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Direct Binding of the N-Terminus of HTLV-1 Tax Oncoprotein to Cyclin-Dependent Kinase 4 Is a Dominant Path To Stimulate the Kinase Activity

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ABSTRACT: The involvement of Tax oncoprotein in the INK4-CDK4/6-Rb pathway has been regarded as a key factor for immortalization and transformation of human T-cell leukemia virus 1 (HTLV-1) infected cells. In both p16 ^{-/-} and ^{+/+} cells, expression of Tax has been correlated with an increase in CDK4 activity, which subsequently increases the phosphorylation of Rb and drives the infected cells into cell cycle progression. In relation to these effects, Tax has been shown to interact with two components of the INK4-CDK4/6-Rb pathway, p16 and cyclin D(s). While Tax competes with CDK4 for p16 binding, thus suppressing p16 inhibition of CDK4, Tax also binds to cyclin D(s) with concomitant increases in both CDK4 activity and the phosphorylation of cyclin D(s). Here we show that both Tax and residues 1–40 of the N-terminus of Tax, Tax40N, bind to and activate CDK4 in vitro. In the presence of INK4 proteins, binding of Tax and Tax40N to CDK4 counteracts against the inhibition of p16 and p18 and acts as the major path to regulate Tax-mediated activation of CDK4. We also report that Tax40N retains the transactivation ability. These results of in vitro studies demonstrate a potentially novel, p16-independent route to regulate CDK4 activity by the Tax oncoprotein in HTLV-1 infected cells.

Our current understanding of cell cycle control in eukaryotes is that a family of conserved serine/threonine protein kinases, the cyclin-dependent kinases (CDKs),¹ together with their negative and positive regulators, play key roles in regulating the proliferative state of a cell by phosphorylating specific substrates at specific points of the cell cycle. Other mechanisms are also involved in this machinery in ways independent of the nuclear division cycle (1, 2). CDK4 and CDK6, together with G1 cyclins (including cyclins D1, D2, D3, and E), act as the driving force for the G1 to S transition (3–5). Upon association with cyclin Ds, CDK4 and CDK6 are activated and phosphorylate the retinoblastoma gene product, pRb, which then triggers the downstream E2Fs-mediated transcription of those genes required for entry into

S phase (6–8). Both INK4 proteins (specific CDK4/6 inhibitors, including p15, p16, p18, and p19) (9–13) and KIP proteins (universal CDK inhibitors, including p21, p27, and p57) (14, 15) inhibit CDK4/6-mediated phosphorylation of pRb, thus contributing to cell cycle control. This signaling pathway has been found to be impaired in hundreds of transformed cell lines and tumors. While most abnormalities in the INK4-CDK4/6-Rb pathway occur at the genetic level, such as amplification of the CDK4 gene or mutation and methylation of the p16 or pRb genes, some oncoproteins are capable of blocking this pathway through protein/protein interactions (16). One of them is the Tax oncoprotein of human T-cell leukemia virus 1 (HTLV-1).

Encoded by HTLV-1 genome DNA exon 2, Tax is a transcription activator crucial for both HTLV-1 viral gene expression and transcription regulation in HTLV-1 infected cells (17–22). On one hand, Tax stimulates transcription of the 21 bp repeat enhancer elements in the long terminal repeat (LTR) of the viral DNA; on the other hand, Tax activates several transcription factors, such as NF- κ B, SRF (serum-response factors), and CREB (cyclic AMP response element binding proteins), which regulate the transcription machinery of infected cells (23–26). Tax is believed to be responsible for adult T-cell leukemia (ATL) and other HTLV-1 related diseases, such as tropical spastic paraparesis (27, 28). It has been reported that Tax binds to p16 in vitro and in vivo and counteracts the CDK4/6 inhibitory activity of p16, resulting in cell cycle progression (16). This finding led to the suggestion that inactivation of p16 through the formation of a Tax/p16 complex might contribute to cellular immortalization and transformation induced by HTLV-1 infection (26). However, in many HTLV-1 infected cells, the p16 gene has been found to be either deleted or expressed

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¹ Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; bp, base pair; CDK, cyclin-dependent kinase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid; GST, glutathione S-transferase; GST-CDK4, recombinant human CDK4 with a GST tag at the N-terminus; GST-p16, recombinant human p16 protein with a GST tag at the N-terminus; GST-p18, recombinant human p18 protein with a GST tag at the N-terminus; HEPES, N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid; HTLV-1, human T-cell leukemia virus 1; INK4, inhibitor of cyclin-dependent kinase 4; IPTG, isopropyl β -D-thiogalactopyranoside; KIP, cyclin-dependent kinase inhibitor protein; LTR, long terminal repeat of HTLV-1 viral DNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; pRb or Rb, human retinoblastoma susceptible gene product; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tax40N, truncated Tax only composed of the first 40 amino acid residues at the N-terminus.

at a very low level (29). Therefore, inactivation of p16 through direct Tax binding may not be the primary mechanism for downregulation of cell cycling in HTLV-1 leukemia. Later a study using p16-null T-cells demonstrated that Tax forms a protein/protein complex with cyclin D3, and expression of Tax protein is correlated with the induction of a novel hyperphosphorylated cyclin D3 protein and an increase in CDK4 kinase activity. This finding further confirmed that expression of Tax protein in HTLV-1 infected cells enhances CDK4 activity and leads to an increase in the phosphorylation of pRb. Nevertheless, the formation of Tax/cyclin D(s) complexes could not be regarded as the major factor contributing to the increase of CDK4 activity since increased phosphorylation of cyclin D3 caused by the physical association of Tax and cyclin D3 could drive the latter into ubiquitin-mediated degradation (30, 31), which presumably leads to a decrease in CDK4 activity, an effect seemingly contrary to the above observations. Therefore, we wondered whether Tax affects CDK4 activity through a dominant alternate path, directly interacting with and activating CDK4.

