

# Role for Akt/Protein Kinase B and Activator Protein-1 in Cellular Proliferation Induced by the Human T-cell Leukemia Virus Type 1 Tax Oncoprotein\*

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Human T-cell leukemia virus type 1 is an oncogenic retrovirus etiologically causal of adult T-cell leukemia. The virus encodes a Tax oncoprotein, which functions in transcriptional regulation, cell cycle control, and transformation. Because adult T-cell leukemia is a highly virulent cancer that is resistant to numerous chemotherapeutic treatments, to understand better this disease it is important to comprehend how human T-cell leukemia virus type 1 promotes cellular growth and survival. Most of the existing data point to Tax activation of NF- $\kappa$ B as important for cellular proliferation and transformation. We show here that Tax, in the absence of NF- $\kappa$ B signaling, can activate activator protein-1 to promote cellular proliferation and survival. Tax is shown to activate activator protein-1 through the phosphatidylinositol 3-kinase/Akt pathway.

Many of the molecular alterations associated with carcinogenesis occur in cell signaling pathways that regulate cell proliferation and differentiation. Human T-cell leukemia virus type 1 (HTLV-1)<sup>2</sup> is the etiological agent for adult T-cell leukemia (ATL), an aggressive human T-cell malignancy (1–4). The mechanisms of ATL leukemogenesis are not yet fully understood. However, viral protein expression early in infection probably plays a major role for disease development (5–7).

HTLV-1 encodes a 40-kDa nuclear oncoprotein, Tax (8, 9). There is strong evidence, including results from transgenic mice, that Tax plays an important role in cellular transformation (10–12). A current view is that cellular transformation by HTLV-1 is linked to the capacity of Tax to deregulate cellular signaling pathways (13–19), to disrupt the cell's genetic integrity (20–22), and to perturb cellular gene expression in part through activation of transcription factors such as NF- $\kappa$ B and activator protein-1 (AP-1) (17, 23, 24). Aberrant activation of these signal transducers and their downstream mediators can provoke uncontrolled cell growth and malignant transformation (14, 15, 17). HTLV-1 infection can also trigger a hyperinflammatory host response leading to connective tissue diseases, such as rheumatoid arthritis, tropical spastic paraparesis, and HTLV-associated myelopathy (25, 26).

Akt or protein kinase B (Akt/PKB) is a serine/threonine protein kinase that functions as a regulator of cell survival and proliferation (27–31). Akt/PKB is activated by PI3K through site-specific phosphorylation. Full activation of Akt requires phosphorylation of Ser<sup>473</sup> (31, 32), and the actual mechanism of activation remains somewhat controversial and is not completely understood. Some have suggested that Akt could be activated in a PI3K-independent manner (31, 32), but the physiological significance of these findings remains poorly established. Akt-binding proteins have also been reported to regulate Akt activity (29). For example, the carboxyl-terminal modulator protein inhibits signaling by direct binding to Akt, leading to reduced phosphorylation at its Ser<sup>473</sup> residue. By contrast, proteins like Hsp90 positively regulate Akt by promoting refolding or stabilization (31).

Akt, in response to growth factors and other extracellular stimuli, regulates normal cellular functions, including nutrient metabolism, cell growth, apoptosis, and survival (27, 29–31). Activated Akt is also thought to trigger transcriptional factors, such as AP-1, in many highly invasive human cancers (32–38). In ATL cells, AP-1 has been found to be constitutively active and may contribute to cellular proliferation and transformation (23, 24, 39). AP-1 is a transcription factor complex composed of the c-Jun and c-Fos proto-oncoproteins (17, 34, 35, 40). Interestingly, HTLV-1 Tax has been reported to induce the expression of various members of the AP-1 family (39, 41–43).

To ask if abnormal activation of Akt may contribute to ATL development, we investigated the role of the HTLV-1 Tax oncoprotein in this cellular pathway. Here, Tax activation of Akt was found to lead to enhanced downstream AP-1 activity. In the absence of NF- $\kappa$ B activation, this Tax-Akt signaling through AP-1 was sufficient to induce cellular proliferation, maintain cell survival, and promote foci formation.

## MATERIALS AND METHODS

**Reagents, Antibodies, and Plasmids**—Recombinant platelet-derived growth factor (PDGF) was purchased from PeproTech. LY294002, a PI3K inhibitor, was purchased from Calbiochem. [<sup>32</sup>P]ATP was from Amersham Biosciences. Monoclonal anti-p110 $\alpha$  and anti-p85 $\alpha$  were purchased from BD Pharmingen. Polyclonal anti-p110 $\alpha$  and anti-p85 $\alpha$  were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Akt and phospho-Ser<sup>473</sup> Akt were detected using anti-Akt or anti-PS473-Akt from Cell Signaling. Tax was detected using either rabbit anti-Tax or mouse monoclonal anti-Tax (National Institutes of Health AIDS Research and Reference Reagent Program). Anti-HA and anti-GFP (Sigma) were used at 1:1000 dilutions. HA-Akt and HA-Akt DN were a kind gift of Dr. Brian A. Hemmings (44). c-Jun dominant negative (c-Jun DN) was a kind gift from M. J. Birrer (45). Tax mutants S258A and M22 were previously described (46, 47).

**Cell Culture, Transfection, and AP-1 Reporter Assays**—Jurkat, H9, A3.01, and CEM-SS are spontaneous human T-cell lines, whereas

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<sup>2</sup> The abbreviations used are: HTLV-1, human T-cell leukemia virus type 1; PKB, protein kinase B; PI3K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; HA, hemagglutinin; GFP, green fluorescent protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; 7-AAD, 7-amino-actinomycin; MEF, mouse embryo fibroblast; ELISA, enzyme-linked immunosorbent assay; AP-1, activator protein-1; CREB, cAMP-response element; C81, C8166-45.

## Akt/Protein Kinase B and AP-1 in Cellular Proliferation

C8166-45 (C81), MT4, and HUT102 are human T-cell lines transfected with HTLV-1 and express the viral Tax protein. All cells grow in suspension and were propagated in RPMI 1640 medium with 10% fetal calf serum (FCS). Mouse embryonic fibroblast (MEF E6i), human fibroblast (BJ), and African green monkey kidney cell line (CV-1) were propagated in Dulbecco's modified Eagle's medium with 10% FCS and transfected according to the manufacturer's protocol using Lipofectamine and Plus reagent (Invitrogen). 3 h after transfection, the cell culture was replaced with fresh medium. To assay luciferase activity, cells were transfected with plasmid DNA mixture containing reporter plasmids, 100 ng of AP-1-Luc, and 100 ng of RSV- $\beta$ -galactosidase. Total amounts of plasmid DNA were normalized by the addition of pCDNA3. Cells were washed twice with  $1\times$  phosphate-buffered saline (PBS) and then lysed in  $1\times$  luciferase lysis buffer (Promega). Luciferase assay substrate (Promega) was used according to the manufacturer's protocol, and activity was measured in an Opticom II luminometer (MGM Instruments).  $\beta$ -Galactosidase activity was measured using Galacto-Star (Tropix), as described by the manufacturer. Luciferase activities were normalized for transfection efficiency based on galactosidase readings. All transfections were performed at least three times. Error bars represent S.D. values.

**Immunofluorescent Microscopy**—Cells were cultured on sterile glass coverslips, washed in PBS, and fixed in 3% paraformaldehyde for 20 min. To prevent nonspecific binding, cells were first incubated with 1% bovine serum albumin in PBS for 30 min. Incubation with primary antibodies was carried out for 1 h at room temperature. Alexa 488- or Alexa 568-conjugated secondary antibodies were then incubated for 45 min at room temperature. DNA was counterstained with Hoechst 33342 (0.1  $\mu$ g/ml). Coverslips were then mounted in Prolong Antifade (Molecular Probes, Inc., Eugene, OR) on glass slides. A Leica TCS-NP/SP confocal microscope was used to visualize the slides.

**Immunoprecipitation**—For co-immunoprecipitations, HTLV-1-transformed C81 and MT4 cell lines or control cell lines (H9 and CEM-SS) were pelleted by centrifugation, washed twice with PBS, and resuspended into radioimmune precipitation buffer (50 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 20 mM  $\beta$ -glycerophosphate, 0.1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ), 1 mM sodium fluoride (NaF), 0.5 mM dithiothreitol, and protease mixture from Roche). Total cell lysates were immunoprecipitated using anti-Tax (National Institutes of Health AIDS Research and Reference Reagent Program) or anti-p85 $\alpha$  or anti-p110 $\alpha$  (Upstate Biotechnology) antibodies with agarose beads overnight at 4  $^{\circ}$ C. The co-immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-P85 $\alpha$ , anti-p110 $\alpha$  (BD Biosciences), and anti-Tax antibodies (National Institutes of Health AIDS Research and Reference Reagent Program) as indicated.

**In Vitro Kinase Assays**—MEFs were transfected with HA-tagged Akt alone or in combination with different Tax plasmids. 24 h after transfection, cells were starved by culturing in Dulbecco's modified Eagle's medium with 1% FCS for 12 h prior to exposure to PDGF (20 ng/ml) and before lysis for kinase assay. Cells were washed in cold PBS and lysed in 50 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 1 mM  $\text{Na}_3\text{VO}_4$ . Equivalent protein concentrations of cell lysates (determined by Bradford assay) were precleared with nonspecific IgG and protein A-Sepharose. After centrifugation, the supernatants were immunoprecipitated with anti-HA antibody. Kinase assays were carried out in kinase assay buffer (25 mM Tris-HCl (pH 7.5), 5 mM  $\beta$ -glycerophosphate, 2 mM dithiothreitol, 0.1 mM  $\text{Na}_3\text{VO}_4$ , 10 mM  $\text{MgCl}_2$ , 200  $\mu$ M ATP) for 30 min at 37  $^{\circ}$ C. GST-GSK-3 (GSK-3) peptide (Cell Signaling) was used as a substrate for Akt. Phosphorylated GSK-3

proteins were electrophoresed in 12% SDS-polyacrylamide gels for detection. The gels were stained with Coomassie Blue and visualized by autoradiography.

