) or mutant Tat (lane 8).

poses v-SCLC cells to apoptosis, we initially performed DDBJ/EMBL/GenBank database searches with the predicted TIP30 protein sequence. These analyses revealed sequence relationships (46% similarity and 22% identity) between TIP30 and a *Caenorhabditis elegans* cAMP-dependent protein kinase catalytic subunit (accession No. gi125214). A weak homology of TIP30 with a reductase was also reported, but such an activity for TIP30 remains elusive (Baker, 1999). However, the N-terminal region of TIP30 contains a sequence motif that is similar to a putative ATP-binding motif often found in serine/threonine kinases (Hanks *et al*., 1988; Bossemeyer, 1994), raising the possibility that TIP30 might be a kinase. Alignments of the putative ATP-binding motif of TIP30 with that of selected kinases are shown in Figure 1A. To investigate this possibility, we tested the ability of bacterially expressed TIP30 to catalyze autophosphorylation. In order to exclude the possibility of TIP30 phosphorylation by a contaminating kinase in the preparation, control kinase assays were performed with an identically expressed and purified mutant TIP30 containing Val and Ala substitutions for two Gly residues (Gly28 and Gly31) within the ATP-binding motif (Figure 1A and B). As shown in Figure 1C, wild-type TIP30 was phosphorylated, whereas mutant TIP30 was not, even at higher concentrations of the mutant protein. This result clearly indicates autophosphorylation of TIP30.

TIP30 phosphorylates the CTD of RNA polymerase II

To ascertain whether TIP30 is a kinase that is capable of phosphorylating other protein substrates, epitope-tagged wild-type and mutant TIP30 proteins were expressed via baculovirus vectors in Sf9 cells, purified to near homogeneity (Figure 2A), and tested in an *in vitro* kinase assay. Since we previously showed an association of TIP30 with an RNA polymerase II–SRB complex (Xiao *et al*., 1998), we first tested the ability of TIP30 to phosphorylate the CTD of the largest subunit of RNA

polymerase II. The analysis in Figure 2B shows that wild-type TIP30, but not mutant TIP30, is able to phosphorylate the CTD of the largest subunit of RNA polymerase II. A phosphoamino acid analysis indicated that both Ser and Thr residues within the CTD were phosphorylated (Figure 2C). Other experiments also showed that TIP30 could not phosphorylate GST (Figure 2D, lane 1), but that it could phosphorylate the largest subunit of a purified native RNA polymerase II (data not shown). TIP30, like the TFIIH-associated CTD kinase (Ohkuma and Roeder, 1994), can also phosphorylate other substrates that include TFIIEα and RAP74, but not TBP, TFIIB and histones (including histone H1), although the significance of these phosphorylations is not clear (data not shown).

Although we cannot yet rule out the formal possibility that the observed CTD phosphorylation is mediated by a contaminating kinase (from Sf9 cells) in the purified TIP30 preparation, this is unlikely in view of the observation that mutation of the presumptive ATP-binding site in TIP30 results in the loss of both CTD phosphorylation by TIP30 purified from Sf9 cells and autophosphorylation by TIP30 purified from bacteria. On the other hand, TIP30 kinase activity might be stimulated by trace amounts of associated proteins from insect cells, consistent with preliminary results indicating associated proteins in affinity-purified TIP30 from HeLa cells (H.Xiao and R.G.Roeder, unpublished data) and with many examples of kinase regulation by associated factors.

The results above suggest that TIP30 is a serine/threonine kinase and the observed phosphorylation of the CTD by TIP30 raised the possibility that HIV-1 Tat might interact with TIP30 and enhance its intrinsic kinase activity. We examined this possibility by asking whether Tat might stimulate phosphorylation of GST–CTD by TIP30. As shown in Figure 2D, wild-type Tat stimulated phosphorylation of the CTD by TIP30 (lanes 3 and 4 versus lane 2), whereas an equivalent amount of the transactivation-defective mutant TatK41T had no effect on CTD phosphorylation (lanes 6 and 7 versus lane 2). As a control, GAL4-VP16, which does not interact with TIP30, had no effect on CTD phosphorylation by TIP30 (Figure 2D, lane 9 versus lane 2). Given the importance of CTD phosphorylation in both transcription elongation and Tat-mediated transactivation (Dahmus, 1995; Chun and Jeang, 1996; Okamoto *et al*., 1996), this result suggests that the interaction of Tat with TIP30 may increase phosphorylation of the CTD of RNA polymerase II in Tat-mediated transcription.