In this paper we report that, in addition to interacting with p16 and cyclin D2, Tax protein directly binds to CDK4 and increases CDK4 activity. It was further demonstrated that the N-terminus of Tax is responsible for CDK4 binding and activation. We also observed that direct binding of Tax to CDK4 counteracts the CDK4 inhibitory activities of both p16 and p18 proteins. Last, it was noticed that the N-terminus of Tax also functions in transactivation.

MATERIALS AND METHODS

Protein Purification and Expression. The full-length Tax gene from HTLV-1 was cloned into the pET-11d expression vector tagged with six His residues at the C-terminus (Novagene) and expressed in *Escherichia coli* BL21(DE3) as previously described (19, 21). Briefly, *E. coli* BL21(DE3) cells harboring the expression vector were incubated in 2 × YT media at 37 °C until the OD₆₀₀ was around 0.6, and then IPTG was added to the final concentration of 40 μM. After incubation at 37 °C for another 6 h, the cells were harvested by centrifugation and frozen at −80 °C for at least 24 h. For purification, cell pellets were resuspended in PBS buffer (pH 8.0) containing 0.3 M NaCl, 0.25 mM PMSF, 0.5 mM β-mercaptoethanol, and 10 mM imidazole and sonicated. After centrifugation, the supernatant was loaded on a Ni-NTA-agarose column (Qiagen) preequilibrated with the above PBS buffer, and then the column was washed with 5 column volumes of the above PBS buffer containing 40 mM imidazole. Tax protein was eluted with a gradient of 80–300 mM imidazole in the above PBS buffer, and after SDS-PAGE, those fractions containing Tax protein were pooled and dialyzed against 20 mM HEPES, 150 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 20% (v/v) glycerol (pH 7.9).

A fragment of Tax gene encoding residues 41–353 (hereafter Tax40C) was amplified by PCR using the primers 5'-CCATGCTTATCCATGGCCATCGTCACGCCCTACTGG-3' (forward) and 5'-CGTAGCCTCGAGTCAGTGATGATGATGATGG-3' (reverse). The resultant PCR product was cloned into pET21d at the *Nco*I and *Xho*I restriction sites to form an expression vector, pET-21d-

Tax40C (this construct includes a C-terminal His₆ tag). Tax40C was expressed and purified as described for the full-length Tax protein.

A fragment of Tax gene encoding the first 40 amino acid residues at the N-terminus (hereafter, Tax40N) was amplified from full-length Tax (cloned into the above pET-11d vector) by PCR using the primers 5'-GGGATCCATGGCCCACT-TCCCAGGGTTTGGACAGAGTC-3' (forward) and 5'-CTAATTCTCGAGTAGGCGGCCGAACATAGTCCCC-CAGAGATG-3' (reverse). Subsequently, this fragment was cloned into the pETGB1 vector at the *Bam*HI and *Xho*I restriction sites (32). The resultant recombinant plasmid, pETGB1-Tax40N, was transformed into *E. coli* BL21(DE3) CodonPlus (Stratagene) cells and overexpressed as a fusion protein, where the GB1 domain enhances protein solubility and the His₆ tag facilitates protein purification. The fusion protein GB1-Tax40N was purified using a Ni-NTA-agarose column as the full-length Tax and further purified on a S-100 column (Pharmacia) using the above HEPES buffer without glycerol.

A Tax40N mutant, C23S, was constructed by the Quick-Change method (Stratagene) using pETGB1-Tax40N as the template. The mutant protein was expressed and purified as Tax40N. Note that all Tax protein and its fragments used in this work contain a His tag at the C-terminus, but only Tax40N and Tax40N C23S contain GB1 fusion protein at the N-terminus (but not in the transactivation assay).

Human CDK4 was cloned into a pGEX-2T vector and expressed in *E. coli* BL21(DE3) CodonPlus cells as a GST fusion protein (33). Bacteria harboring pGEX-2T-CDK4 were incubated in LB media at 27 °C until OD₆₀₀ reached 0.6. After IPTG induction at the final concentration of 0.1 mM, bacteria were further incubated at 27 °C for 12 h. For purification, the cell lysate was loaded on a reduced glutathione-agarose column (Sigma) preequilibrated with the above PBS buffer. After being washed with PBS buffer, bound proteins were eluted with PBS buffer containing 20 mg/mL freshly prepared reduced glutathione. After addition of ATP and MgSO₄ to final concentrations of 100 and 10 mM, respectively, the eluent was incubated at 25 °C for 24 h (34). Subsequently, the eluent was dialyzed against PBS buffer to remove extra salts and glutathione and further purified on a Q Sepharose Fastflow column (Pharmacia) with a NaCl gradient from 0 to 600 mM in 50 mM Tris-HCl (pH 8.4). After analysis by SDS-PAGE, the fractions containing GST-CDK4 were dialyzed against the above HEPES buffer.

The preparation of CDK4 (with a His₆ tag at the C-terminus)/cyclin D2 holoenzyme, GST-p16, GST-p18, GST-Rb, free p16, and p18 were as previously described (33, 35, 36).

