THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 3, Issue of January 17, pp. 1487–1493, 2003
Printed in U.S.A.

Human T-lymphotropic Virus Type I Tax Activates I-κB Kinase by Inhibiting I-κB Kinase-associated Serine/Threonine Protein Phosphatase 2A*

Received for publication, October 17, 2002
Published, JBC Papers in Press, November 4, 2002, DOI 10.1074/jbc.M210631200

De-Xue Fu‡, Yu-Liang Kuo‡, Bao-Ying Liu‡, Kuan-Teh Jeang§, and Chou-Zen Giam‡¶

From the ‡Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814 and §Molecular Virology Section, Laboratory of Molecular Microbiology, NIAID, National Institutes of Health, Bethesda, Maryland 20892-0460

I-κB kinase (IKK) is a serine/threonine kinase that phosphorylates I-κBα and I-κBβ and targets them for polyubiquitination and proteasome-mediated degradation. IKK consists of two highly related catalytic subunits, α and β, and a regulatory γ subunit, which becomes activated after serine phosphorylation of the activation loops of the catalytic domains. The human T-lymphotropic retrovirus type-I trans-activator, Tax, has been shown to interact directly with IKKγ and activates IKK via a mechanism not fully understood. Here we demonstrate that IKK binds serine/threonine protein phosphatase 2A (PP2A), and via a tripartite protein-protein interaction, Tax, IKKγ, and PP2A form a stable ternary complex. *In vitro*, PP2A down-regulates active IKK prepared from Tax-producing MT4 cells. In the presence of Tax, however, the ability of PP2A to inactivate IKK is diminished. Despite their interaction with IKKγ, PP2A-interaction-defective Tax mutants failed to activate NF-κB. Our data support the notion that IKKγ-associated PP2A is responsible for the rapid deactivation of IKK, and inhibition of PP2A by Tax in the context of IKK·PP2A·Tax ternary complex leads to constitutive IKK and NF-κB activation.

NF-κB/Rel family of transcription factors are controlled by inhibitory I-κB proteins I-κBα and I-κBβ and the I-κB-like domains in NF-κB1 and NF-κB2 that sequester NF-κB/Rel in the cytoplasm as multiprotein complexes (for reviews, see Ref. 1–3). Upon activation by extracellular stimuli such as interleukin-1, tumor necrosis factor-α, bacterial lipopolysaccharide, or by human T-lymphotropic virus type I (HTLV-I)[1] Tax, I-κBα and I-κBβ become serine-phosphorylated and polyubiquinated and are rapidly degraded via proteasome-mediated proteolysis, resulting in heightened nuclear levels of NF-κB and increased expression of a plethora of cellular genes under NF-κB regulation, including the genes of many cytokines and their receptors, adhesion molecules, and immune modulators (1–3). Dysregulation and/or hyperactivation of the NF-κB/I-κB regulatory pathway as caused by chromosomal translocation (4), oncogene transduction (5), viral infection, or targeted gene disruption (6, 7) leads to cancers of the hematopoietic cells or chronic inflammatory diseases.

The phosphorylation of I-κBα and I-κBβ is mediated by a kinase called I-κB kinase (IKK) (8, 9). The core IKK enzyme consists of two highly homologous catalytic subunits α and β of 85 and 87 kDa in sizes, respectively, and a 48-kDa regulatory subunit, IKK-γ/NEMO (referred to as IKK-γ herein) (9, 10). Both IKK-α and IKK-β contain NH_2-terminal kinase domains followed by leucine zippers and helix-loop-helix domains that mediate protein-protein interactions important for IKK oligomerization and kinase activity (8, 9). Likewise, IKK-γ also contains extensive helical regions and leucine zipper domains that engage in protein-protein interaction (9, 10). *In vivo* the IKK holoenzyme exists as a large protein complex of at least 700–900 kDa in size (8, 9). It is not clear what other protein components are present in the holo-IKK enzyme complex in addition to IKK-α, IKK-β, and IKK-γ. Several members of the I-κB and NF-κB/Rel families of proteins and mitogen-activated protein kinase phosphatase-1 (MKP), MEK kinase, and NF-κB inducing kinase (NIK) have been reported to interact with IKK, although these proteins have not been found to co-elute with the 900-kDa IKK complex chromatographically (9).