The kinase activity of TIP30 is essential for its ability to stimulate transcription

Our previous study has shown that TIP30 acts as a coactivator to increase Tat-mediated transcription. To determine the role of the TIP30 kinase in its coactivator activity, we tested whether alterations in the ATP binding motif of TIP30 that inhibit its kinase activity also abolish its ability to stimulate Tat-mediated transcription. Plasmids expressing Flag-tagged wild-type and kinase-defective TIP30 were co-transfected with or without a plasmid expressing Tat into Jurkat cells. Consistent with our previous results, wild-type TIP30 stimulated Tat-activated transcription whereas the mutant TIP30 did not (Figure 3A). Instead, it slightly inhibited Tat-activated

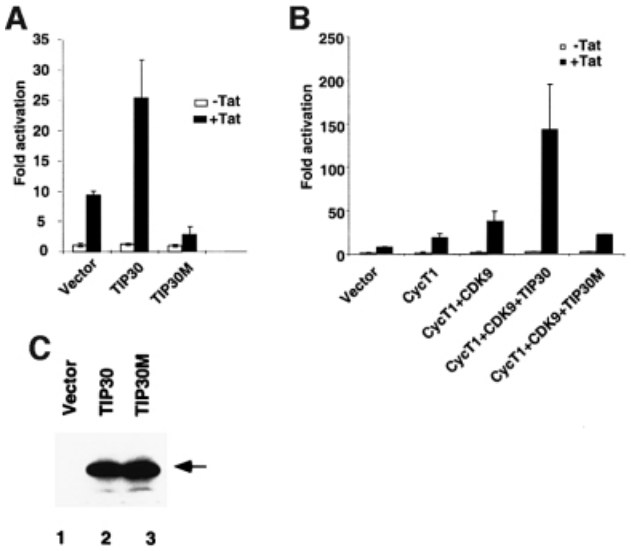

Fig. 3. The kinase activity of TIP30 is required for enhancing Tat-activated transcription. (**A**) Inhibition of Tat-activated transcription by overexpression of TIP30M in Jurkat cells. Jurkat cells were co-transfected with a reporter pGL/HIV-1Luc (1 μg), a control pSV-β-Gal (0.5 μg) and pSVTat (40 ng), in the presence of either pCIN4-TIP30 (2 μg) or pCIN4-TIP30M (2 μg) or pCIN4 (2 μg). Results represent the averages of three experiments. (**B**) TIP30 cooperates with CycT1 and CDK9 to stimulate Tat-dependent transcription. NIH 3T3 cells were co-transfected with plasmids pGL/HIV-1lLuc (200 ng), control pSV-β-Gal (200 ng) directing β-galactosidase expression and plasmid expressing Tat (20 ng), CycT1 (200 ng), CDK9 (200 ng), TIP30 (200 ng) or TIP30M (200 ng) as indicated. Results represent averages of two experiments. (**C**) Immunoblot analysis of wild-type and mutant TIP30 expression. Aliquots of lysates (10 μg) from NIH 3T3 cells transfected with empty vector, pCIN4-TIP30 or pCIN4-TIP30M were analyzed by Western blotting with anti-TIP30 antibody (Xiao *et al*., 1998).

transcription. Furthermore, in rodent cells in which vectors expressing CDK9 and the human-specific Tat coactivator CycT1 elevated the Tat response from 9- to 30-fold, ectopically expressed wild-type TIP30, but not the mutant TIP30, cooperated with CycT1 and CDK9 to effect a larger (140-fold) Tat response (Figure 3B). An immunoblot analysis showed that both wild-type and mutant TIP30 were expressed in transfected cells at similar levels (Figure 3C). These results suggest that the kinase activity of TIP30 is essential for its observed coactivator function.