**Cell Proliferation and Apoptosis**—Cell proliferation was determined by using either direct cell counting or the Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol. Briefly, after transfection, 50,000–100,000 cells/well were plated in a 96-well plate. After 24, 48, or 72 h, 10  $\mu$ l of Cell Counting Kit-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt) (Dojindo) was added to each well and incubated for 1–4 h. The cell viability in each well was determined by reading the optical density at 450 nm. After treatment with either LY294002 (20  $\mu$ M) or mTOR inhibitor rapamycin (100 ng/ml) for 8 h, cells were harvested, and apoptosis was determined using an annexin V/7-amino-actinomycin (7-AAD) apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol. Briefly, annexin V is a  $\text{Ca}^{2+}$ -dependent phospholipid-binding protein that has a high affinity for phosphatidylserine and is useful for identifying apoptotic cells with exposed PS. 7-AAD is a standard flow cytometry viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for annexin V and negative for 7-AAD are beginning to undergo apoptosis. Cells that stain positive for both annexin V and 7-AAD are either in the end stage of apoptosis, undergoing necrosis, or already dead. Cells that stain negative for both annexin V and 7-AAD are alive and not experiencing measurable apoptosis.

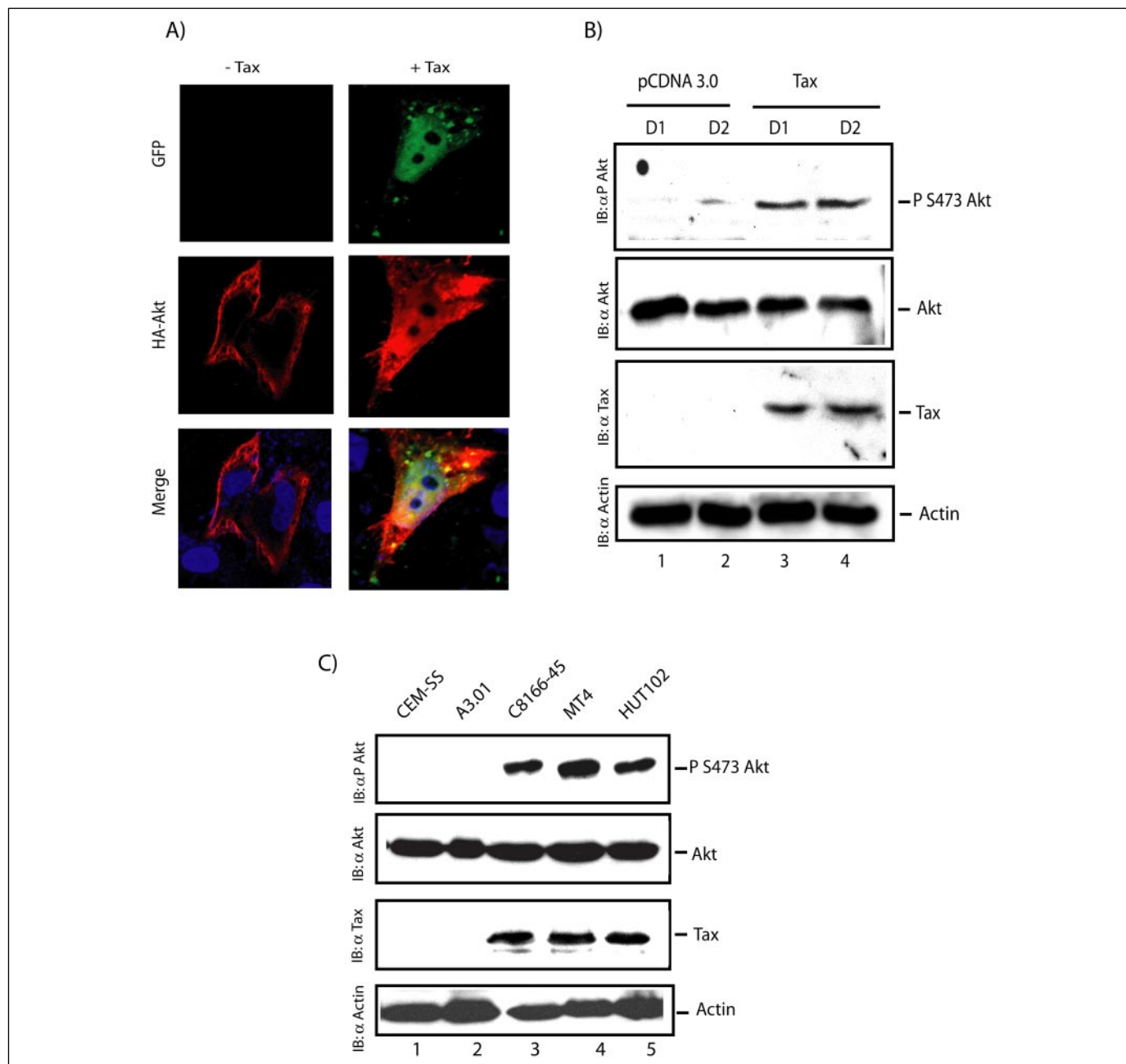
**Focus Formation Assays**—CV-1 cells were transfected with 0.5  $\mu$ g of Akt WT alone or in combination with different Tax plasmids (0.5  $\mu$ g). 24 h after transfection, cells were removed from the confluent layer with trypsin and plated at  $1\times 10^5$  cells/dish in Dulbecco's modified Eagle's medium, 1% FCS. The cultures were maintained in the same medium, with medium changes every 3 days, until the appearance of cell foci was evident (between 21 and 35 days after transfection).

**Enzyme-linked Immunosorbent Assay (ELISA)**—Jurkat and C81 cells ( $5\times 10^6$  cells/ml) were cultured in RPMI supplemented with 5% fetal calf serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin sulfate. The cells were cultured either unstimulated or stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (Sigma). Aliquots of the cell culture supernatant were removed and assayed for levels of PDGF using the human PDGF-BB Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol. The detection limit for PDGF-BB was 10 pg/ml.

## RESULTS

**Tax Activates Akt/Protein Kinase B**—Alterations in many signaling pathways that regulate cell proliferation are seen in transformed cells. Akt or protein kinase B (Akt/PKB) is a serine/threonine kinase that impacts numerous cellular processes. PI3K is an upstream regulator of Akt (31, 32, 48, 49), and dysregulation of the PI3K-Akt pathway is frequent in many human cancers. Abnormal activation of transcription factors downstream of PI3K/Akt such as NF- $\kappa$ B and AP-1 has also been linked to the promotion of cellular transformation (31, 32, 48, 49).

The HTLV-1 Tax oncoprotein is known to activate NF- $\kappa$ B, and this activation has long been considered to be the primary impetus for ATL transformation (50). Whether other factors besides NF- $\kappa$ B contribute to ATL have not been well defined. In a Rat-1 cell culture model of Tax transformation, the PI3K/Akt pathway was reported to be highly activated (51). Here, the contribution in human and mouse cells of the