The transactivator/oncoprotein of HTLV-I, Tax, has been shown to activate IKK constitutively (11–16). Tax-mediated IKK activation is due in part to a direct interaction between Tax and IKK-γ (14, 16, 18). The molecular mechanism via which Tax affects IKK activation after its association with IKK-γ remains incompletely understood, however. We have found recently that Tax can interact directly with the catalytic subunit of the major serine/threonine protein phosphatase 2A (PP2Ac) *in vivo* and *in vitro*.[2] Furthermore, Tax acts as a noncompetitive inhibitor of PP2A in *in vitro* assays where ^{32}P-labeled glycogen phosphorylase *a* is used as a PP2A substrate.[2] Consistent with the notion that Tax inhibits the activity of PP2A, MEK phosphorylation and to a lesser extent cAMP-response element-binding protein and ATF-1 phosphorylation in Tax-expressing HTLV-I-transformed cells and in Tax-transfected human embryonic kidney 293 cells are greatly elevated.[2] Most interestingly, PP2A interaction-defective mutants of Tax fail to stimulate MEK phosphorylation and are unable to activate NF-κB, thus suggesting a link between the inhibition of PP2A by Tax and NF-κB activation.[2]

* This work was supported by National Institutes of Health Grants RO1 CA48709 and RO1 CA/GM 75688. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Tel.: 301-295-9624; Fax: 301-295-1545; E-mail: giam@bob.usuf2.usuhs.mil.

[1] The abbreviations used are: HTLV, human T-lymphotropic virus type I; IKK, I-κB kinase; PP2A, protein phosphatase 2A; GST, glutathione *S*-transferase; LTR, long terminal repeat; OA, okadaic acid; MEK; mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; CREB, cAMP-response element-binding protein; p/CAF, p300 CREB-binding protein-associated factor.

[2] Y.-L. Kuo, L.-C. Wang, M.-H. Liang, Y. Tang, D.-X. Fu, B.-Y. Liu, R. Harrod, H.-J. Kung, H.-M. Shih, and C.-Z. Gram, submitted for publication.

PP2A is a major serine/threonine protein phosphatase in all eukaryotic cells (for comprehensive reviews, see Refs. 19–23). It is crucial for the negative regulation of multiple cellular processes. The holo-PP2A enzyme is a heterotrimer that consists of a core enzyme formed by the highly conserved A subunit (60 kDa, 2 human isoforms) and C (catalytic) subunit (36 kDa, 2 human isoforms) together with many different regulatory B-subunits that derive from multiple genes (three distinct families: B/B55/PR55 (3 genes), B′/B56/PR61 (>3 genes), and B″/PR72/PR130 (1 gene)), and their alternatively spliced mRNAs (19–21, 23, 24). The crystal structure of the A-subunit shows it to contain 15 tandem helical repeats (HEAT motifs) that assume a shape that resembles a horseshoe (25). It serves as a scaffold to which C- and B-subunits are attached (19–21, 23). The heterodimer formed by A- and C-subunits and the heterotrimer containing all three subunits are termed core enzyme and holoenzyme, respectively (19–21, 23). A role of PP2A in controlling the signal transduction pathway that leads to IKK/NF-κB activation has been reported previously (8). Okadaic acid, an inhibitor of PP2A, has been shown to activate NF-κB *in vivo* (26, 27). Furthermore, PP2A can inactivate the kinase activity of IKK *in vitro* (8). Here we provide evidence to show that via a tripartite interaction, Tax, PP2A, and IKKγ form a ternary complex. In *in vitro* IKK assays, Tax reduces the ability of PP2A to inactivate IKK. Consistent with the notion that activation of IKK requires that Tax bind to both IKKγ and PP2A, several Tax mutants that are abrogated or attenuated for PP2A binding fail to activate NF-κB even though they continue to bind IKKγ. These results indicate that, in the context of the IKK·PP2A·Tax complex, PP2A activity is inhibited or diminished. In essence, IKK is activated by serine phosphorylation of its activation loop upon extracellular stimulation. In normal cells, phospho-IKK becomes rapidly inactivated by IKKγ-associated PP2A, returning IKK to a resting state. In HTLV-I infected or transformed cells, PP2A inhibition by IKKγ-bound Tax maintains IKK in the phosphorylated and active form, causing constitutive phosphorylation and degradation of I-κB, which in turn leads to nuclear presence of NF-κB/Rel and potent activation of genes under NF-κB/Rel control.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—The anti-Tax monoclonal antibody 4C5 is of IgG2a subtype and reacts with amino acid residues 333–353 of Tax.[3] The anti-PP2A C-subunit monoclonal antibody (the COOH-terminal amino acid residues 295–309 of the catalytic subunit of human PP2A) and anti-IKKγ (amino acids 1–419, representing full-length human IKKγ) monoclonal antibody were from Upstate Biotechnology, Inc. and Santa Cruz Biotechnology, Inc., respectively. The purified PP2A was derived from human red blood cells and consists of both A and C subunits (Upstate Biotechnology). Jurkat and the HTLV-I-transformed T-cell line MT-4 were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml penicillin (Invitrogen) in the presence of 10% CO_2.