Peptide Synthesis. Six peptides were chemically synthesized as previously described (37). Briefly, 200 mg of TentaGel S NH₂-SS resin swelled in dimethylformamide (DMF) was applied to six 10 mL reactors. Peptides were synthesized from the C-terminus to the N-terminus utilizing a resin-bound initiator methionine. Each synthesis cycle consisted of two steps, coupling Fmoc-protected amino acids to the resin-bound peptides and subsequent deprotection in 20% piperidine. After 15 cycles, a cocktail solution (90% trifluoroacetic acid, 2% anisole, 5% thioanisole, and 3% ethanedithiol) was used to remove all protective groups, and then the products were cleaved from the resin in a cycliza-

tion/release reaction. The flow-through was dried under vacuum for 6 h and further purified by reverse-phase HPLC. All of the resin and reagents were purchased from Advanced ChemTech, except that dimethylformamide, trifluoroacetic acid, and piperidine were from Sigma.

Pull-Down Assays. To investigate the interaction between His₆-tagged Tax proteins (including full-length Tax, Tax40N, and Tax40C) and GST-CDK4, a 2.5 mL assay mixture containing 0.1 μ M Tax proteins and 0.05 μ M GST-CDK4 in PBS buffer was incubated at 4 °C for 12 h and then loaded on a 300 μ L Ni-NTA-agarose column which had been equilibrated with PBS buffer containing 0.15 M KCl, 0.5 mM β -mercaptoethanol, and 20 mM imidazole (pH 8.0). Subsequently, the column was washed with 1.0 mL of PBS buffer containing 20 and 50 mM imidazole separately and then eluted with 100 μ L of PBS buffer with 1.0 M imidazole. The eluent was analyzed by Western blot using an anti-human CDK4 antibody (Santa Cruz). Experiments using free p16, instead of GST-CDK4, were done in the same way except that the concentration of p16 was 0.2 μ M, and anti-human p16 antibody (BD PharMingen) was used for Western blotting.

Peptide competition assays were conducted in a manner analogous to pull-down assays except that varying amounts of competitor peptides were included in the reaction mixtures.

To investigate the interactions between the preassembled GST-p16 (or p18)-CDK4-cyclin D2 complex and Tax proteins, 0.2 mL of PBS buffer (pH 7.5) containing 0.1 μ M GST-p16 (or p18) and 0.2 μ M CDK4-cyclin D2 was incubated at 4 °C for 2 h and then loaded on a minicolumn containing 250 μ L of PBS-equilibrated reduced glutathione-agarose. After being washed with 1.0 mL of PBS buffer, the resin was resuspended in 1.0 mL of PBS buffer containing various amounts of Tax proteins and incubated at 4 °C for another 12 h. The resin was repacked in a minicolumn and washed with 2.0 mL of PBS buffer, and the bound proteins were assessed by Western blot using anti-human CDK4 antibody.

In Vitro Kinase Assay. The in vitro kinase assay involved 3 units of the CDK4/cyclin D2 complex (about 0.3 μ g of protein) and varying concentrations of the proteins or peptides under investigation in kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2.5 mM EGTA, 0.1 mM Na₃VO₄, 1 mM NaF, 10 mM β -glycerophosphate, and 1 mM DTT) supplemented with 0.2 mM AEBSF, 2.5 mg/mL leupeptin, and 2.5 mg/mL aprotinin in a total volume of 15.0 μ L. One unit of CDK4 kinase is defined as the amount of CDK4 that catalyzes the incorporation of 1 μ mol of phosphate into pRb at 30 °C within 15 min (35). After preincubation at 30 °C for 30 min, GST-Rb379–928 (100 ng) and 5 μ Ci of [γ -³²P]ATP were added into each reaction mixture. Following incubation at 30 °C for another 15 min, the reaction mixtures were separated by SDS-PAGE, and the CDK4 activity was determined by quantitating the incorporation of ³²P into substrate pRb using a PhosphorImager (Molecular Dynamics, Inc.). Measurements were repeated in duplicate.

Transactivation Assay. A system modified from the MATCHMAKER LexA yeast two-hybrid system (BD Clontech) was used to evaluate the basic transactivation activities of full-length and truncated Tax proteins (38). In this assay, full-length or truncated Tax genes were cloned into a pLexA

vector at the *Eco*RI and *Xho*I sites as binding domain fusion plasmids, and p8op-*lacZ* acted as a reporter plasmid. p8op-*lacZ* carries the *lacZ* reporter gene under the control of the LexA operator and the minimal TATA region from the GAL1 promoter and exhibits zero transcriptional activity in the absence of a LexA-fused activator. When pLexA-Tax plasmids were transformed into the host, EGY48 harboring p8op-*lacZ*, and expressed by galactose induction, the resultant LexA-fused Tax proteins bound to p8op-*lacZ* and turned on the *lacZ* gene through transactivation. Quantitation of the β -galactosidase activity of the *lacZ* gene product enabled us to assess the transactivation activities of Tax proteins. All transformation, expression, and β -galactosidase activity liquid assay experiments were performed according to the manufacturer's instructions. Fragments of Tax encoding Tax40N and Tax40C were amplified by PCR using methods similar to those described in Protein Expression and Purification, and three mutants, S10A, C29S, and C36S, were generated by the QuickChange method using pLexA-Tax40N as template.

RESULTS AND DISCUSSION

Tax Directly Binds to CDK4 through Its N-Terminus and Activates CDK4. It has been well established that Tax protein is involved in the CDK4-Rb pathway in HTLV-1 infected cells (16, 29, 39). In both p16 +/+ and -/- infected cells, expression of Tax activated CDK4 and stimulated the phosphorylation of Rb, which drove the cells into cell cycle progression. However, the mechanism by which Tax activates CDK4 needs to be further investigated since indirect regulatory paths alone, such as Tax binding to p16 and cyclin D(s), cannot account for the observed dramatic activation of CDK4. Direct interactions between Tax and CDK4 seem to be the most likely alternative. Since most of CDK4 exists in complexes with cyclins, Rb, and inhibitors such as INK4 and KIP proteins (40), it has not been feasible to examine direct Tax/CDK4 interactions in vivo.