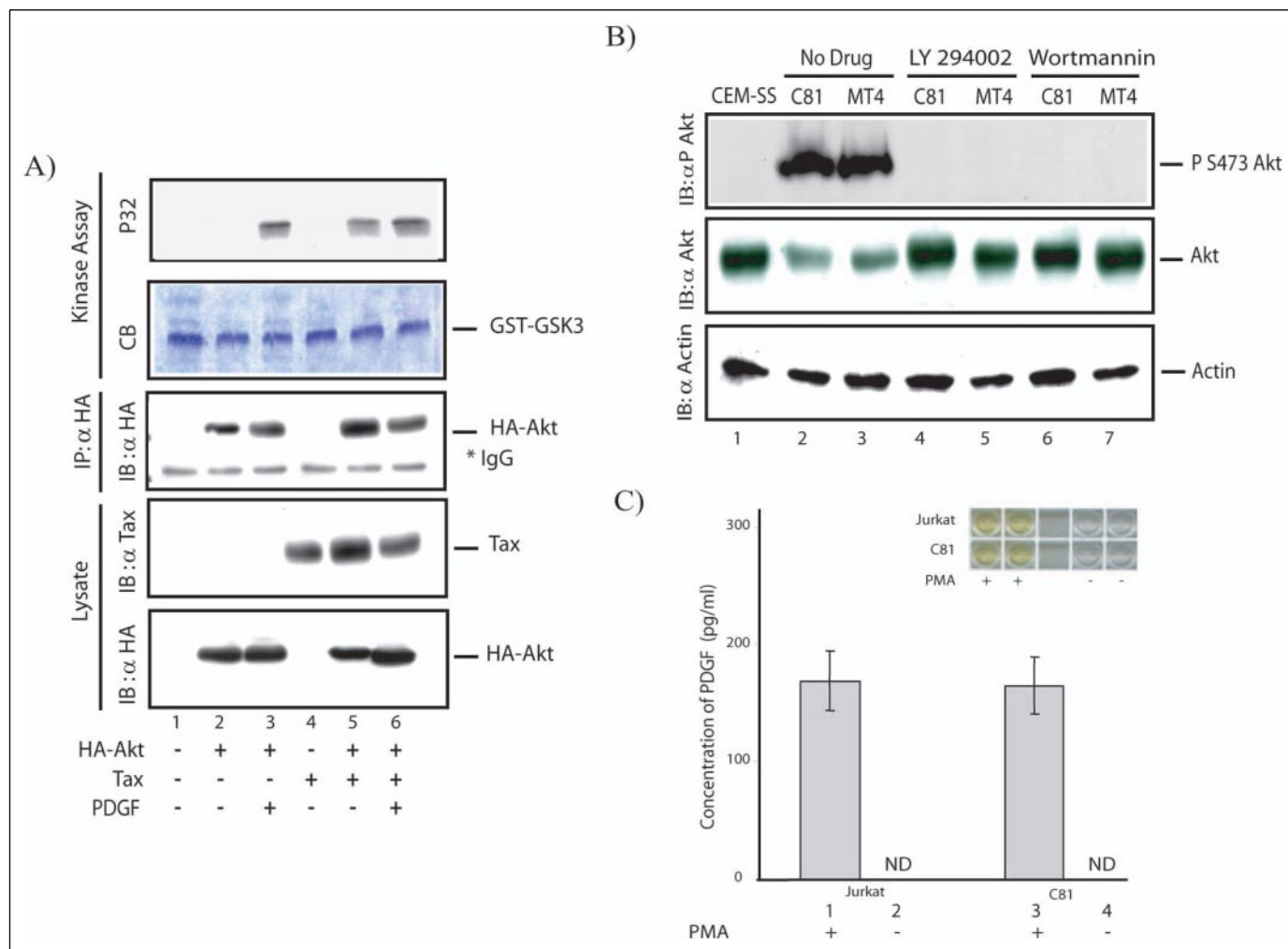
**FIGURE 1. HTLV-1 Tax induces activation of Akt.** *A*, Tax induces nuclear localization of Akt. MEF cells in low serum medium (1% FCS) were transfected with GFP-Tax and HA-Akt. Anti-HA was used to reveal the subcellular localization of HA-Akt. In the absence of Tax, Akt localizes to the membrane and cytoplasm of cells (*middle and lower panels*). In the presence of Tax, Akt translocates to the nucleus (*middle and lower panels*). *B*, MEF cells in low serum medium (1% FCS) were transfected in duplicate (D1 and D2) with either an empty vector pCDNA 3.0 (*lanes 1 and 2*) or 2  $\mu$ g of Tax-expressing vector (*lanes 3 and 4*) for 48 h. Activated Akt was detected using anti-phospho-Ser<sup>473</sup> Akt (P S473 Akt). The amounts of Akt and Tax in the cell extracts were verified using anti-Akt or anti-Tax (*middle panels* as indicated, respectively). Equal amounts of protein in the cell extract were verified using anti-actin (*lower panel*). *IB*, immunoblot. *C*, Akt is phosphorylated in HTLV-1-transformed T-cells. All cells were cultured in low serum medium (1% FCS). Control cells (CEM-SS (*lane 1*) and A3.01 (*lane 2*)) and HTLV-1-transformed cells (C8166-45 (*lane 3*), MT4 cells (*lane 4*), and HUT102 (*lane 5*)) were subjected to Western blot using anti-phospho-Ser<sup>473</sup> Akt (*top panel*), anti-Akt (*second panel*), anti-Tax (*third panel*), and anti-actin (*bottom panel*). Differences in the amount of phospho-Ser<sup>473</sup> Akt were noted between the control cells and the HTLV-1 cells. Detection of phospho-Ser<sup>473</sup> Akt can be seen in CEM-SS cells when these cells were cultured under high serum conditions (e.g. 10% FCS; data not shown).

PI3K/Akt pathway to Tax-induced changes in cell growth was investigated.

Nuclear translocation of Akt has been previously described in cells treated with growth factor, and this occurrence is thought to reflect Akt activation (31, 32, 48, 49). Interestingly, when we transfected fresh primary mouse embryonic fibroblasts (MEFs) with GFP-Tax and an HA-Akt plasmid, Akt staining consistent with activation was observed in the nucleus (Fig. 1A). To confirm that this nuclear localization indeed reflected activation, we checked the phosphorylation profile of Akt (using anti-phospho-

Ser<sup>473</sup> antibody) in Tax-transfected primary MEFs (Fig. 1B) and in three Tax-expressing HTLV-1 transformed T-cell lines (C8166-45, MT4, and HUT102) (Fig. 1C). In both settings, when compared with controls (Fig. 1, B, lanes 1 and 2, and C, lanes 1 and 2), phosphorylation of Akt at Ser<sup>473</sup>, as a measure of activation, was greatly enhanced in the Tax-expressing cells (Fig. 1, B, lanes 3 and 4, and C, lanes 3–5).

*Activation of Akt by Tax Is Growth Factor-independent but PI3K-dependent*—Constitutive activation of Akt/PKB is frequent in highly invasive cancers (31, 52, 53). Some investigators have suggested that growth



**FIGURE 2. Tax activates Akt in a PDGF-independent manner to phosphorylate GSK3.** *A*, Akt-phosphorylated GSK3 peptide *in vitro* in a PDGF-independent manner. MEF cells in low serum medium (1% FCS) were transfected with either pCDNA 3.0 (lane 1) or HA-Akt (lanes 2, 3, 5, and 6) and/or Tax-expressing vector (lanes 4–6) for 48 h. Where indicated, PDGF (20 ng/ml) was added for 6 h to the medium. HA-tagged Akt protein was immunoprecipitated with anti-HA-agarose beads and probed with anti-HA. In addition to HA-Akt, a background signal due to the IgG heavy chain was detected (\*IgG). The presence of recombinant GST-GSK3 was assessed by Coomassie Blue staining (CB; Kinase Assay panels). PKB/Akt activity was measured by *in vitro* kinase assay (see P32 bands in the top panel). Amounts of HA-Akt and Tax in cell extracts were detected using anti-HA and anti-Tax (Lysate panels). *IB*, immunoblot. *B*, Akt activation in Tax-expressing T-cells is PI3K-dependent. CEM-SS (lane 1), C8166–45 (C81) (lanes 2, 4, and 6), and MT4 cells (lanes 3, 5, and 7) were cultivated in RPMI1640, 1% FCS for 48 h and treated with PI3K inhibitors (LY294002 and wortmannin) for 12 h before analysis. Activated Akt was detected using anti-phospho-Ser<sup>473</sup> Akt antibody. Amounts of total Akt in the cell lysates were detected using anti-Akt (middle panel). Equal amounts of protein in the cell extracts were verified using anti-actin (lower panel). *C*, ELISA for PDGF in cell culture supernatants. Jurkat and C81 cells were either unstimulated (lanes 2 and 4) or stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) (lanes 1 and 3). Aliquots of the cell culture supernatant were removed and assayed for levels of PDGF using an ELISA. Experiments were conducted in triplicate two times. Mean  $\pm$  S.E. values are shown. A scan from a representative ELISA is also shown in the inset. ND, not detectable. The detection limit for PDGF-BB was 10 pg/ml.

factor (*i.e.* PDGF)-independent activation of Akt plays an important role in the resistance of cancers to chemotherapy (31, 37, 52, 53). Adult T-cell leukemia (ATL) is a particularly aggressive form of cancer that resists chemotherapy (54). We thus wondered if Akt activity in the setting of the HTLV-1 Tax oncoprotein is growth factor-independent.

We transfected primary MEFs cultured in low serum medium with combinations of a HA-Akt plasmid with/without a Tax plasmid and with/without PDGF treatment. 48 h after transfection of cells, HA-tagged Akt was immunoprecipitated with anti-HA, and Akt activity was measured by *in vitro* kinase assays (Fig. 2A). Under such conditions of relative serum deprivation, Akt immunoprecipitated from primary MEF was inactive for *in vitro* phosphorylation of a glycogen synthase 3-kinase, GS3K, peptide (Fig. 2A, lane 2).

PDGF is a survival factor that inhibits apoptosis and promotes cell proliferation through activation of the PI3K/Akt pathway (31, 55). When we added PDGF (20 ng/ml) to our cells cultured in low serum medium, this addition was sufficient to activate Akt as measured by

phosphorylation of GS3K (Fig. 2A, lane 3). Interestingly, when HA-Akt was immunoprecipitated from Tax-transfected MEFs cultured in low serum medium, this moiety capably phosphorylated GS3K peptide *in vitro* (Fig. 2A, lane 5) without needing PDGF treatment. These results are consistent with the chemotherapy-resistant profile of ATL cells and suggest that Tax activates Akt in a growth factor-independent manner.