The kinase activity of TIP30 is essential for mediating apoptosis in serum-deprived cells

To confirm further that TIP30 is able to predispose cells to apoptosis, we generated NIH 3T3 and v-SCLC cell lines that stably overexpress either the wild-type TIP30 or the kinase-defective TIP30 and control cell lines containing an empty vector. A Western blot analysis of the levels of ectopically expressed TIP30 in these cell lines is shown in Figure 4A. We then examined proliferation of cells overexpressing TIP30 by counting live cells (Trypan blue negative) after growth in low-serum medium for 3 days. Expression of the mutant TIP30 had no effect on cell growth in comparison with cells containing an empty vector; however, expression of TIP30 resulted in a significantly lower number of live cells (Figure 4B). Since TIP30 was implicated previously in the regulation of apoptosis, it is conceivable that inhibition of growth in

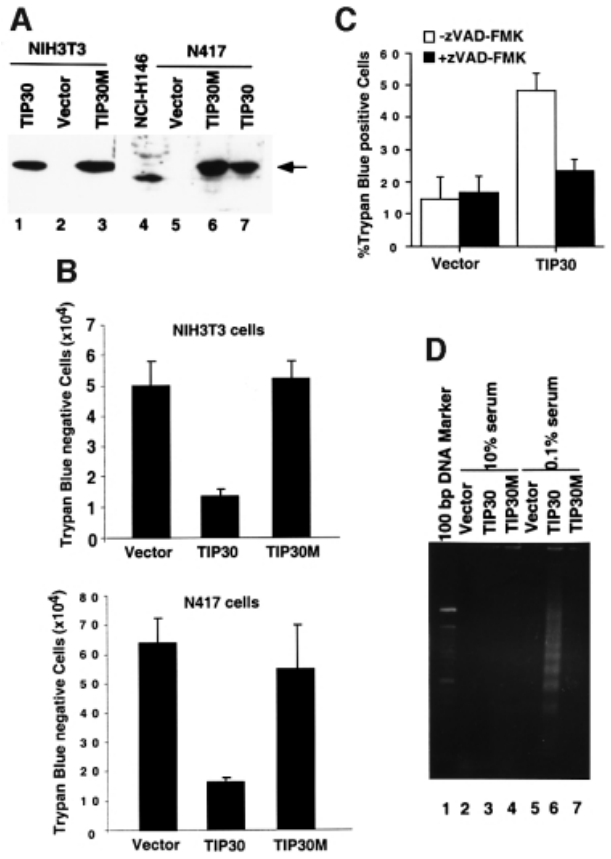

Fig. 4. (**A**) Immunoblot analysis of TIP30 and TIP30M expression in stable cell lines. Aliquots (100 μg) of lysates from each cell line as indicated were analyzed by Western blotting with anti-TIP30 antibody. H146 is a less metastatic SCLC cell line. (**B**) TIP30 expression inhibits NIH 3T3 (upper panel) and N417 (bottom panel) cell growth. NIH 3T3 (2×10^4) or N417 cells (2×10^5) expressing TIP30 or TIP30M, or containing an empty vector, were seeded on six-well plates in DMEM or RPMI medium supplemented with 10% FCS. After 24 h, cells were washed with serum-free medium and grown in medium supplemented with 0.1% bovine calf serum for 3 days. Cells were then trypsinized and stained with Trypan blue. Trypan blue-negative cells were counted. The average numbers represent three independent experiments. (**C**) Inhibition of TIP30-mediated cell death by the caspase inhibitor zVAD-FMK. NIH 3T3 cell lines containing an empty vector or TIP30-expressing plasmid were assayed for cell death with or without 50 μM zVAD-FMK in the medium during serum deprivation as described in Figure 4B. Trypan blue-positive and -negative cells were counted. (**D**) DNA fragmentation analysis. Fragmented DNA was purified from different NIH 3T3 cells that were grown in DMEM medium supplemented with 10% FCS or 0.1% bovine calf serum for 3 days as described (Shtivelman, 1997). Aliquots of DNA were separated on a 1.5% agarose gel and then stained with ethidium bromide.