We examined this issue with two approaches: in vitro binding assay using free GST-fused CDK4 expressed in *E. coli* and in vitro activity assay using the CDK4/cyclin D2 complex expressed in insect cells. The Tax protein and its fragments used in such assays all contain a His tag at the C-terminus, and some also contain a fusion protein as described in Materials and Methods. GST-CDK4 was used in the binding assay due to poor solubility and stability of free CDK4 expressed in *E. coli*. It has been reported that there is no interaction between Tax and the GST tag (16). In an assay mixture containing Tax and GST-CDK4, GST-CDK4 was detected as a pull-down product [Figure 1(I), lane 2], suggesting that Tax interacts directly with CDK4. We then constructed a series of deletion mutants (all containing a His tag at the C-terminus) to map out the CDK4 binding domain. The N-terminal domain of Tax covering the first 40 amino acid residues, Tax40N (fused to GB1), is the smallest domain retaining CDK4 binding ability (lane 3) while there is no detectable interaction between CDK4 and Tax40C, Tax without the first 40 amino acid residues (lane 4). As a control, it was shown that GB1 protein itself does not show any interaction with CDK4 (lane 5).

We also checked the interactions between Tax proteins and free p16. As shown in Figure 1(II), p16 was detected in

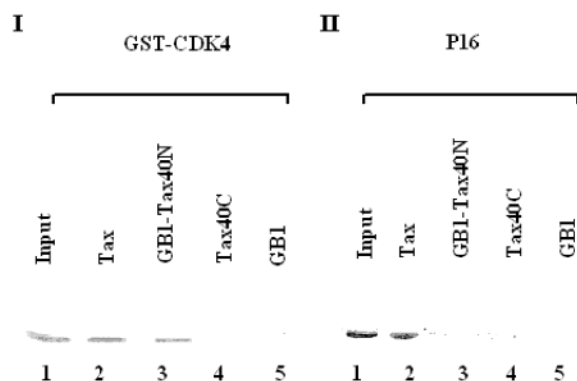


FIGURE 1: Pull-down assays to investigate the binding of Tax proteins to CDK4 and p16. Tax and its fragments contain His tags for immobilization on the nickel column. (I) 2.5 mL of the assay mixture containing 0.1 μ M Tax proteins and 0.05 μ M GST-CDK4 was incubated at 4 °C for 12 h and then loaded on a minicolumn with 200 μ L of Ni-NTA-agarose. After the column was washed with 1.0 mL of PBS buffer containing 0.15 M KCl, 0.5 mM β -mercaptoethanol, and 50 mM imidazole (pH 8.0), bound proteins were eluted with 100 μ L of PBS buffer containing 1.0 M imidazole and blotted with anti-human CDK4 antibody (sc-260, Santa Cruz). Lanes: 1, the input containing 10% of the amount of GST-CDK4 used in other lanes; 2, 0.1 μ M Tax/0.05 μ M GST-CDK4; 3, 0.1 μ M GB1-Tax40N/0.05 μ M GST-CDK4; 4, 0.1 μ M Tax40C/0.05 μ M GST-CDK4; 5, mock lane, GB1 with a His₆ tag was used as control. (II) The experiments were performed in the same way as in (I) except that 0.2 μ M p16 (untagged) was included in the reaction mixtures instead of 0.05 μ M GST-CDK4. Lanes: 1, the input containing 5% of the amount of p16 used in other lanes; 2, 0.1 μ M Tax/0.2 μ M p16; 3, 0.1 μ M GB1-Tax40N/0.2 μ M p16; 4, 0.1 μ M Tax40C/0.2 μ M p16; 5, mock lane, GB1 was used as control.

the pull-down product of the Tax/p16 mixture (lane 2) but not in the pull-down products of the GB1-Tax40N/p16 and Tax40C/p16 mixtures (lanes 3 and 4, respectively), suggesting that Tax interacts with p16 differently than it does with CDK4. Intact molecules of Tax appear to be required for p16 binding, whereas the fragment containing the 40 N-terminal residues is sufficient for CDK4 binding. In addition, it has been demonstrated in a previous study that Arg62 of Tax is required for binding to cyclin D(s) (29). Mutation at this position disrupts the Tax/cyclin D complex, which suggests that Tax40N cannot bind to cyclin D2. Taken together, these results show that GB1-Tax40N binds to CDK4 but not to p16 or cyclin D2.

To understand the biological significance of the direct interaction between Tax and CDK4, *in vitro* assays of CDK4/cyclin D2 activities were performed by use of the proteins expressed in insect cells as described previously (35). As shown in Figure 2, the presence of Tax activates CDK4 activity to 500% (curve A), while GB1-Tax40N did the same to 400% (curve B). As a control, GB1 did not exhibit any influence on CDK4 activity at a concentration up to 500 nM (curve D). Since GB1-Tax40N cannot bind to cyclin D2, the above enhancement of CDK4 activity should be attributed to direct interactions between Tax and CDK4. These results not only suggest that Tax activates CDK4 via direct binding with CDK4 but also identify residues 1–40 at the N-terminus as the primary site of binding. The significance of curve C will be explained later.