The above transfection findings indicate that Akt might be constitutively active in ATL cells regardless of growth factor stimulation. To check this prediction, we cultured HTLV-1-transformed T-cell lines, C8166–45 (C81) and MT4, in reduced serum (1% FCS) for 24 h and then assessed their Akt status by Western blotting using anti-phospho-Ser<sup>473</sup>. Both C81 and MT4 cells maintained high levels of ambiently phosphorylated Akt (Fig. 2B, lanes 2 and 3), whereas control CEM-SS T-cell line, negative for Tax expression, showed no detectable phospho-Akt (Fig. 2B, lane 1). We next asked whether this Akt-phosphorylation in Tax-expressing cells is PI3K signaling-dependent. PI3Ks are heterodimeric lipid kinases composed of regulatory and catalytic subunits

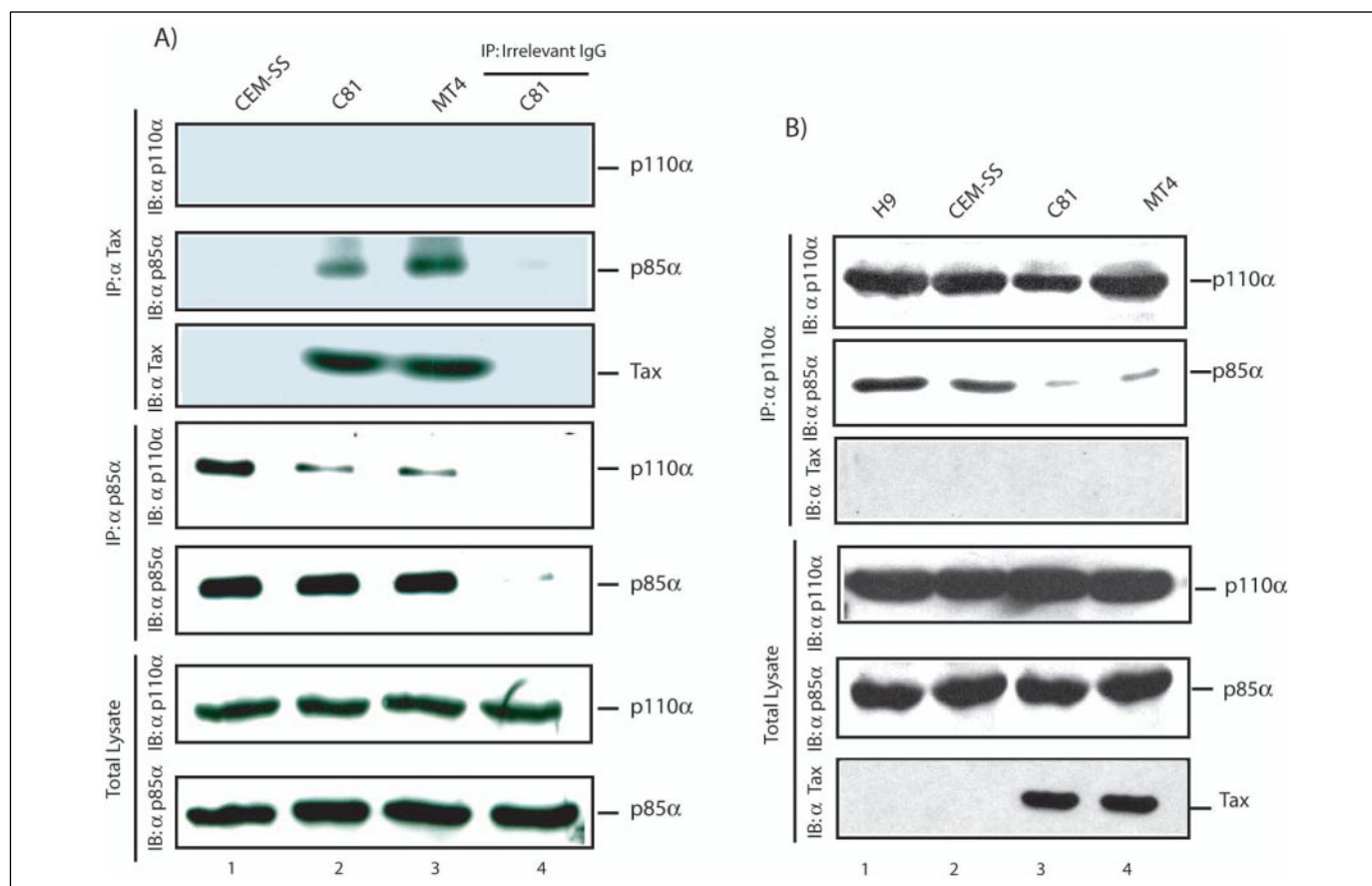


FIGURE 3. Tax interacts with PI3K in T-cells. A, Tax and cell endogenous p85 $\alpha$  were immunoprecipitated (IP) from CEM-SS (lane 1), C81 (lane 2), and MT4 cells (lane 3) using rabbit anti-Tax and rabbit anti-p85 $\alpha$  antibodies. Isotypic control using an irrelevant rabbit IgG was also performed using C81 lysate (lane 4). Immunoprecipitated PI3K subunits were detected using anti-p110 $\alpha$  or anti-p85 $\alpha$  as indicated. Equal amounts of p110 $\alpha$  and p85 $\alpha$  in the cell extracts were verified using anti-p110 $\alpha$  and anti-p85 $\alpha$  (Total Lysate panels). B, immunoblot. Cell endogenous p110 $\alpha$  was immunoprecipitated from H9 (lane 1), CEM-SS (lane 2), C81 (lane 3), and MT4 cells (lane 4) using rabbit anti-p110 $\alpha$ . The presence of p85 $\alpha$  subunit and Tax in the immunoprecipitates (IP, *ap110 $\alpha$*  panels) was queried by immunoblotting (IB) using anti-p85 $\alpha$  or anti-Tax antibodies. The presence of p110 $\alpha$ , p85 $\alpha$ , and Tax in the cell extracts was verified with anti-p110 $\alpha$ , anti-p85 $\alpha$ , and anti-Tax as indicated (Total Lysate panels).

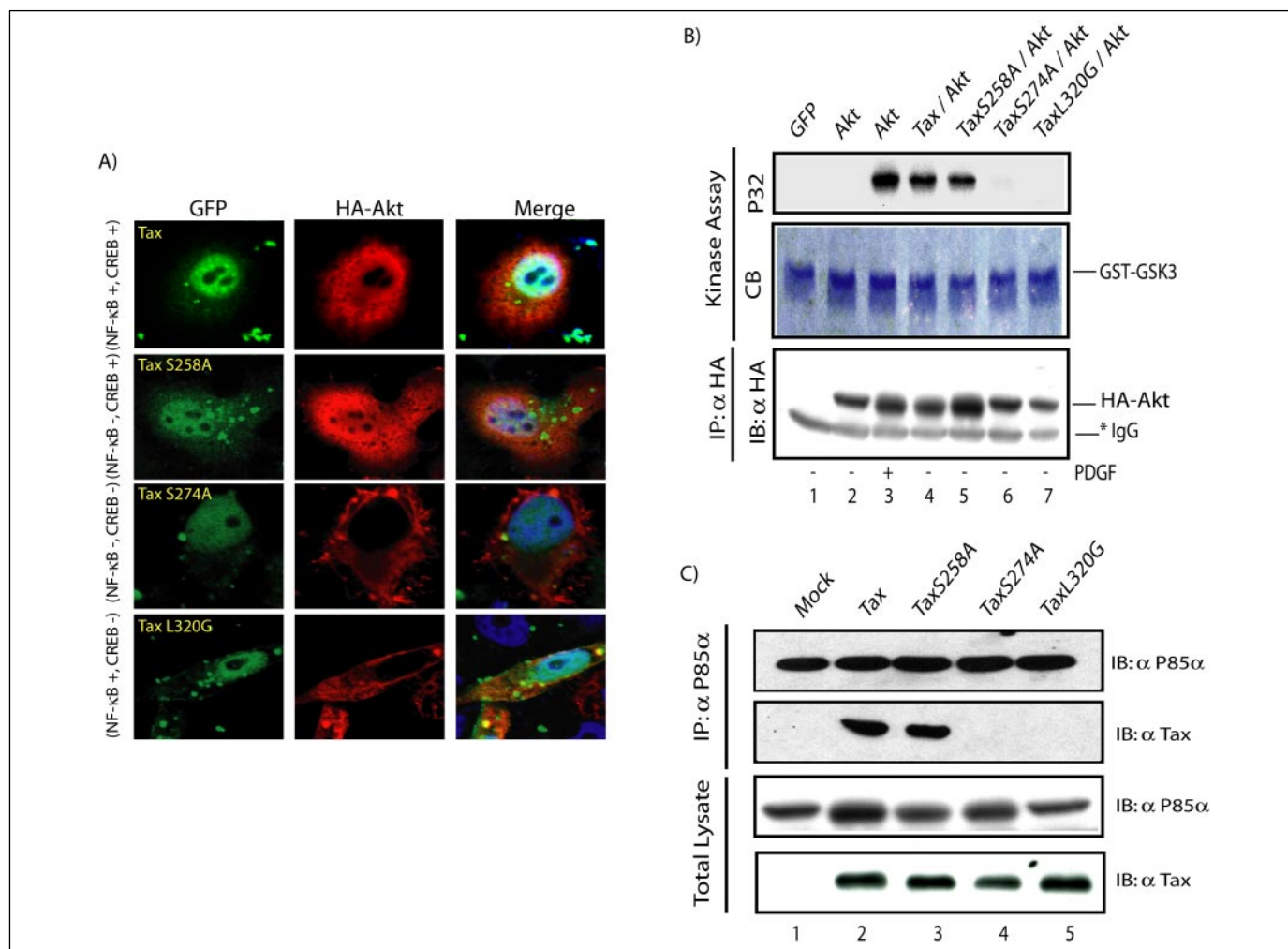
that are encoded by different genes. The regulatory subunits of PI3Ks are encoded by one of the three genes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), which are also subject to alternative splicing. The p110 catalytic subunit is also encoded by three genes ( $\alpha$ ,  $\beta$ , and  $\delta$ ). Class IA PI3Ks (p110 $\alpha$ -p85 $\alpha$ ) are activated by receptor tyrosine kinases, and deregulation of their function has been implicated in several human cancers (15, 53).

To address this question, C81 and MT4 cells were treated for 12 h with two different PI3K inhibitors, either LY294002 (a reversible inhibitor of the PI3K ATP binding site) or wortmannin (an irreversible inhibitor that binds to the p110 $\alpha$  catalytic subunit of PI3K) (32). Treatment of cells with either of the two PI3K inhibitors effectively abrogated Akt phosphorylation in the HTLV-1-transformed cells (Fig. 2B, lanes 4–7), supporting a PI3K-dependent Tax activity.