cells overexpressing TIP30 results from cell death. Indeed, as demonstrated in Figure 4C, cultures of cells expressing TIP30 showed a significantly higher proportion (50%) of dead cells than did cultures of control cells (15%) in low serum. Importantly, this TIP30 effect on cell death was inhibited by addition of the caspase-specific inhibitor VAD-FMK to the cell cultures (Figure 4C). DNA fragmentation (Figure 4D) and TUNEL (data not shown) analyses confirmed that the observed cell death resulted from apoptosis. NIH 3T3-TIP30 cells showed the DNA fragmentation pattern that is characteristic of apoptotic cells, whereas the NIH 3T3-Vector and NIH 3T3-TIP30M cells did not (Figure 4D). Furthermore, and as expected,

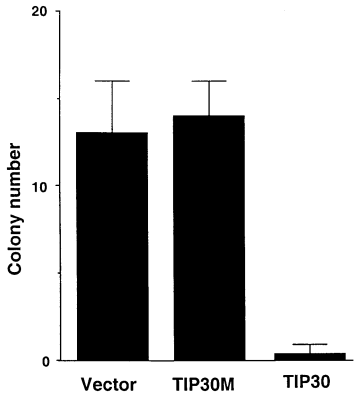

Fig. 5. Growth of N417 clones in semi-solid medium. The numbers represent average numbers of colonies from three independent experiments.

N417-TIP30 cells displayed higher sensitivity to the apoptotic effects of serum deprivation than did the N417-TIP30M and N417-Vector cells (data not shown). Taken together, these results indicate that TIP30 requires its kinase activity for sensitizing cells to apoptosis under low-serum growth conditions.

The kinase activity of TIP30 is required for suppression of colony formation under anchorage-independent conditions

Since it has been demonstrated previously that ectopic expression of TIP30 can inhibit colony formation of v-SCLC cells on soft agar (Shtivelman, 1997), we examined whether the kinase activity of TIP30 also plays a role in this event. As shown in Figure 5, cells expressing wild-type TIP30 resulted in significantly fewer colonies when grown on soft agar medium than did cells expressing mutant TIP30 or containing an empty vector. This result suggests that the kinase activity of TIP30 could also be involved in suppression of metastasis.

Expression of apoptotic genes Bad and Siva is induced by overexpression of wild-type, but not kinase-defective, TIP30

Having demonstrated that the kinase activity of TIP30 is required both for enhancing Tat-mediated transcription and for sensitizing cells to apoptosis, we hypothesized that TIP30 might directly regulate transcription of genes that facilitate apoptosis. To identify potential TIP30-targeted genes, we examined the expression of known apoptosis-related genes, as well as cancer-related genes, in v-SCLC cells. Poly(A) mRNAs were isolated from the v-SCLC-TIP30 and v-SCLC-TIP30M cell lines and used to generate probes for cDNA microarray analysis. The human cancer cDNA array contained 588 genes that are known to be involved in the regulation of tumor growth, metastasis, cell cycle, DNA repair and apoptosis, as well as nine control genes for normalization. Of the 597 genes tested, only ~12 genes were expressed at visibly higher levels (relative to the control genes) in v-SCLC-TIP30 cells (Figure 6A versus B; indicated in Figure 6C) than in v-SCLC-TIP30M cells (Figure 6B). As summarized in Figure 6D, these included the apoptotic genes Bad (Yang *et al*., 1995) and Siva (Prasad *et al*., 1997), as well as genes involved in DNA repair and tumor invasion. The