Plasmid Construction and Recombinant Protein Purification—The glutathione *S*-transferase (GST) fusion of wild-type and mutant forms of Tax, GST-Tax, GST-H43Q, GST-K85N, GST-M22, GST-M47, were constructed as reported previously (28). GST-IκBα-(1–54) and Ser-32 and Ser-36 phosphorylation sites mutant GST-IκBα-(1–54)AA were generous gifts of Drs. DiDonato and Karin (8). GST-IKKγΔC was constructed by replacing in GST-Tax an *Nco*I, *Sma*I fragment that contains the coding sequence for wild-type Tax with an *Nco*I, *Sma*I fragment that encodes the NH_2-terminal 306 amino acid residues of IKKγ. GST fusion proteins were expressed and prepared by standard protocols and stored frozen in buffer D (20 mM Hepes (pH 7.9), 100 mM KCl, 0.2% (v/v) 2-mercaptoethanol, 1 μM phenylmethylsulfonyl fluoride, and 20% glycerol) at −80 °C.

Immunoprecipitation and Western Blotting—Immunoprecipitations were carried out using extracts prepared from Jurkat and HTLV-I-transformed MT-4 cells. Ten million cells were harvested, washed 3 times, each with 10 ml of phosphate-buffer saline, and lysed by repeated passage through a 27.5-gauge syringe in 1 ml of lysis buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride) containing 50 μg/ml each of the protease inhibitors pepstatin, leupeptin, bestatin, and aprotinin (Roche Molecular Biochemicals). Cell debris was removed by centrifugation at 12,000 rpm in a microcentrifuge for 10 min at 4 °C. Immunoprecipitation was carried out with 200 μg of proteins in 500 μl of cell lysates. Briefly, cell lysates were precleared by incubation with 100 μl of a 50% slurry of protein G-agarose (Invitrogen) and 2 μg of normal mouse antiserum in 5 μl for 30 min at 4 °C followed by centrifugation at 12,000 rpm for 5 min. After preclearing, 2 μg of a Tax monoclonal antibody (4C5), PP2A C-subunit monoclonal antibody, or IKKγ monoclonal antibody were added to each sample. The reactions were preincubated at 4 °C for 1 h. After the addition of 30 μl of protein G-agarose to each reaction, the mixtures were incubated overnight. On the following day, immune complexes were pelleted by centrifugation at 3000 rpm for 5 min at 4 °C, washed 3 times with 0.8 ml of lysis buffer, resuspended in 30 μl of SDS-PAGE loading buffer, heated at 95 °C for 3 min, and centrifuged, and then 15 μl of sample were loaded and resolved on a 12% SDS-polyacrylamide gel containing a 4% stacking gel. Proteins resolved in the gels were transferred to nitrocellulose membranes (Schleicher & Schuell). For Western blotting, membranes were incubated with blocking buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% Tween, 0.02% sodium azide, 5% nonfat dry milk) for 3 h and then probed in the same buffer for 2 h with mouse monoclonal antibodies against PP2Ac, IKKγ, or Tax. After washing, the blots were incubated for 1 h in the same buffer containing an anti-mouse horseradish peroxidase-conjugated, secondary antibody (diluted 1:1000, Santa Cruz Biotechnology), washed three times, developed using a chemiluminescent substrate (SuperSignal; Pierce), and exposed to x-ray films.