We next checked whether the observed Akt activation in Tax-expressing cells could be explained by a viral oncoprotein-induced increase in autocrine secretion of PDGF (56, 57). To address this possibility, the levels of PDGF in the supernatant of unstimulated Jurkat and HTLV-1-transformed T-cell line, C81 (Fig. 2C), were assessed by ELISA. No difference in PDGF levels was detected between the supernatants, suggesting that Tax does not significantly induce PDGF secretion from HTLV-1-transformed cells. That the ELISA assay was working correctly was verified by its ability to measure robust PDGF secretion after Jurkat and C81 cells were treated with phorbol 12-myristate 13-acetate (Fig. 2C).

*Tax Interacts with the p85 $\alpha$  Subunit of PI3K*—Activation of Akt in cells is in part regulated by a series of “on” signals generated by increased inositol 1,4,5-trisphosphate levels and “off” signals that dictate Akt Ser<sup>473</sup> dephosphorylation (31, 32). Enhanced function of upstream regulators of Akt has been described for many tumors (31, 32, 48). The finding that Tax activation of Akt is growth factor-independent but PI3K-dependent suggests a novel mechanism of action by this HTLV-1 oncoprotein through PI3K. PI3K activity is tightly regulated in normal cells by various mechanisms. The current view is that a preformed p110 $\alpha$ -p85 $\alpha$  complex is present in the cytoplasm of resting cells. By binding to the p110 $\alpha$  catalytic subunit, the p85 $\alpha$  regulatory subunit not only stabilizes p110 $\alpha$  but also inhibits its lipid kinase activity (31, 58). The inactive cytoplasmic p110 $\alpha$ -p85 $\alpha$  dimer is poised for activation in response to appropriate stimuli. For receptor tyrosine kinases, this cue comes from ligand-mediated activation of the kinase activity and recruitment of the p110 $\alpha$ -p85 $\alpha$  complex to the receptor by interaction of the Src homology 2 domain(s) of p85 $\alpha$  with phosphotyrosine moieties on the receptors (59). The receptor tyrosine kinase-p85 $\alpha$  interaction frees the inhibitory effect of p85 $\alpha$  from the p110 $\alpha$  kinase activity (58). Based on this model, we wondered whether the observed Tax activation of Akt might result from Tax binding to PI3K separating p85 $\alpha$  from p110 $\alpha$ .

Next, Tax binding to PI3K was tested in C81 and MT4 cells (Fig. 3). We immunoprecipitated Tax from cell lysates (*top panels*) and sub-



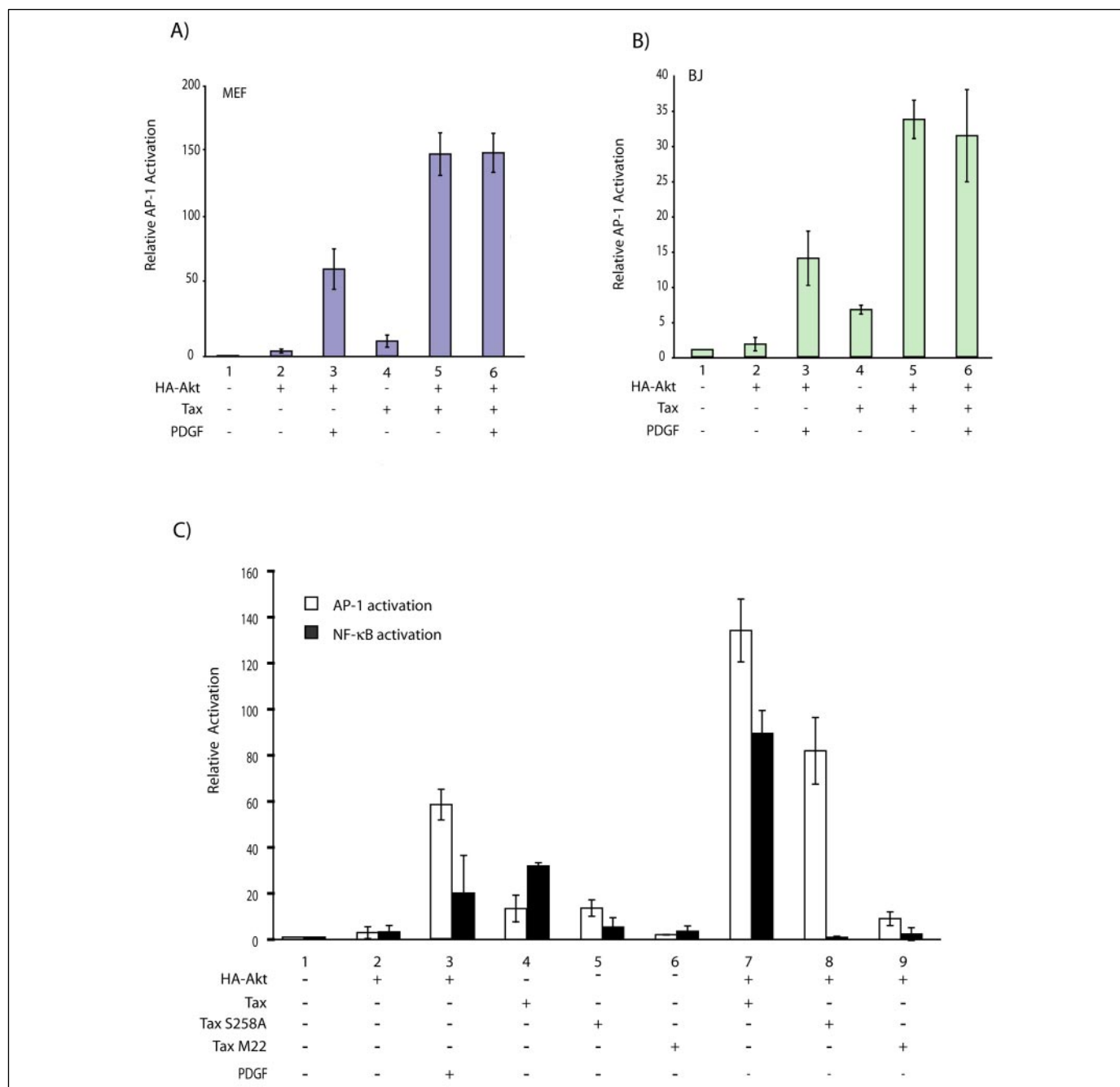
**FIGURE 4. Characterization of Tax mutants for the ability to activate Akt.** A, MEF cells in 1% FCS were transfected with HA-Akt and different GFP-Tax plasmids as indicated. Anti-HA was used to reveal the subcellular localization of HA-Akt. DNA was counterstained with Hoechst 33342. Both Tax and Tax S258A, an NF- $\kappa$ B activation-deficient mutant, induced the nuclear relocalization of Akt (two top panels), whereas neither Tax S274A (a mutant deficient in both CREB and NF- $\kappa$ B activation) nor Tax L320G, a CREB activation-deficient mutant, promoted the nuclear localization of Akt (lower two panels). B, Akt activity was measured using an *in vitro* kinase assay (see "Materials and Methods"). MEF cells were transfected with either a GFP control plasmid (lane 1) or an HA-Akt plasmid (lanes 2–7) without (lanes 2 and 3) or with different GFP-Tax plasmids (0.5  $\mu$ g; as indicated for lanes 4–7) for 48 h in low serum medium. Where indicated, PDGF (20 ng/ml) was added for 6 h to the culture medium. HA-tagged Akt protein was immunoprecipitated with anti-HA (IP: $\alpha$ HA). Amounts of HA-Akt in the immunoprecipitate were detected by immunoblotting with anti-HA (IB: $\alpha$ HA). Background IgG heavy chain signal was also detected (lower panel, \*IgG). Immunoprecipitates were used to phosphorylate *in vitro* GST-GSK3 peptide. Phospho-peptides are shown in the top panel (P32). Amounts of recombinant GST-GSK3 used in the kinase assays were verified by Coomassie Blue staining (CB). C, MEF cells in low serum medium were transfected with different Tax plasmids (2  $\mu$ g) (as indicated for lanes 2–5) for 48 h. Cell endogenous p85 $\alpha$  was immunoprecipitated using rabbit anti-p85 $\alpha$  antibodies. The presence of p85 $\alpha$  subunit and Tax in the immunoprecipitates (IP) was queried by immunoblotting (IB) using anti-p85 $\alpha$  and anti-Tax antibodies. Presence of p85 $\alpha$  and Tax in the cell extract was verified with anti-p85 $\alpha$  and anti-Tax (Total Lysate panels).

jected to Western blot the precipitates searching for Tax-associated PI3K p85 $\alpha$  or p110 $\alpha$  subunit. Tax was found to bind p85 $\alpha$  but not p110 $\alpha$  (Fig. 3A, lanes 2 and 3). Interestingly, when we queried for the status of PI3K p110 $\alpha$ /p85 $\alpha$  dimerization by immunoprecipitating either its p85 $\alpha$  (Fig. 3A) or its p110 $\alpha$  (Fig. 3B) subunit, we observed in Tax-expressing T-cell lines (C81 and MT4), compared with control CEM-SS cells, a reduced association of p110 $\alpha$  with p85 $\alpha$  (Fig. 3, A, lanes 2 and 3, and B, lanes 3 and 4), despite total levels of p110 $\alpha$  and p85 $\alpha$  in all cells being similar (Fig. 3, A and B, Total Lysate). One interpretation of these results is that Tax binding to p85 $\alpha$  disrupts the p110 $\alpha$ -p85 $\alpha$  complex. Tax disruption of p110 $\alpha$ -p85 $\alpha$  association agrees with recent studies showing that the stable association of regulatory p85 $\alpha$  and catalytic p110 $\alpha$  subunits is critical for normal regulation (60, 61). Thus, binding of PI3K by oncogenic v-Ras was also previously found to free and activate the p110 $\alpha$  subunit from the p110 $\alpha$ -p85 $\alpha$  complex (62, 63).