Peptides Encompassing the Middle Region of Tax40N Retain the CDK4-Activating Activity. To map out those important segments of Tax40N for CDK4 activation, 6

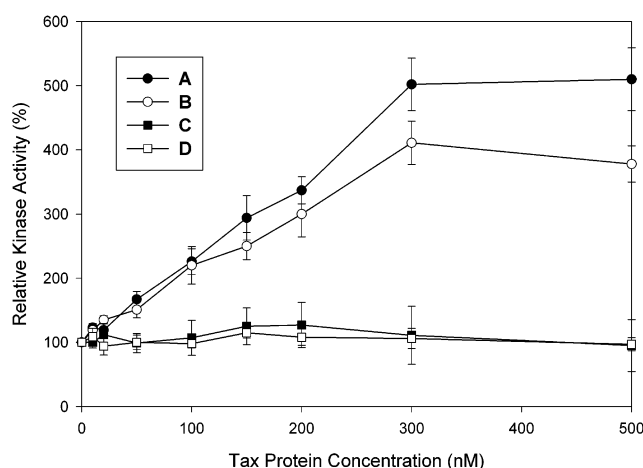


FIGURE 2: *In vitro* kinase activity assay to examine Tax-mediated activation of CDK4. The CDK4/cyclin D2 complex and GST-Rb were expressed as described previously (33). Each assay mixture contained 3 units of CDK4/cyclin D2, 100 ng of GST-Rb379–928, 5 μ Ci of [γ -³²P]ATP, and a varying amount of Tax protein. The reaction was performed at 30 °C, and the incorporation of ³²P into GST-Rb379–928 was quantified with a PhosphorImager. Key: A, Tax; B, GB1-Tax40N; C, GB1-Tax40N C23S; D, GB1 as control. All measurements were performed in duplicate, and changes higher than 50% were regarded as significant. The kinase activity in the absence of Tax is used as the 100% reference point.

peptides of 15 amino acid residues (except P1, which includes residues 2–15) were synthesized and their interactions with CDK4 were investigated. These peptides were designed to start from the N-terminus and move toward the C-terminus with a “window” of 5 residues. We first examined the CDK4-activating ability of these peptides. As shown in Figure 3(I), peptides 2 (⁶⁶GFGQSLFLGYPVYVF²⁰), 3 (¹¹LLFGYPVPYVFGDCVQ²⁵), and 4 (¹⁶PVYVFGDCVQGDWCP³⁰) retained the CDK4-activating ability, while peptides 1 (⁰²AHFPFGQSLFLGY¹⁵), 5 (²¹GDCVQGDWCPISGL³⁵), and 6 (²⁶GDWCPISGGLCSARL⁴⁰) exhibited little activation. Out of all six peptides, peptide 3 (encompassing residues 11–25) retained the highest CDK4-activating activity (nearly 300% at the concentration of 1.5 μ M), suggesting that this segment could be responsible for CDK4 activation. This was further confirmed by peptide competition assays. As can be seen in Figure 3(II), peptide 3 disrupted the interaction between Tax40N and CDK4, while peptide 1 failed at a concentration up to 2.0 μ M. Therefore, the segment spanning from residue 11 to residue 25 could be responsible for CDK4 binding and activation.

Direct Binding of Tax to CDK4 Counteracts the Inhibitory Activity of INK4 Protein p16. It has been reported that Tax protein downregulates INK4 proteins through two distinct mechanisms: functional suppression of p15 and p16 through direct binding and transcriptional repression of p18 and p19 (39). To address the question of how direct binding of Tax to CDK4 affects the inhibitory activities of INK4 proteins, we examined CDK4 activity in the presence of both Tax proteins and INK4 proteins. In the presence of 150 nM p16 (which inhibited 80% of CDK4 activity), addition of Tax protein into the assay mixture led to increases in CDK4 activity [Figure 4(I), curve A]. When Tax was in excess, it totally counteracted the inhibitory effect of p16 and activated the CDK4 activity to ca. 400%. In principle, the above counteraction could result from interactions between Tax and