Glutathione S-Transferase Pull-down Assay—For GST pull-down experiments, ~500 ng each of purified GST, GST-Tax, or GST-IKKγ was incubated with 300 ng of purified PP2A or PP2A and Tax in 30 μl of 1× binding buffer (25 mM HEPES (pH 7.9), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 10% glycerol, 0.15% Nonidet P-40, 0.25 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) for 30 min at 30 °C. Forty microliters of a 50% slurry of prewashed glutathione-Sepharose 4B (Amersham Biosciences) were added to each binding reaction, and samples were incubated for 1 h with agitation at 4 °C. After incubation, the protein-bound Sepharose beads were washed 3 times with 800 μl of 1× binding buffer, pelleted by centrifugation at 1200 rpm for 5 min, resuspended in 30 μl of SDS-PAGE loading buffer, and heated to 95 °C, and 15 μl from each reaction were resolved by 12% SDS-PAGE followed by immunoblotting, as described. To remove the GST moiety from GST-IKKγ, the fusion protein was proteolysed with 0.2 unit of thrombin (Sigma) overnight at 4 °C, and the treated protein was then incubated with GST-Tax as described above. Under these conditions, GST-IKKγ was converted completely to IKKγ by thrombin, and the residual thrombin in the reaction mixture did not interfere significantly with the binding reactions.

In Vitro I-κB Kinase Assay—To measure IKK activity, 10^7 MT-4 cells were harvested by centrifugation and extracted in 1 ml of a lysis buffer containing 20 mM Tris-HCl (pH 7.6), 20 mM glycerol phosphate, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 0.5% Nonidet P-40, 0.1 mM sodium vanadate, 10 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin. After a brief centrifugation, extracts were incubated for 2 h with agitation at 4 °C with 0.5 μg of an antibody against IKKγ followed by protein G-Sepharose (Invitrogen) precipitation. Each IKK immune complex reaction was carried out using 1 μg of recombinant GST protein fused to amino acid residues 1–54 of IκBα (GST- IκBα-(1–54)) as the substrate and 10 μM [γ-^{32}P]ATP in a final volume of 30 μl of a reaction buffer containing 30 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 1 mM dithiothreitol at 25 °C for 30 min. The reactions were terminated by the addition of SDS-PAGE loading buffer. IKK activity as measured by the phosphorylation level of IκBα was evaluated by SDS-PAGE followed by autoradiography. When applicable, 5 ng of PP2A and 0.1–0.6 μg of Tax were added into a 30-μl reaction.

RESULTS

IKKγ Interacts with PP2A and Tax in Vivo—Several cellular kinases and regulatory proteins whose activities are regulated by serine/threonine phosphorylation form protein complexes with PP2A (29–34). In this manner, PP2A is localized to its targets at once and can affect rapid on/off control of regulatory

[3] D.-X. Fu, Y.-L. Kuo, B.-Y. Liu, K.-T. Jeang, and C.-Z. Giam, unpublished results.

processes immediately after signaling events are initiated. Because Tax interacts with both PP2A and IKKγ, we wondered if PP2A also forms a complex with IKK. This might explain the rapidity with which the IKK/NF-κB pathway is returned to the inactive state soon after its activation. Furthermore, because Tax constitutively activates IKK, we wondered if, in the context of the IKK-PP2A-Tax complex, the phosphatase activity of PP2A may be inhibited by Tax. Because IKK is activated through serine phosphorylation of its activation loop by upstream kinases, a block in its de-phosphorylation by Tax would maintain phospho-IKK in an active state and can explain the constitutive activation of IKK by Tax.