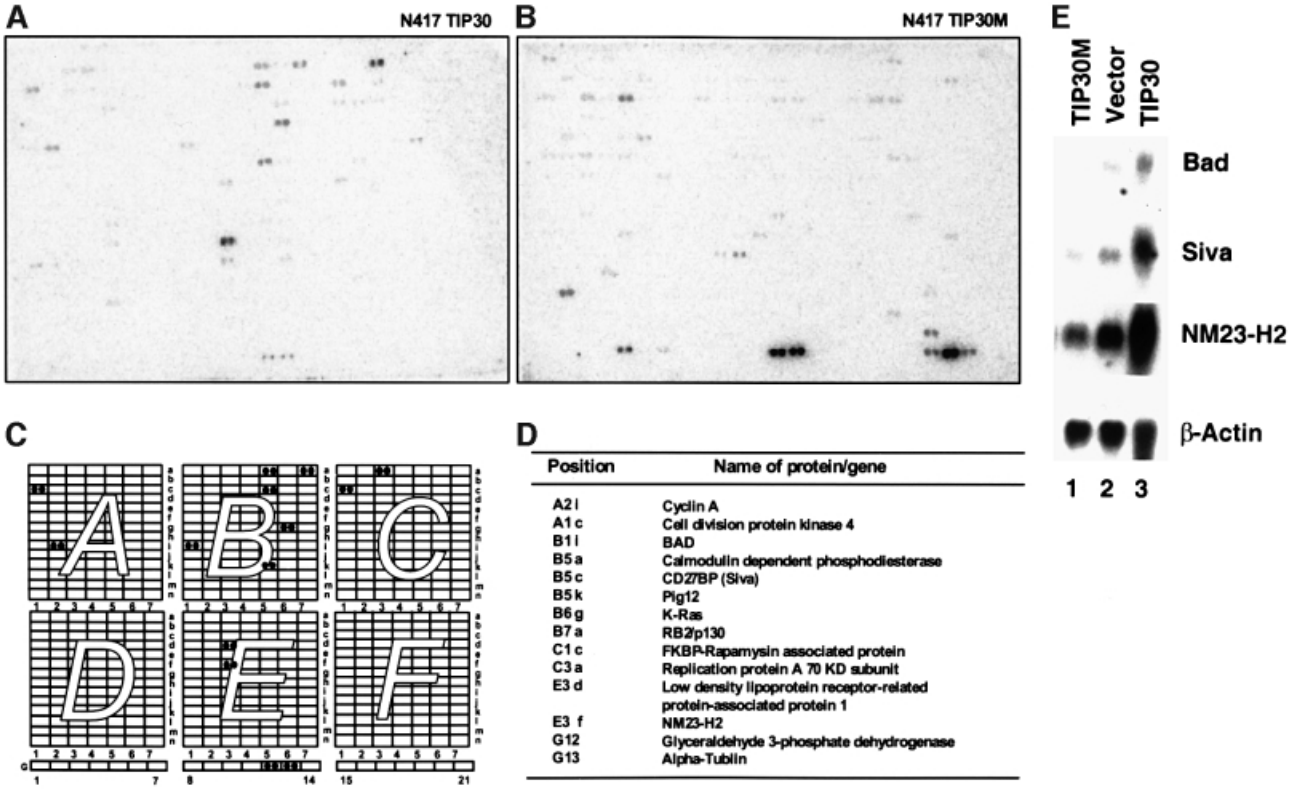

Fig. 6. Identification of TIP30-responsive genes. The human cancer cDNA array membranes that were hybridized with probes derived from N417-TIP30 (**A**) or N417-TIP30M (**B**) were examined with Molecular Dynamic Storm 840. The positions of cDNAs are shown in (**C**). The two housekeeping genes and the TIP30-responsive genes are listed in (**D**). (**E**) Northern blot analyses of Bad, Siva and NM23-H2 mRNA expressions in N417 clones. Total RNAs from different N417 clones were analyzed by Northern blotting with probes as indicated.

elevated expression of Bad, Siva and NM23-H2 (Postel *et al*., 1993) genes was also confirmed by Northern blot analysis (Figure 6E).