**Tax-Akt Activation Leads to Downstream AP-1 Activity**—Akt stimulates several downstream signaling pathways (NF- $\kappa$ B, CREB, and

AP-1) whose dysregulation can significantly impact cancer progression. We are interested in understanding which Akt/PKB downstream pathway is perturbed in HTLV-1-infected cells. In the literature, expression of Tax has been documented to activate primarily two major pathways, CREB and NF- $\kappa$ B (17). Several Tax mutants have been constructed that specifically activate CREB but are silent for NF- $\kappa$ B or *vice versa* (64). To understand if Tax activation of Akt segregates with either its CREB or NF- $\kappa$ B function, the ability by three GFP-tagged Tax point mutants with defined CREB and NF- $\kappa$ B phenotypes to activate Akt was examined. GFP-Tax S258A is NF- $\kappa$ B<sup>-</sup>/CREB<sup>+</sup>; GFP-Tax S274A is NF- $\kappa$ B<sup>-</sup>/CREB<sup>-</sup>; and GFP-Tax L320G is NF- $\kappa$ B<sup>+</sup>/CREB<sup>-</sup> (46) (Fig. 4A).

MEFs were transfected with HA-Akt plasmid plus either GFP-Tax or GFP-Tax mutants, and 48 h after transfection cells were fixed and stained for Akt (anti-HA) and DNA (Fig. 4A). Among the cells transfected with Tax mutants, GFP-Tax S258A promoted the nuclear migration of Akt, whereas GFP-Tax S274A and GFP-Tax L320G did not (Fig.

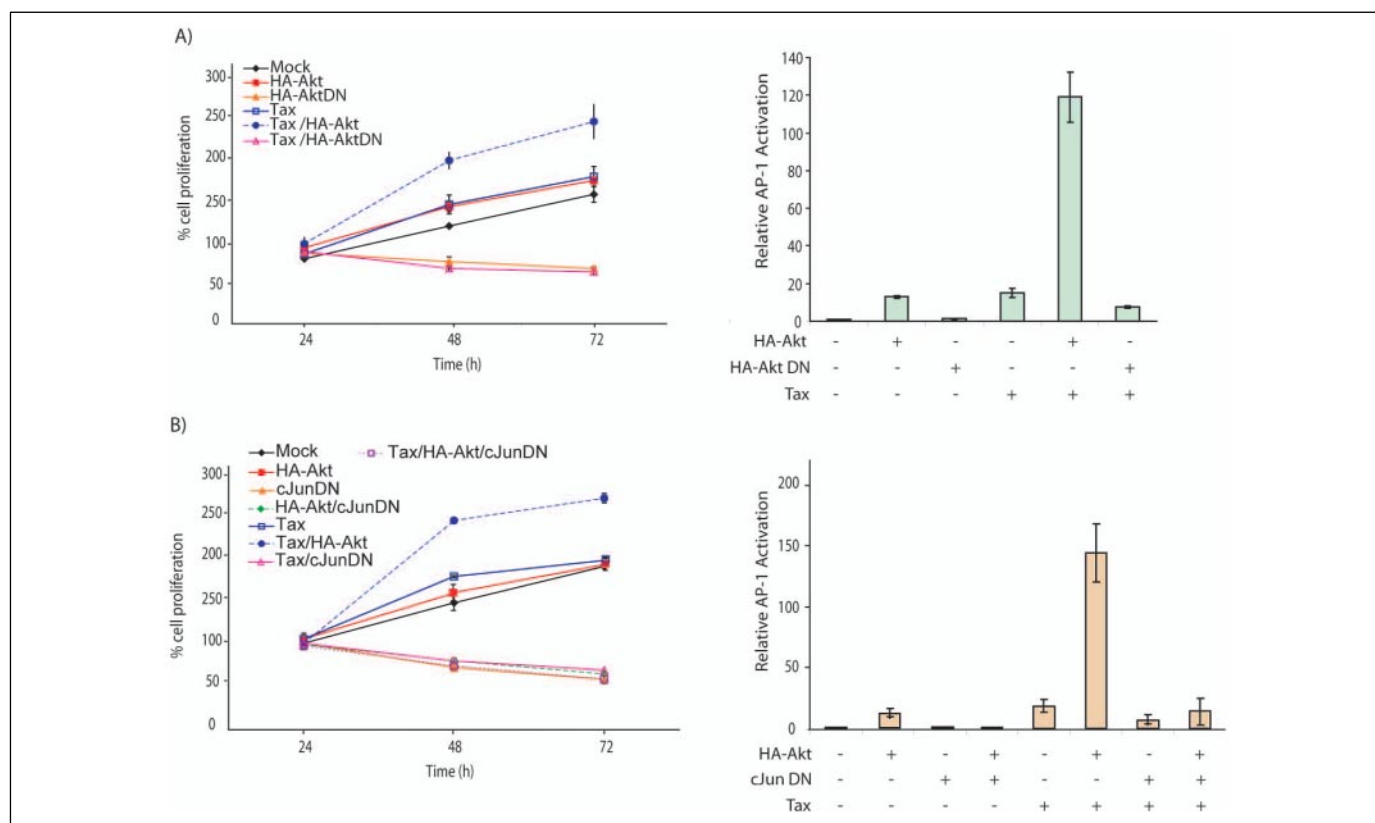


**FIGURE 5. Akt activates AP-1 in the presence of Tax in a PDGF-independent manner.** MEFs (A) and primary human fibroblasts (BJ) cells (B) were co-transfected with AP-1-Luc plasmid (activator protein-1-dependent luciferase reporter) and pCDNA 3.0 (lane 1), or HA-Akt (1  $\mu$ g) (lanes 2–6) and a Tax-expressing vector (1  $\mu$ g) (lanes 4–6). 3 h after transfection, cell transfection medium was replaced with Dulbecco's modified Eagle's medium, 1% FCS. Where indicated, PDGF (20 ng/ml) was added to the medium for 6 h. The same amounts of cellular extracts were subjected to luciferase assays (see "Materials and Methods"). C, MEFs were transfected with pCDNA 3.0 and either AP-1-Luc or NF- $\kappa$ B-Luc plasmids. Co-transfections with HA-Akt (1  $\mu$ g), Tax (1  $\mu$ g), Tax S258A (1  $\mu$ g), or Tax M22 (1  $\mu$ g) are indicated. 3 h after transfection, cell media were replaced with Dulbecco's modified Eagle's medium, 1% FCS. Where indicated, PDGF (20 ng/ml) was added to the medium for 6 h. The same amounts of cellular extracts were subjected to a luciferase assay. The bars represent three independent experiments with S.D. values.

4A). Similarly, when GSK3 phosphorylation was assayed as a measure of Akt activation, Tax and Tax S258A were found to promote phosphorylation (Fig. 4B, lanes 4 and 5), whereas Tax S274A and Tax L320G (Fig. 4B, lanes 6 and 7) did not. At first glance, the ability of Tax to signal through CREB (see GFP-Tax and GFP-Tax S258A), but not NF- $\kappa$ B, seemed required to sustain Akt activation. However, when we used another Tax mutant, Tax M22 (NF- $\kappa$ B<sup>-</sup>/CREB<sup>+</sup>) (47, 65), this mutant, although capable of CREB signaling, was unable to mediate Akt activation (data not shown). Elsewhere, Tax S258A (46), but not Tax M22

(65), was shown to be capable of immortalizing primary T-lymphocytes. Taken together, these results correlate the ability of Tax to activate Akt with immortalization of T-cells by HTLV-1.

The Tax mutants provided opportunities to further investigate the link between activation of Akt and the ability of Tax to bind p85 $\alpha$ . To address this, cell endogenous p85 $\alpha$  was immunoprecipitated from Tax- or Tax mutant-transfected MEFs, and Western blotting was performed to query for association with p85 $\alpha$  by Tax and Tax mutants. Indeed, Tax and Tax S258A, but not Tax S274A nor Tax L320G, bound p85 $\alpha$  (Fig.



**FIGURE 6. AP-1 activation through the Akt/PKB pathway is important for proliferation of Tax-transfected cells.** *A*, overexpression of Akt dominant negative (DN) mutant inhibited cell growth. CV-1 cells were transfected with AP-1-Luc plasmid and either HA-Akt, HA-Akt DN, or Tax plasmid. Cells were monitored in parallel for proliferation (*left*) using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) coloration (Dojindo), according to the manufacturer's protocol, and for AP-1 activation by luciferase reporter assay (*right*). *B*, overexpression of c-Jun DN inhibited cell growth in Tax-expressing cells. CV-1 cells were transfected with AP-1-Luc plasmid and either HA-Akt, c-Jun DN, or Tax plasmid. Cells were assayed in parallel for proliferation (*left*) using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt) coloration, according to the manufacturer's protocol, or for AP-1 activation, based on the luciferase reporter assay (*right*).