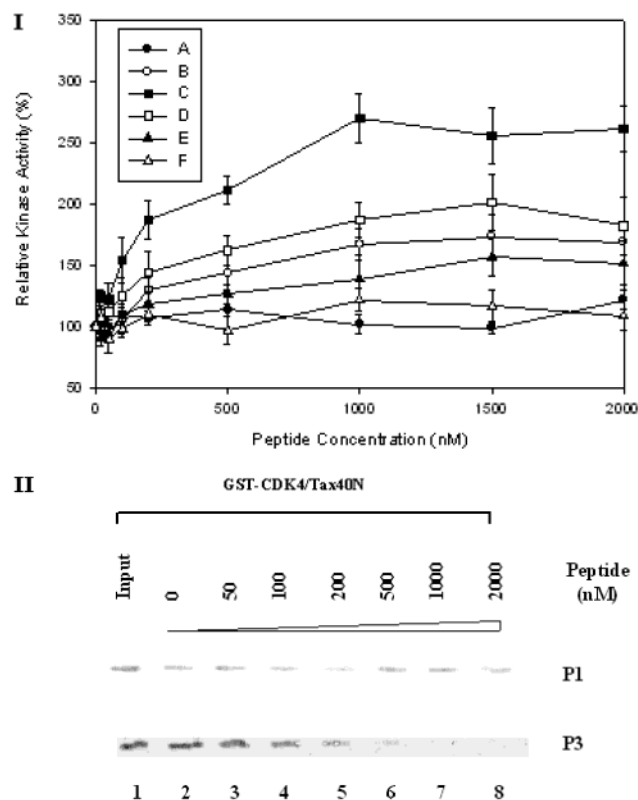


FIGURE 3: Abilities of Tax40N-derived peptides to activate CDK4/cyclin D2 activities and to compete with Tax40N for GST-CDK4 binding. (I) In vitro kinase activity assay. Experiments were performed as described in Figure 2 except that Tax40N-derived peptides were used in the assay mixtures. Key: A, peptide 1 (residues 2–15); B, peptide 2 (residues 6–20); C, peptide 3 (residues 11–25); D, peptide 4 (residues 16–30); E, peptide 5 (residues 21–35); F, peptide 6 (residues 26–40). (II) Competition assay. The experiments were performed as described in Figure 1(I) for the pull-down assays containing GB1-Tax40N and GST-CDK4, except that varying amounts of peptides were included in the reaction mixtures. Only data from peptides 1 and 3 (P1 and P3, respectively) are represented in the figure. Lanes: 1, the input containing 10% of the amount of GST-CDK4 used in other lanes; 2–8, 0.1 μ M GB1-Tax40N/0.05 μ M GST-CDK4/peptides at concentrations of 0, 50, 100, 200, 500, 1000, and 2000 nM, respectively.

all three components, p16, cyclin D2, and CDK4. To test whether the counteraction effect is caused by direct binding between CDK4 and Tax, the experiment was repeated with GB1-Tax40N, which can only bind CDK4 as described by the results in the preceding sections. As shown in curve B, the effect of GB1-Tax40N (300% maximum) is similar to, though somewhat lower than, that of the full-length Tax protein, which is similar to the results of CDK4-activation experiments shown in Figure 2. This result suggests that direct binding of Tax to CDK4 is the major factor in p16 counteraction. As a control, it was shown that GB1 has no detectable counteraction effect (curve E).

Different Behavior between p16 and p18. Although the in vitro inhibitory effects of p16 and p18 toward CDK4 activities are essentially indistinguishable (33), Tax and GB1-Tax40N recovered p18-inhibited CDK4 activity to only about 100% (curves C and D, respectively). This result suggests that the counteracting effect caused by Tax and GB1-Tax40N against p16 is higher than that against p18. Since GB1-Tax40N cannot bind to p16, p18, or cyclin D2, the difference in counteraction could reflect the fact that direct binding of

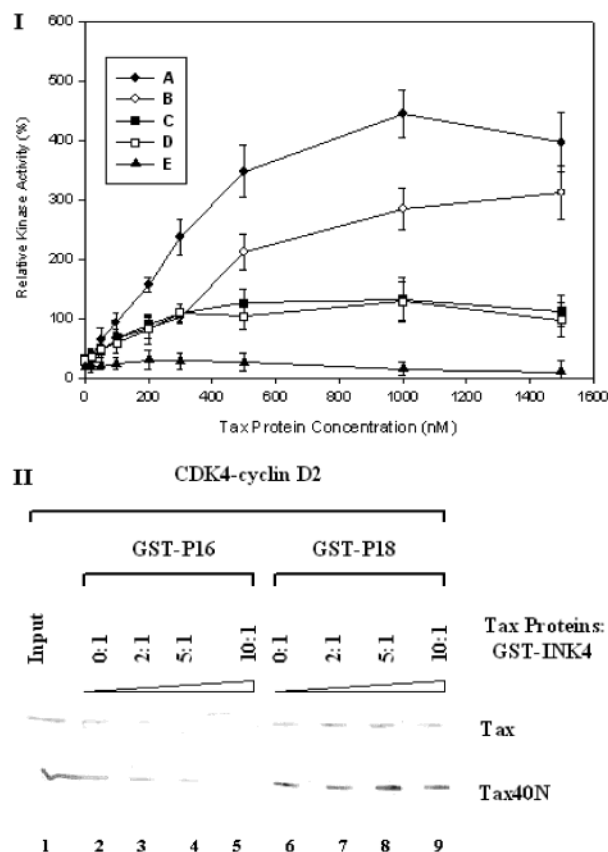


FIGURE 4: Tax counteraction of CDK4 inhibition by p16 and p18. (I) In vitro kinase activity assay. Experiments were performed as described in Figure 2 except that each reaction mixture contained 150 nM p16 or p18 (untagged). Key: A, Tax and 150 nM p16; B, GB1-Tax40N and 150 nM p16; C, Tax and 150 nM p18; D, GB1-Tax40N and 150 nM p18; E, GB1 and 150 nM p16. (II) Competition assays. GST-tagged INK4 proteins (0.1 μ M) were incubated with CDK4/cyclin D2 (0.2 μ M) to form INK4-CDK4-cyclin D2 ternary complexes, and the resultant complexes were immobilized to reduced glutathione-agarose. After unbound CDK4-cyclin D2 was washed away, the resins were incubated with various amounts of Tax proteins, and after washing, bound proteins were blotted against anti-human CDK4 antibody. As indicated, Tax and GB1-Tax40N were used as competitors in the upper and lower rows, respectively. Lanes: 1, the input containing 5% of CDK4-cyclin D2 used in other lanes; 2, no competitor/GST-p16 (0.1 μ M) (0:1); 3, competitor (0.2 μ M)/GST-p16 (0.1 μ M) (2:1); 4, competitor (0.5 μ M)/GST-p16 (0.1 μ M) (5:1); 5, competitor (1.0 μ M)/GST-p16 (0.1 μ M) (10:1); 6, no competitor/GST-p18 (0.1 μ M) (0:1); 7, competitor (0.2 μ M)/GST-p18 (0.1 μ M) (2:1); 8, competitor (0.5 μ M)/GST-p18 (0.1 μ M) (5:1); 9, competitor (1.0 μ M)/GST-p18 (0.1 μ M) (10:1).