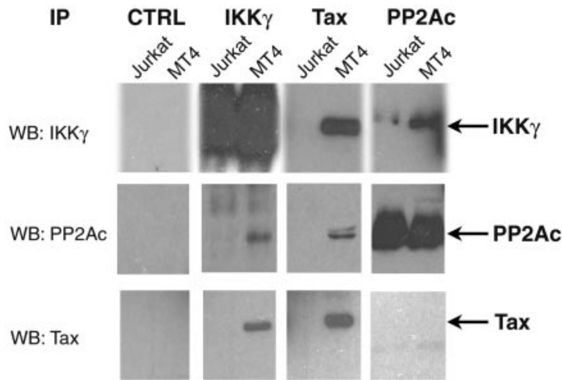

FIG. 1. **IKKγ interacts with the catalytic subunit of PP2A and Tax *in vivo*.** Immunoprecipitations were carried out as detailed in "Experimental Procedures" using extracts prepared from Jurkat and the HTLV-I-transformed cell line MT-4. One-half of 1 ml of cell extracts were immunoprecipitated with 2 μg of nonspecific mouse IgG (*CTRL*) and antibodies against IKKγ, Tax, and the catalytic subunit of PP2A (PP2Ac) as labeled at the *top*. The immunoprecipitates (*IP*) were resolved on a SDS, 12% PAGE and analyzed by Western blotting with IKKγ, Tax, and PP2Ac monoclonal antibodies (*WB: IKKγ*, *WB: PP2Ac*, and *WB: Tax*), respectively. The data shown are representative of three independent experiments.

To determine whether IKK, PP2A, and Tax form a ternary complex *in vivo*, cell extracts were prepared from a Tax-expressing HTLV-I-transformed human T-cell line, MT-4, and a control human T-cell line, Jurkat, and subjected to immunoprecipitation using antibodies against Tax, the catalytic subunit of PP2A (PP2Ac), and IKKγ, respectively. As expected, mouse monoclonal antibody against IKKγ precipitated IKKγ easily (Fig. 1, *lanes 3* and *4*, *upper panel*). From Tax-positive MT-4 cell extracts, both the PP2A c-subunit (PP2Ac) and Tax were readily co-precipitated (*lane 4*, *middle* and *lower panels*, respectively). In Jurkat extracts, a low but detectable trace of PP2Ac was also present (*lane 3*, *middle panel*). Likewise, Tax monoclonal antibody 4C5 co-precipitated Tax, PP2Ac, and IKKγ (*lane 6*, *lower*, *middle*, and *upper panels*). The 4C5 antibody is highly specific for Tax. It did not react with either PP2Ac or IKKγ, as indicated by immunoprecipitations done with Tax-null Jurkat extracts (*lane 5*). Finally, a mouse monoclonal antibody against the COOH-terminal region of PP2Ac co-precipitated IKKγ from both Jurkat and MT-4 extracts. Interestingly and in agreement with the immunoprecipitation performed with anti- IKKγ (*lanes 3* and *4*), the amount of IKKγ that co-precipitated with PP2Ac is lower when Jurkat extracts were used (compare *lanes 7* and *8*, *upper panel*). The PP2Ac antibody failed to co-immunoprecipitate Tax, however (*lanes 7* and *8*, *bottom panel*). We think this is due to a disruption of the