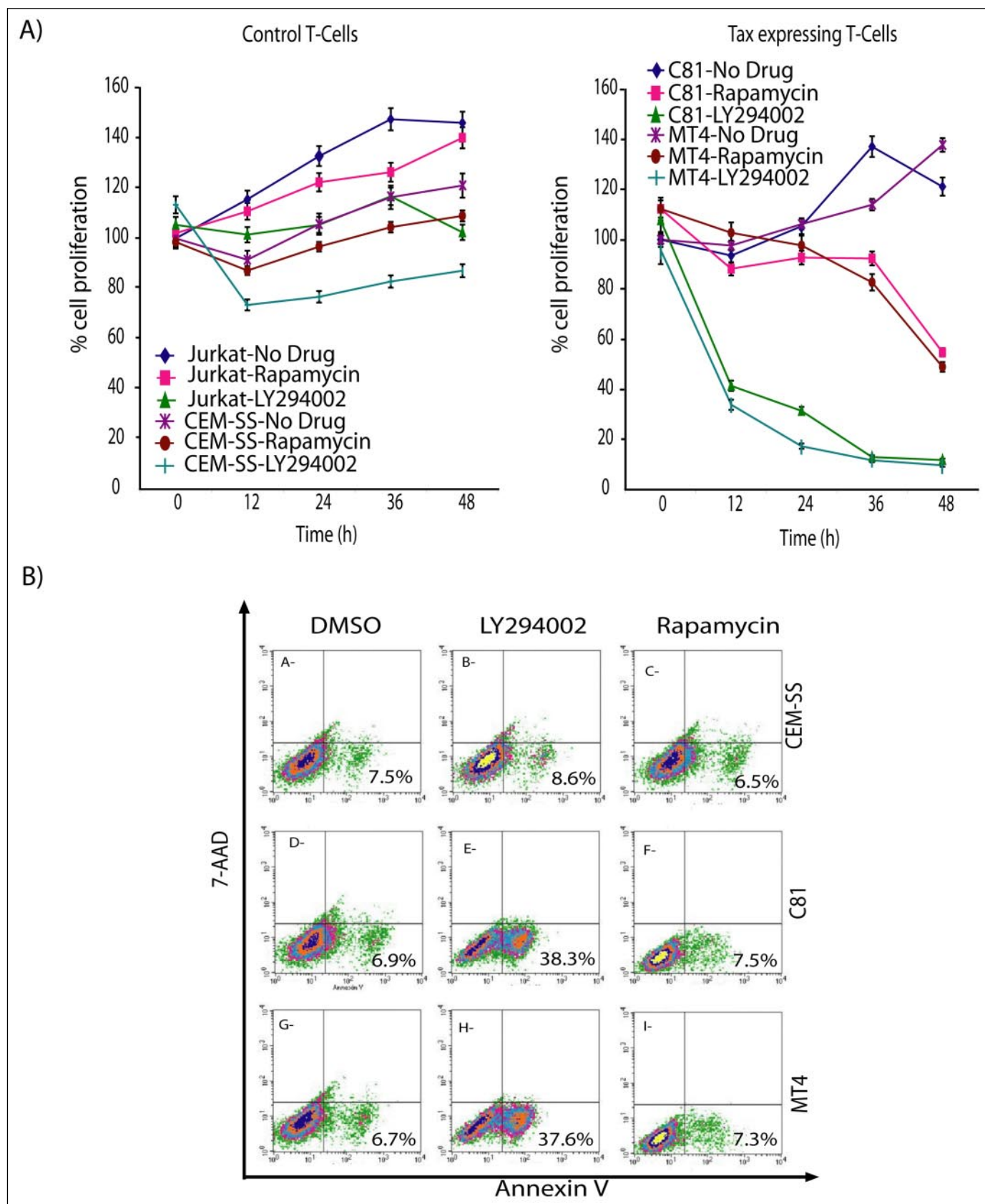
4C, lanes 2 and 3). These results are consistent with the binding of Tax to p85 $\alpha$  and its ability to activate Akt.

Because neither CREB nor NF- $\kappa$ B signaling seemed sufficient to explain the consequence of Tax-mediated Akt activation and because Akt has been shown to activate AP-1 (38, 66, 67), we investigated whether Tax-mediated Akt activation might result in heightened AP-1 activity. We transfected AP-1-Luc reporter plasmid plus HA-Akt into either MEFs (Fig. 5A) or primary human fibroblasts (BJ; Fig. 5B) with (Fig. 5, A, lanes 4–6, and B, lanes 4–6) or without (Fig. 5, A, lanes 1–3, and B, lanes 1–3) Tax. In both MEF and BJ cells, co-expression of Tax and Akt significantly (>40-fold) enhanced AP-1 activity. The abilities of Tax S258A and Tax M22 (both NF- $\kappa$ B<sup>-</sup>/CREB<sup>+</sup>) to signal through AP-1 were also examined. We transfected MEFs, either with an AP-1-Luc or a NF- $\kappa$ B-Luc reporter plus HA-Akt (Fig. 5C, lanes 2, 3, and 7–9) with either Tax (Fig. 5C, lanes 4 and 7), Tax S258A (Fig. 5C, lanes 5 and 8), or Tax M22 (Fig. 5C, lanes 6 and 9). Both Tax and Tax S258A (Fig. 5C, lanes 7 and 8), but not Tax M22 (Fig. 5C, lane 9), significantly activated AP-1 when co-expressed with Akt. Because Tax and Tax S258A, but not Tax M22, have been previously shown to possess cell immortalization function, the current results are consistent with a linkage between Tax-Akt-AP-1 activity and immortalization.

**Akt Regulates Cell Growth and Cell Survival in Tax-expressing Cells**—The above findings prompted the notion that Tax-Akt signaling may have consequences for cellular proliferation. To address this question, we used CV-1, an African Green monkey kidney cell line that proliferates indefinitely in a contact-inhibited manner in tissue culture. In our hands, CV-1 cells divide very slowly when cultured in 1% FCS.

Into CV-1 cells cultured in low serum, we transfected either a HA-tagged Akt (HA-Akt) or a HA-tagged Akt dominant negative mutant (HA-Akt DN) with or without co-transfected Tax (Fig. 6A, left) and compared resulting cellular proliferation. When maintained in low serum, CV-1 cells proliferated very slowly in the absence of exogenously transfected Tax or Akt (Fig. 6A, mock). When Akt alone, Tax alone, or Akt plus Tax were transfected, the cells all grew significantly better (Fig. 6A, left). Moreover, suppression of cell endogenous Akt activity by exogenous transfection of a dominant negative Akt mutant (Akt DN) abolished proliferation of Tax-transfected cells (Fig. 6A, Tax/Akt DN). To confirm that the Akt DN mutant was functionally active, CV-1 cells transfected in parallel with a reporter plasmid was assayed for AP-1-driven luciferase activity. These transfections verified that the HA-Akt DN plasmid did dominantly repress Tax activation of AP-1 (Fig. 6A, right).

The proliferative effect of Akt in Tax-expressing cells could occur through a variety of downstream signaling molecules. It was important to check whether the observed growth effect is specifically mediated through AP-1. Because c-Jun is an important AP-1 component, we employed c-Jun DN (45) (Fig. 6B) to help define the downstream cell proliferative activity of Akt. Transfection of c-Jun DN into CV-1 cells produced suppression of Akt-mediated proliferation (Fig. 6B, left) in a manner very similar to that achieved with Akt DN plasmid (Fig. 6A, left). Control experiments verified that c-Jun DN was indeed functionally active in suppressing Tax plus Akt activation of AP-1 (Fig. 6B, right). Taken together, these results indicate that the primary cell proliferative signal downstream of Akt in Tax-expressing cells is AP-1.



**FIGURE 7. PI3K/Akt inhibitor LY294002 abolished Tax-dependent T-cell growth.** *A*, control CEM-SS and Jurkat (*left*) or Tax-expressing C81 and MT4 (*right*) cells were treated as indicated with either PI3K inhibitor LY294002 (20  $\mu$ M) or mTOR inhibitor rapamycin (100 ng/ml) for 48 h. Cell proliferation was measured every 12 h using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) coloration (Dojindo) according to the manufacturer's protocol. *B*, CEM-SS, C81, and MT4 cells were treated with either Me<sub>2</sub>SO (*DMSO*; *left column*), LY294002 (20  $\mu$ M) (*middle column*), or rapamycin (100 ng/ml) (*right column*) for 8 h. Apoptosis was determined by fluorescence-activated cell sorting analyses of cells after annexin V and 7-AAD staining.