Discussion

An interesting and potentially important feature of TIP30 is that it displays a serine/threonine kinase activity. Although it is possible that TIP30 may have other physiological substrates, we speculate that one target for TIP30 is the CTD of the largest subunit of RNA polymerase II. Besides TIP30, other cellular kinases that include the SRB10 and 11 components of the RNA polymerase II holoenzyme/mediator (Liao *et al*., 1995), c-ABL tyrosine kinase (Baskaran *et al*., 1997), TFIIH (Lu *et al*., 1992), P-TEFb (Marshall *et al*., 1996) were previously reported to be capable of phosphorylating the CTD on the largest subunit of RNA polymerase II, suggesting multiple pathways for regulation of RNA polymerase II phosphorylation. Among these kinases, TFIIH, P-TEFb and TIP30 are able to interact with the activation domain of Tat (Blau *et al*., 1996; Cujec *et al*., 1997; Mancebo *et al*., 1997; Parada and Roeder, 1997; Zhu *et al*., 1997; Xiao *et al*., 1998) and participate in Tat-mediated transcription. However, both TFIIH and P-TEFb have been implicated, by immunodepletion studies, in basal transcription of the HIV-1 and adenovirus major late promoters in nuclear extracts (Parada and Roeder, 1997; Zhu *et al*., 1997). These observations are consistent with previous findings that TFIIH serves as a basal transcription factor and P-TEFb as a general elongation factor in systems reconstituted with partially purified factors. In contrast, TIP30 appears not to be necessary for basal transcription from the adenovirus major late promoter or for activation by GAL4-SP1 or GAL4-VP16 in nuclear extracts, suggesting that TIP30 might be more of a gene-specific cofactor (Xiao *et al*., 1998). This is also consistent with our observation that ectopic expression of TIP30 only induces expression of a subset of genes. It is possible that phosphorylation of RNA polymerase II by TIP30 would increase expression of this subset of genes, whereas phosphorylation of RNA polymerase II by P-TEFb and TFIIH is required for constitutive or basal expression of most, if not all, cellular genes. Therefore, TIP30 might represent one of the first examples of the regulation of apoptosis by a mechanism involving the kinase activity of a transcriptional coactivator.

Apoptosis is facilitated by the cell death machinery that acts downstream of the death signals in the cytosol (Green and Reed, 1998). Growing evidence has indicated that there are multiple genetically programmed pathways that can be triggered by a variety of physiological death signals, as well as pathological cellular insults that activate those death molecules to execute apoptosis (Dragovich *et al*., 1998; Vaux and Korsmeyer, 1999). While pro- and anti-apoptotic molecules may be regulated via mechanisms involving post-translation modification and/or conformation changes, as well as translocations of these molecules, they may also be regulated at the level of transcription. Biochemical and genetic studies have suggested that some of the transcription factors do not induce apoptosis *per se*, but instead maintain appropriate expression of pro-

and anti-apoptotic genes in order to establish the sensitivity of cells to apoptosis (Vaux and Korsmeyer, 1999). Thus, an obvious prediction that can be made from this scenario is that loss or inactivation of those transcription factors might reduce the sensitivity of cells to death signals and decrease their ability to undergo apoptosis, thus leading in turn to tumor progression.

Consistent with this idea, it was demonstrated previously that the SCLC cells lacking TIP30 were more resistant to death-inducing signals such as growth factor withdrawal and chemotherapeutic drugs than the TIP30-expressing SCLC cells (Shtivelman, 1997). Our present data clearly support a role for TIP30 in apoptosis, as proposed by Shtivelman (1997). It appears that ectopic expression of TIP30 elevates the mRNA levels of pro-apoptotic genes, as exemplified by Bad and Siva. Since Bad is associated with BCL-2 and induces apoptosis (Yang *et al*., 1995) and since Siva participates in the CD27-mediated apoptosis (Prasad *et al*., 1997), it is likely that TIP30 sensitizes cells to apoptosis, at least in part, by elevating expression of Siva and Bad. Higher levels of these two proteins would presumably alter cell growth and commit cells to an apoptotic pathway. Regardless of whether these genes are direct or indirect targets of TIP30-mediated transcriptional activation, our data have provided a plausible explanation for TIP30-induced apoptosis and implicated a novel mechanism for regulation of apoptosis in cells.