GB1-Tax40N to CDK4 in the presence of p16 is different from that in the presence of p18. To address this premise, we examined the interactions between Tax and the pre-assembled GST-p16 or GST-p18-CDK4-cyclin D2 complex using a pull-down assay (13). As shown in Figure 4(II), when Tax was present in the pull-down mixture with a Tax:GST-p16 molar ratio of 2:1, CDK4 was still detected in the pull-down product (lane 3, upper row). However, when Tax was in excess (Tax:GST-p16 = 5:1 and 10:1), no CDK4 was detected in the pull-down products (lanes 4 and 5, upper row), suggesting that the GST-p16/CDK4-cyclin D2 ternary complex was disrupted by an excess amount of Tax. In contrast, CDK4 was detected in all pull-down products from GST-p18/CDK4-cyclin D2/Tax mixtures (lanes 7–9, upper row), indicating that the GST-p18/CDK4-cyclin D2 ternary

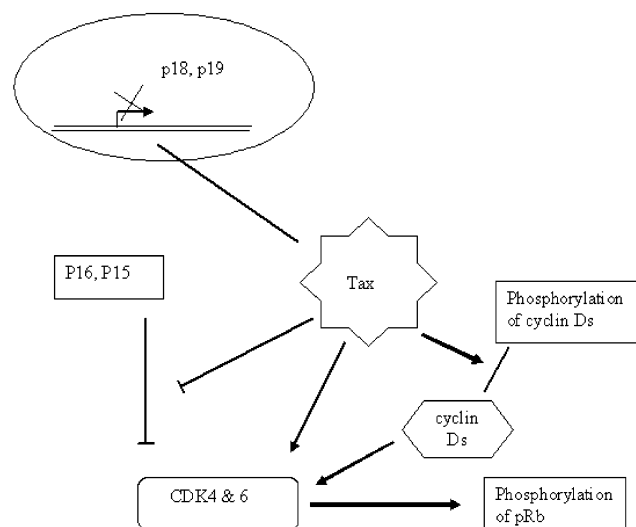


FIGURE 5: Regulatory paths of Tax-mediated activation of CDK4. Arrows stand for positive regulation, and bars indicate negative effect. The cross represents a transcriptional repression in the nucleus.

complex remained intact when Tax existed in excess (Tax: GST-p18 = 10:1). Similar observations were obtained when GB1-Tax40N was present in the pull-down mixtures (lanes 3–5 and 7–9, lower row) except that CDK4 was present in the pull-down product when the GB1-Tax40N:GST-p16 molar ratio was 5:1 (lane 4, lower row). These results indicate that direct binding of Tax and GB1-Tax40N can disrupt the preassembled GST-p16-CDK4-cyclin D2 ternary complex more effectively than they can do to the GST-p18-CDK4-cyclin D2 ternary complex. While this is an interesting and potentially significant observation, its molecular basis remains to be established by further investigation.

Direct Binding of Tax to CDK4 Is the Major Contributor to Tax-Mediated Activation of CDK4. There are four different mechanisms for the involvement of Tax in the activation of CDK4, including (1) transcriptional repression of p18 and p19 genes (39), (2) silencing p16 and p15 through direct protein/protein interactions (16, 39), (3) direct binding to cyclin Ds (29), and (4) direct activation of CDK4 through protein/protein interactions as described in this work (Figure 5). While the first mechanism is at the gene level, the latter three are protein/protein interactions between Tax and three components of one system, the INK4-CDK4-cyclin D2 ternary complex (41) or the CDK4-cyclin D2 binary complex plus INK4 (40, 41). Therefore, it is of interest to investigate which protein/protein interaction contributes most to the activation of CDK4. As was seen earlier, Tax brought about an increase of 500% in CDK4 activity as a consequence of Tax/cyclin D2 and Tax/CDK4 interactions [Figure 1(I), curve A]. In comparison, GB1-Tax40N increased CDK4 activity up to 400% [Figure 1(I), curve B], and this activation should only be attributed to the interaction between Tax and CDK4 since GB1-Tax40N is incompetent in binding cyclin D2. These results suggest that direct interaction between Tax and CDK4 affects CDK4 activity more than binding of Tax to cyclin D2. Furthermore, Tax counteracted the inhibition of p16 and stimulated CDK4 activity to 400% [Figure 4(I), curve A]. This overall effect can arise from interactions between Tax and all three components of the p16-CDK4-cyclin D2 system. The difference between the effect of Tax

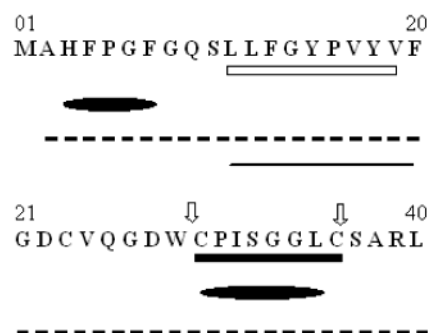


FIGURE 6: Structural and functional motifs within the N-terminus of Tax oncoprotein. The solid line represents the CDK4 binding and activating segment, and black circles indicate CREB binding motifs. The core of the zinc finger and the MHC I binding motif are represented as filled and empty rectangles, respectively. The nuclear localization signal segment is specified as a dotted line, while arrows represent dominant negative mutation sites (43).

and GB1-Tax40N, which counteracted the inhibition of p16 and stimulated the activity of CDK4 to 300% [Figure 4(I), curve B], could be interpreted to suggest that direct binding to CDK4 (which is the only likely mechanism for GB1-Tax40N) is the dominant path in activating CDK4.