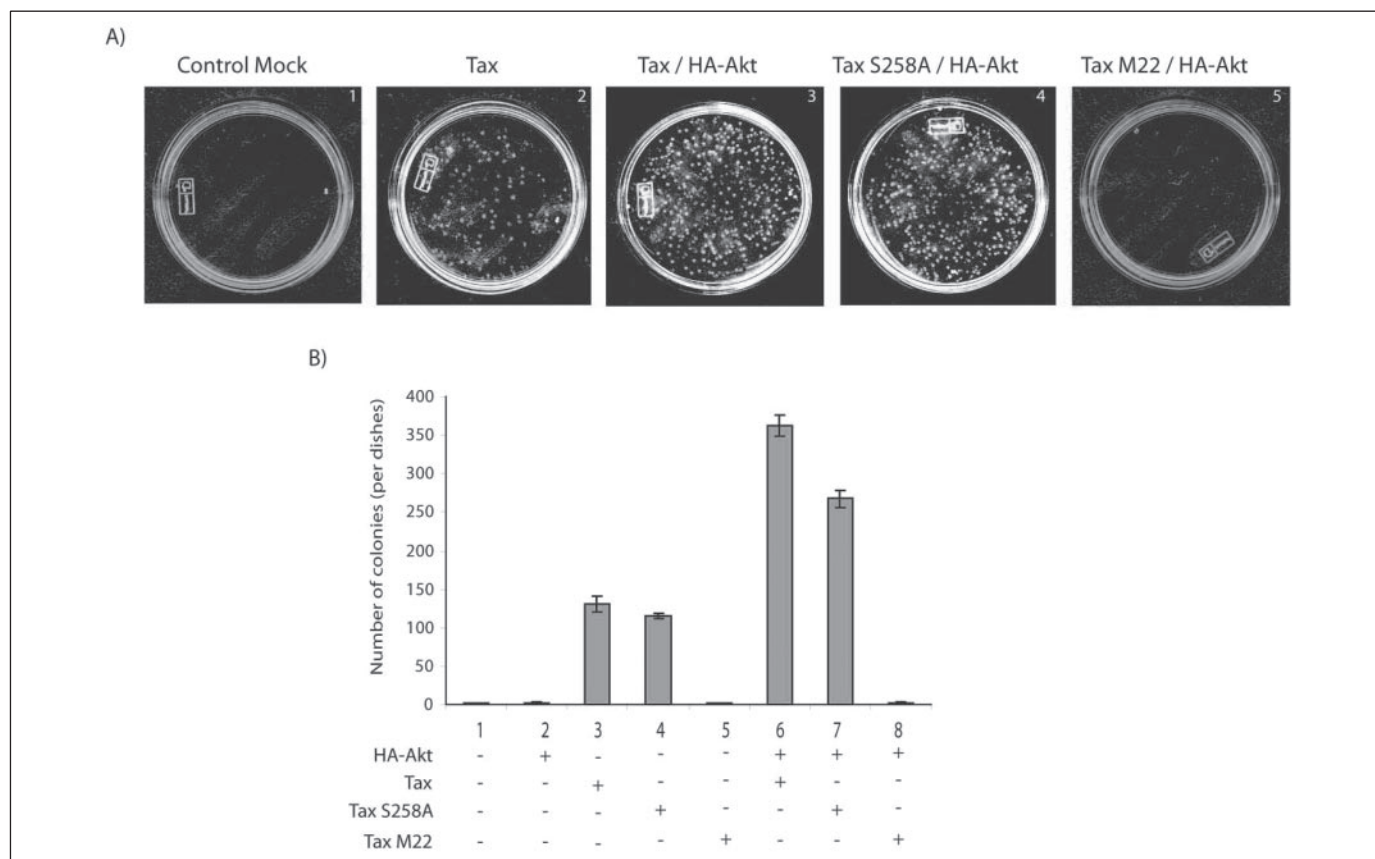


FIGURE 8. **Activation of AP-1 through PI3K/Akt is important for Tax-mediated cell proliferation.** A, Tax-induced foci formation in cells. CV-1 cells were transfected with either HA-Akt alone (0.5  $\mu$ g), Tax alone (0.5  $\mu$ g), or HA-Akt (0.5  $\mu$ g) plus the indicated Tax plasmids (0.5  $\mu$ g). 24 h after transfection, cells were plated in 5-cm dishes and maintained in 1% FCS. After visible colonies were observed (35 days after transfection), the plates were washed and fixed. Plates from representative experiments are shown. NF- $\kappa$ B-deficient Tax mutant S258A, but not M22, was able to induce foci formation in CV-1 cells. B, CV-1 cells were transfected with 0.5  $\mu$ g of HA-Akt alone or in combination with the indicated Tax plasmids (0.5  $\mu$ g). Cells were maintained in 1% FCS and fixed 35 days after transfection. Foci formation was quantified by counting the number of colonies per dish after 35 days of transfection.

We next checked to see if Akt plays a similarly important proliferative role in HTLV-1-transformed T-cells. Control T-cells (Jurkat and CEM-SS) and HTLV-1-transformed T-cells for 48 h were treated with either an inhibitor that acts upstream of Akt, LY294002 (a PI3K inhibitor), or one that acts downstream in the PI3K/Akt pathway, rapamycin (an mTOR inhibitor). mTOR (mammalian target of Rapamycin) is an atypical serine/threonine kinase, which controls cell growth by regulating translation, transcription, and ribosome biogenesis (68). The proliferation of inhibitor-treated cells was measured (Fig. 7A). Compared with control  $\text{Me}_2\text{SO}$  treatment (*No Drug*) or to inhibitor-treated control T-cells, proliferation of HTLV-1-transformed cells decreased more significantly when treated with LY294002 than with rapamycin (Fig. 7A, right).

Independently, we also assessed inhibitor-treated cells for viability. Annexin V staining of control T-cells (CEM-SS) and HTLV-1 cells treated with  $\text{Me}_2\text{SO}$ , LY294002, or rapamycin was assessed. Consistent with the proliferation results (Fig. 7A), drug-treated C81 and MT4 cells were significantly increased in Annexin V-positive cells. For example, whereas 8.6% of LY294002-treated CEM-SS cells showed early apoptosis (Annexin V-positive/7-AAD-negative), the same treatment provoked 38.3 and 37.6% of C81 and MT4 cells, respectively, into incipient apoptosis (Fig. 7B). This finding indicates that Akt function may be selectively important for the survival of these HTLV-1-transformed T-cells as compared with control T-cells.

*PI3K/Akt Activation of AP-1 Is Important for Tax Induction of Focus Formation*—In a previous report, Liu *et al.* (51) demonstrated the ability of Tax to transform rodent fibroblasts through the PI3K/Akt pathway.

However, in that study, the PI3K/Akt downstream signal, which mediated transformation, was not clarified. Above, the Tax M22 mutant deficient for NF- $\kappa$ B signaling (65) activated neither Akt nor AP-1 (Fig. 5C), whereas the Tax S258A mutant, which is also deficient for NF- $\kappa$ B signaling (46), maintained both Akt and AP-1 activation (Figs. 4 and 5C). These two mutants thus provided us with tools to ask whether Akt/AP-1 activation by Tax in the absence of NF- $\kappa$ B signaling is linked to cellular proliferation. To answer this question, CV-1 cells were transfected with either HA-Akt alone or HA-Akt in combination with various Tax plasmids. CV-1 cells are contact-inhibited in reduced serum (1% FCS), and we asked if contact-uninhibited focus formation would be observed in transfected cells (Fig. 8A). Both Tax and Tax S258A, but not Tax M22, induced foci formation with or without cotransfection with Akt (Fig. 8B). Although Akt did not by itself induce foci in CV-1 cells, its co-expression with Tax WT or Tax S258A increased the number of foci otherwise seen with Tax WT alone or Tax S258A alone. Additionally, Akt co-expression accelerated foci initiation. Thus, whereas foci induced by Tax were visible 32–35 days after transfection, Tax/Akt foci were noted by 21 days after transfection. An interpretation of the Tax S258A results suggests a role for AP-1, independent of NF- $\kappa$ B activation, in HTLV-1 induction of cellular proliferation and transformation.

## DISCUSSION

The PI3K/Akt signaling pathway is a key regulator of numerous physiological cellular processes, which include proliferation, metabolism, and apoptosis (28, 31, 69–74). Disruption of PI3K/Akt signaling has

been reported in the malignant progression caused by two well known transforming viruses, hepatitis B virus and human papilloma virus (32, 37, 48, 52). The hepatitis B virus oncoprotein HBx and the human papilloma virus proteins E6 and E7 have been implicated in alterations of the cellular PI3K/Akt pathway (75, 76). Further supporting its importance in cancer, several reports have shown that PI3K/Akt activation contributes to chemotherapeutic resistance of leukemias (15, 53, 77).

In tumor cells, the regulation of Akt is complex. Akt is phosphorylated at Ser<sup>473</sup> in many tumors through a yet unclear mechanism (15, 73). Here, we show that Tax interacts with PI3K and possibly through this interaction heightens the level of inositol 1,4,5-trisphosphate, perhaps leading to the phosphorylation of Ser<sup>473</sup> in Akt. Treatment with PI3K inhibitor strongly inhibited the amount of phosphorylated Akt and caused death of Tax-expressing cells. Because the same treatment had a reduced effect on Tax-nonexpressing cells, this suggests that Akt activation may be important to HTLV-1 transformation.

If Tax activates PI3K/Akt, how does this activity integrate into the general scheme of the effects of Tax on signal transduction? Previously, it was understood that Tax regulates cellular metabolism largely through CREB/ATF and NF- $\kappa$ B (8, 78). Most studies have hypothesized Tax/NF- $\kappa$ B as the major axis for cellular proliferation and transformation (8, 78). What has not been established clearly is the role, if any, played by AP-1 in HTLV-1-induced changes in cellular metabolism. AP-1 is an important transcription factor that governs the expression of many genes (34). Whereas the importance of AP-1 in tumor promotion in other systems is well defined (34, 35, 40), its role in ATL is not understood. We used a Tax mutant that activates PI3K/Akt/AP-1 but not NF- $\kappa$ B to show here that AP-1 activation, in the absence of NF- $\kappa$ B signaling, is important for Tax-mediated cell growth. Thus, we compared the phenotype of two Tax mutants that both activate CREB/ATF but not NF- $\kappa$ B (Tax S258A and Tax M22), with one (Tax S258A), but not the other (Tax M22), being functional for AP-1. Tax S258A has been previously described to be immortalizing for primary T-lymphocytes (46), whereas Tax M22 was reported to be unable to immortalize T-cells (65). Here we show that Tax S258A, but not Tax M22, induced AP-1 activation through the PI3K/Akt pathway (Fig. 5C) and promoted cellular growth and formation of foci (Fig. 8B). Those results indicate that AP-1, in the absence of NF- $\kappa$ B, can contribute a discrete role to the growth-promoting properties of Tax.

Despite advances in therapeutic drugs for treating cancers, ATL prognosis remains poor, with an overall survival after diagnosis averaging only 6 months. Interestingly, drug treatment to disrupt PI3K/Akt signaling has been shown to inhibit the motility of highly invasive breast cancer cells (14, 79). In this regard, our current findings suggest that therapeutic approaches targeting the PI3K/Akt pathway, using inhibitors such as LY294002 (80), merit consideration for treating ATL.

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