Our present data also raise a number of intriguing questions. First, with which cellular transcriptional activator(s) does TIP30 interact? Since TIP30 was isolated by virtue of an interaction with Tat, it is possible that a cellular transcriptional activator, possibly analogous in function to Tat, might also interact with TIP30 to activate transcription. Thus, identification of such an activator would contribute to a better understanding of TIP30-mediated apoptosis. Secondly, what are the genes and signals that are upstream of TIP30? To date, many transcription factors have been demonstrated to participate in the regulation of apoptosis. Among these, the tumor suppressor p53 has been shown to delay cell division in order to repair DNA damage or to induce apoptosis and to eliminate cells and, when overexpressed, to induce a number of genes that include Bax (Miyashita and Reed, 1995) and pigs 1–12 (Polyak *et al*., 1997). Although TIP30 induces one p53-targeted gene, pig 12, p53 clearly regulates the expression of many other genes that are different from TIP30-targeted genes. Myc is another important transcription factor that can control the decision of a cell to undergo apoptosis (Evan *et al*., 1992). While the mechanism underlying Myc-mediated apoptosis remains unclear, Myc was reported to affect p53-mediated apoptosis and to cooperate with Ras in inducing apoptosis (Hermeking and Eick, 1994; Wagner *et al*., 1994; Kauffmann-Zeh *et al*., 1997). Similar to TIP30-mediated apoptosis, Myc-mediated apoptosis is also promoted by serum deprivation (Evan *et al*., 1992). v-SCLC N417 cells were reported to have higher level of Myc but no expression of wild-type p53 (Adachi *et al*., 1996; Shtivelman, 1997). It is tempting to speculate that p53 and Myc might act as upstream regulators in a TIP30-mediated signal pathway. If so, TIP30-mediated transcription regulation would provide a novel mechanism for p53- and Myc-mediated apoptosis. Nevertheless, the validation of this proposal requires further experimentation. Finally, since two malignant tumors, v-SCLC and neuroblastoma, do not express TIP30 and since introduction of TIP30 into v-SCLC cells inhibits their metastatic potential in mice (Shtivelman, 1997), identification of the kinase activity of TIP30 and its downstream target genes provides clues for further studies of the mechanism of metastasis suppression. It is conceivable that TIP30 somewhat directly induces apoptotic pathways and thereby results in inhibition of metastasis. However, the finding that several DNA repair and metastasis-related genes (including RPA70, NM23-H2 and RAP) are induced by TIP30 (Figure 6D) indicates that other cellular responses may also be involved in the inhibition of tumor growth and metastasis by TIP30. Unraveling TIP30-mediated signal pathway(s) might provide novel connections between networks of signal transduction pathways for controlling apoptosis, metastasis, DNA repair response and proliferation within cells.

Materials and methods

Plasmids and cell lines

The bacterial expression plasmid pRSET-his-TIP30M was generated by directly changing DNA sequences of the TIP30 gene on pREST-his-TIP30 (Xiao *et al*., 1998) with the site-directed mutagenesis kit (Stratagene). The oligonucleotide (5′-TTGGGCGCCAGCGTAGAAAC-CGCTAGAGTGCTCTTA-3′) was used for mutagenesis according to the manufacturer's instructions (Stratagene).