Tax40N Is a Relatively Independent Domain with Multiple Functions. Numerous proteins and DNA sequences have been reported to interact with Tax protein and some interactions involve the N-terminus of Tax (21, 42, 43). As shown in Figure 6, Tax40N is a part of the nuclear localization sequence (ranging from residue 2 to residue 50) and contains the core of a zinc finger (Cys29...Cys36), which is important for DNA binding. It also contains three protein binding motifs including the CREB binding segment (spanning residues 2–34), MHC-I binding motif (class I major histocompatibility complex binding, covering residues 11–19), and CDK4 binding motif (residues 11–25), and these motifs overlap each other to some degree. In addition to our earlier observation that Tax40N itself activated CDK4, chromatographic behavior suggested that GB1-Tax40N is likely to be structured: in S100 gel-filtration chromatography, GB1-Tax40N was eluted as a monomer at the retardation range for a typical folded protein. The removal of GB1 from GB1-Tax40N led to aggregation.

To further confirm this premise, a LexA-based assay was developed to examine the transactivation activities of Tax proteins. As shown in Figure 7, the N-terminus of Tax is responsible for this LexA-based transactivation. Tax40N exhibited a transactivation activity comparable to that of intact Tax, while the C-terminus, Tax40C, showed little transactivation activity similarly to the negative control CDK4. Mutations at Cys residues at the zinc finger, Tax40N C29S and C36S, led to loss of the transactivation activity, suggesting that the zinc finger is essential for this LexA-based transactivation. Interestingly, a previous study showed that residues important for HTLV-1 LTRs transactivation are not limited to the N-terminus (43). Like mutations at Cys29 and Cys36, mutations at residues distant from the N-terminus, such as Ser274, Leu296, and Leu320, also led to loss of LTR transactivation activity. Moreover, some residues at the N-terminus contributed differently to LTR-based transactivation and LexA-based transactivation. As an example, mutation S10A turned off LTR transactivation (43) but did

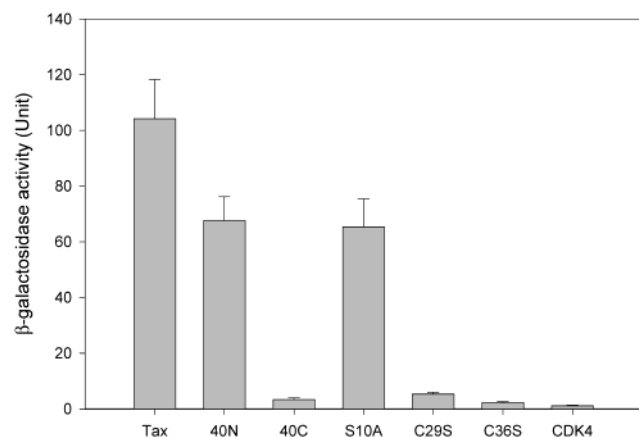


FIGURE 7: Transactivation assays. pLexA plasmids with Tax genes were transformed into yeast EGY48 harboring the reporter plasmid, p8op-*lacZ*, and transformants containing both pLexA and p8op-*lacZ* were selected on synthetic dropout (SD) medium deficient for uracil (Ura) and tryptophan (Trp). Ura/Trp positives were restreaked onto SD-Ura/-Trp/X-gal/galactose/raffinose media. The β -galactosidase activity of the *lacZ* gene product, which represented the strength of Tax proteins in transactivation, was determined in a liquid β -galactosidase enzymatic activity assay using ONPG (Sigma) as substrate. CDK4 was used as a negative control, and all three mutants were constructed using Tax40N (not GB1-fused) as the template. Assays were performed in triplicates.

not affect LexA-mediated transactivation as shown in Figure 7.

While this work was in progress, Grassmann and colleagues reported studies showing that physical interaction between Tax and CDK4 stimulates the phosphorylation of Rb in vivo, and mutations at the N-terminus of Tax disrupt the Tax-CDK4 complex and eliminate the Tax-mediated activation of CDK4 (44). Our results of in vitro studies generally support their in vivo observations. For example, in their study, the C23S mutation disrupted the interaction between Tax and CDK4. In our study, Tax40N C23S did not show any detectable CDK4-activating activity (Figure 2, curve C), and P3, the peptide retaining the highest CDK4-activating activity out of all six peptides, contained C23 (Figure 3). However, our results have provided additional mechanistic insights and molecular basis for the function of Tax. First, we used purified Tax40N protein to demonstrate that the N-terminus is not only essential but also the primary effector for CDK4 activation. Second, we mapped out the motif responsible for binding to CDK4 through peptide competition experiments. Third, using Tax40N, we singled out the effect caused by binding of Tax to CDK4 and demonstrated that direct binding between Tax and CDK4, rather than interactions between Tax/cyclin D2 and Tax/INK4, contributes to the greatest extent to Tax-mediated activation of CDK4. Fourth, we examined the counteraction of Tax against CDK4-specific inhibitors, p16 and p18, rather than p21 (a universal inhibitor of all CDKs). Last but not least, we also demonstrated the function of Tax40N in LexA-mediated transactivation, which further supports the independence of Tax40N in structure and function.

In conclusion, besides binding to both p16 and cyclin D2, Tax binds to CDK4 and enhances the kinase activity. This direct CDK4 binding appears to be the predominant mechanism for Tax-mediated CDK4 activation, which could contribute to HTLV-1-related tumorigenesis. Due to the complexity of the involvement of Tax in the INK4-CDK4/

6-Rb pathway, more questions need to be clarified, such as the structural basis of Tax binding to three components of the INK4/CDK4-cyclin D2 system, the coordination among these interactions, and the correlation between the INK4-CDK4-Rb pathway and other Tax-involved pathways. A combination of cell biology and biochemical and biophysical approaches will be necessary to address these questions.

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