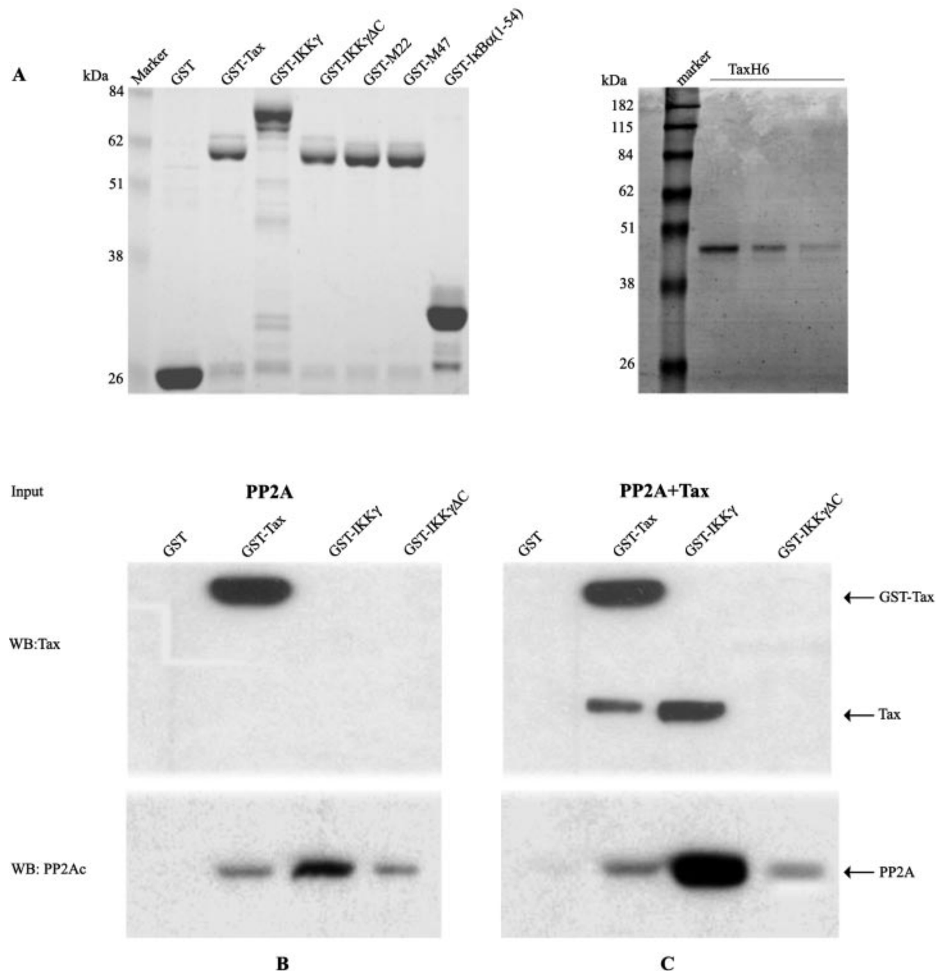

FIG. 2. *A*, profile of purified Tax and GST fusion proteins. GST, GST-Tax, GST-IKKγ, GST-IKKγΔc, GST-M22, GST-M47, GST- IκBα-(1–54) were expressed and purified as previously reported (28). Procedures for the expression and purification of hexahistidine-tagged Tax protein are as published (45). After electrophoresis in a SDS, 12% polyacrylamide gel, the proteins were stained with Coomassie Brilliant Blue R-250 (Sigma). Molecular mass markers are as shown. *B* and *C*, IKKγ, Tax, and PP2A form a ternary complex *in vitro*. Purified GST, GST-Tax, GST-IKKγ, or GST-IKKγΔC (500 ng each) was incubated with 300 ng of purified PP2A (*PP2A panel*) (*B*) or both 300 ng each of purified PP2A and Tax (*PP2A+Tax panel*) (*C*) as described under "Experimental Procedures." The proteins bound to the glutathione-Sepharose were resolved in SDS-12% PAGE and Western blotted (*WB*) with either 4C5 Tax-monoclonal antibody (*WB: Tax*, *upper panels*) or PP2Ac monoclonal antibody (*WB: PP2Ac*, *lower panels*). The data shown are representative of two independent experiments.