The insect cell expression vectors pVL-Flag-TIP30 and pVL-Flag-TIP30M were generated by inserting DNA fragments encoding Flag-TIP30 and Flag-TIP30M (isolated from pFlag-TIP30 and pFlag-TIP30M) into the vector pVL1392 at the *Eco*RI–*Bam*HI site. pFlag7-TIP30 and pFlag7-TIP30M were constructed by insertion of DNA fragments encoding TIP30 and TIP30M (from pRSET-his-TIP30 and pRSET-his-TIP30M) into pFlag-7 (Chiang and Roeder, 1993) at *Nde*I and *Bam*HI sites. Recombinant baculoviruses were generated according to the manufacturer's instructions (Novagen). The mammalian expression vectors pCIN4-TIP30 and pCIN4-TIP30M were created by cloning *Eco*RI–*Bam*HI fragments of Flag-TIP30 into pCIN4 (Invitrogen). NIH 3T3, H146 and N417 were obtained from the American Tissue Culture Collection. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) or RPMI supplemented with 10% fetal calf serum (FCS) and antibiotics. TIP30-expressing and control cell lines were established as described previously (Shtivelman, 1997; Xiao *et al*., 1998). pCIN4-CycT1 was created by insertion of PCR-amplified human CycT1 DNA encoding the entire open reading frame into pCIN4 at *Eco*RI and *Bam*HI sites. pGST-CycT1 (Wei *et al*., 1998) was used as a DNA template for PCR. Oligonucleotides for PCR were 5′-GGAATTCCATGGAGGGAG-AGAGGAAGAACA-3′ and 5′-CGGGGATCCTTACTTAGGAAGGG-GTGGAAG-3′. The plasmid for expressing CDK9 has been described (Mancebo *et al*., 1997). pGL2/HIV-1Luc was described previously (Bassuk *et al*., 1997)

Kinase assay

Recombinant proteins were mixed in 20 µl of reaction buffer containing 10 mM Tris pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol and 5 µCi [γ-^{32}P]ATP (3000 Ci/mmol). Reactions were incubated at 37°C for 30 min and then stopped by adding 10 µl of 3× SDS sample buffer. The kinase products were analyzed by SDS—PAGE followed by autoradiography. GST–CTD was expressed and purified as described (Peterson *et al*., 1992). CTD peptides were purchased from Research Genetic Inc. (Huntsville, AL). Phosphoamino acid analysis was performed as described (Ausubel *et al*., 1995).

Transient transfection and luciferase assay

Jurkat cells at a density of 2×10^6 per well in six-well dishes were co-transfected with plasmid DNAs with Superfectin according to the manufacturer's instructions (Qiagen). NIH 3T3 cells in six-well dishes were transfected with lipofectamine as described (Xiao *et al*., 1998). All transfections were balanced to an equal amount of DNA with the vector pCIN4. Forty-eight hours after transfection, cells were collected and

assayed for luciferase activity with Promega's luciferase assay kit and for β-galactosidase activity as described previously (Xiao *et al*., 1998).

Soft agar assays

The N417 transfectants (5×10^3) were grown in RPMI medium containing 0.53% agarose and 10% FCS, on top of a layer containing 0.4% agarose for 21 days. The clones whose diameters were >1 mm were counted.

Microarray and Northern blot analyses

Poly(A) mRNA was purified from the N417-TIP30 and N417-TIP30M cell lines that were cultured in RPMI containing 10% FCS with the RNA/DNA Midi and oligo direct mRNA mini kits according to the manufacturer's instructions (Qiagen). The cDNA probes were generated and hybridized with the atlas human cancer cDNA expression array filters according to the manufacturer's instructions (Clontech). For Northern blot analyses, total RNA was isolated from cells cultured in RPMI containing 10% FCS by using an RNA purification kit (Qiagen). Ten micrograms of RNA were electrophoresed on a 1% agarose–formaldehyde gel and transferred to nitrocellulose filters as described (Ausubel *et al*., 1995). The filters were hybridized with probes in ExpressHyb hybridization buffer and washed according to the manufacturer's instructions (Clontech). The cDNAs encoding human Bad and Siva were amplified by RT–PCR from human placental poly(A) mRNA and subcloned into pRSET-His Vector (Hoffmann and Roeder, 1996) at *Nde*I and *Bam*HI sites. NM23-H2 cDNA probe was derived from the plasmid (IMAGE: 1651303) containing the NM23-H2 gene, which was obtained from the American Tissue Culture Collection. Probes were labeled with [α-^{32}P]dCTP with the Megaprime DNA labeling system RPN1606 (Amersham).

Acknowledgements

We thank Zhengxing Wang for affinity-purified RNA polymerase II and valuable suggestions. This work was supported by a grant to R.G.R. from the National Institutes of Health (AI37327) and by the Tebil Foundation. H.X. was supported in part by a fellowship from the National Institutes of Health.

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Received November 8, 1999; revised December 9, 1999; accepted January 10, 2000