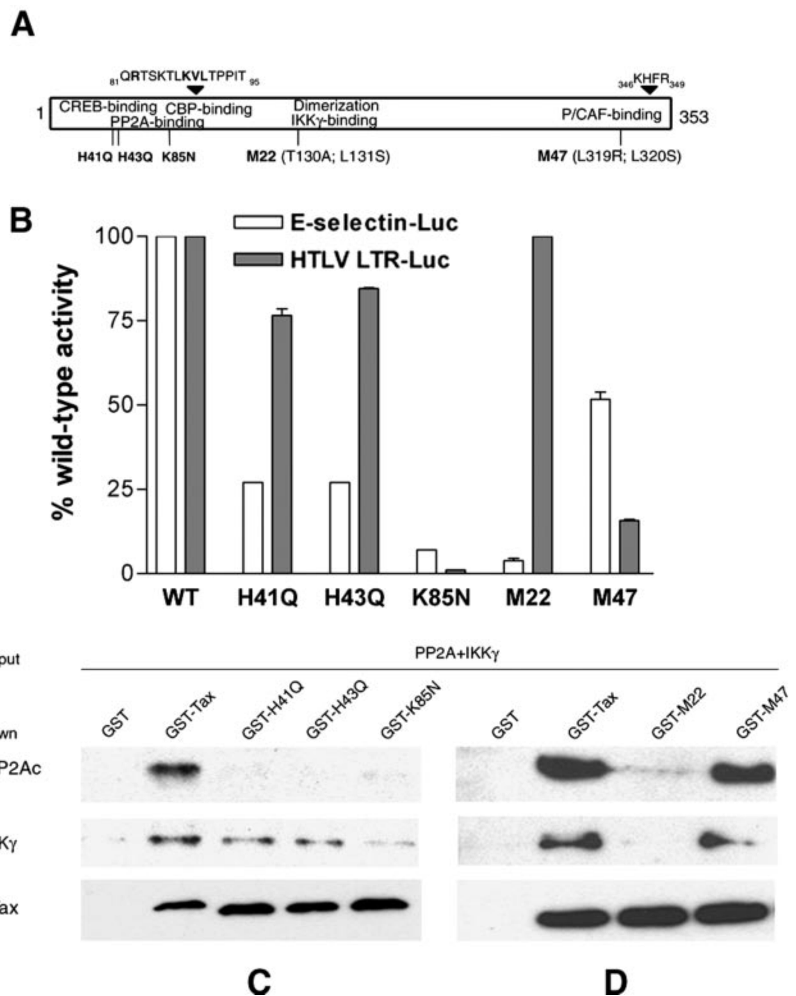

FIG. 3. **Tax binding to both PP2A and IKKγ is required for NF-κB activation.** *A*, domain organization of Tax. *Arrows* mark major trypsin sensitive sites of Tax at Lys-88 and Lys-346, or Arg-349. H41Q, H43Q, and K85N denote three mutants attenuated or abrogated for PP2A binding. M22 is defective in dimer formation, IKKγ binding, and NF-κB activation. M47 is defective in P/CAF binding and LTR transactivation. *B*, transactivation properties of Tax mutants. DNA transfection and luciferase assays were as previously reported (17). Typically, 0.5 μg of CMV-Tax (or mutants) plasmid and 0.5 μg of the reporter were used to transfect 3×10^5 human embryonic kidney 293 cells in each well of a 12-well plate. DNA transfections were carried out in triplicate. The means of luminescence units from each set of three luciferase assays were then used to compute fold trans-activation of each Tax construct for the HTLV-I LTR-Luc (*solid bar*) or the E-selectin-Luc (*open bar*) reporter. *C*, *in vitro* interaction of PP2A and IKKγ with Tax mutants H41Q, H43Q, and K85N. One-half of 1 μg each of GST, GST-Tax, GST-H41Q, GST-H43Q, or GST-K85N was incubated with 0.3 μg each of purified PP2A and IKKγ as described under "Experimental Procedures." Proteins bound to the glutathione-Sepharose 4B were resolved by SDS, 12% PAGE and Western-blotted with PP2Ac, IKK, and Tax (4C5) antibodies (*WB: PP2Ac*, *WB: IKKγ*, *WB: Tax*), respectively. The data shown are representative of three independent experiments. *D*, *in vitro* interaction of PP2A and IKKγ with M22 and M47 mutants. Pull-down experiments were performed as in *C*. The data shown are representative of three independent experiments. *WT*, wild type.

protein-protein interaction between Tax and PP2Ac by the PP2Ac antibody. The COOH-terminal region of PP2Ac is involved in extensive protein-protein interaction; the carboxyl leucine residue undergoes a methyl esterification (35–37) critical for C subunit assembly with A and B subunits (36, 38, 39) to form the core and holoenzymes. It is likely that this region is also important for binding Tax. Together, these data indicate that PP2A interacts with IKK directly. Furthermore, Tax interacts with IKKγ and PP2A *in vivo* in a stable ternary complex.

IKKγ, Tax, and PP2A Form a Stable Multiprotein Complex in Vitro—To characterize further the interaction among Tax, IKKγ, and PP2A, GST pull-down experiments were performed using purified PP2A, consisting principally of both the catalytic C-subunit (PP2Ac) and the regulatory A-subunit, derived from human red blood cells (Upstate Biotechnology) in the presence or absence of Tax protein purified from an *Escherichia coli* expression system. Care was taken to ensure comparable levels of purified GST, GST-Tax, and GST-IKKγ fusion proteins (Fig. 2*A*) were used. In binding reactions containing PP2A only, GST-Tax, GST-IKKγ, and GST-IKKγΔC, the GST fusion of an IKKγ mutant deleted for the COOH-terminal Tax binding domain (amino acid residues 307–419), all displayed efficient binding to PP2Ac (Fig. 2*B*, *lower panel*). Under these experimental conditions, ~2 and 4% of input PP2Ac became bound to GST-Tax and GST- IKKγ, respectively. The interactions appear specific since under the same binding condition, GST control did not bind PP2Ac. Interestingly, in reactions where both PP2A and Tax were added, the binding of PP2A to GST-IKKγ became significantly enhanced (compare Fig. 2, *B* and *C*, *GST-IKKγ lanes*, *lower panel*). By contrast, Tax did not increase PP2A binding to GST-IKKγΔC (Fig. 2*C*, compare the *GST-IKKγ* and *GST-IKKγΔC lanes* in the *lower panel*) whose Tax binding site is deleted and is unable to bind Tax (*upper panel*), suggesting that when Tax is bound to IKKγ, it can further stabilize or enhance IKKγ-PP2A interaction. As anticipated, GST-IKKγ interacted with Tax (Fig. 2*C*, *GST-IKKγ lane*, *upper panel*). In agreement with earlier results, GST-Tax interacted with PP2A (*GST-Tax lane*, *lower panel*). Because Tax exists as homodimer, GST-Tax and Tax interaction was also seen (*GST-Tax lane*, *upper panel*). The exogenously added Tax (300 ng) did not significantly compete for PP2Ac binding to GST-Tax (500 ng), most likely because the interaction between Tax and PP2Ac was not saturating (only 2% input PP2Ac was pulled down by GST-Tax). No binding of Tax or PP2A to GST was detected. Together, these data support the immunoprecipitation results shown above and indicate that PP2A is associated with IKK. Furthermore, in the presence of Tax, a stable IKK·PP2A·Tax ternary complex can form. Finally, even though IKKγΔC mutant is defective in binding Tax (18, 40), it is still able to associate with PP2A, much like the full-length IKKγ, suggesting that PP2A binds to a region in IKKγ upstream of the COOH-terminal Tax binding site.

Tax-PP2A Interaction Is Necessary for NF-κB Activation—If indeed the inhibition of PP2A by Tax in the context of the IKK·PP2A·Tax complex leads to a constitutive activation of IKK, then one might expect that both Tax-PP2A and Tax-IKKγ interactions would be necessary for Tax-mediated activation of IKK and NF-κB. Using yeast 2-hybrid analysis, we have previously mapped the domain critical for PP2Ac binding to the

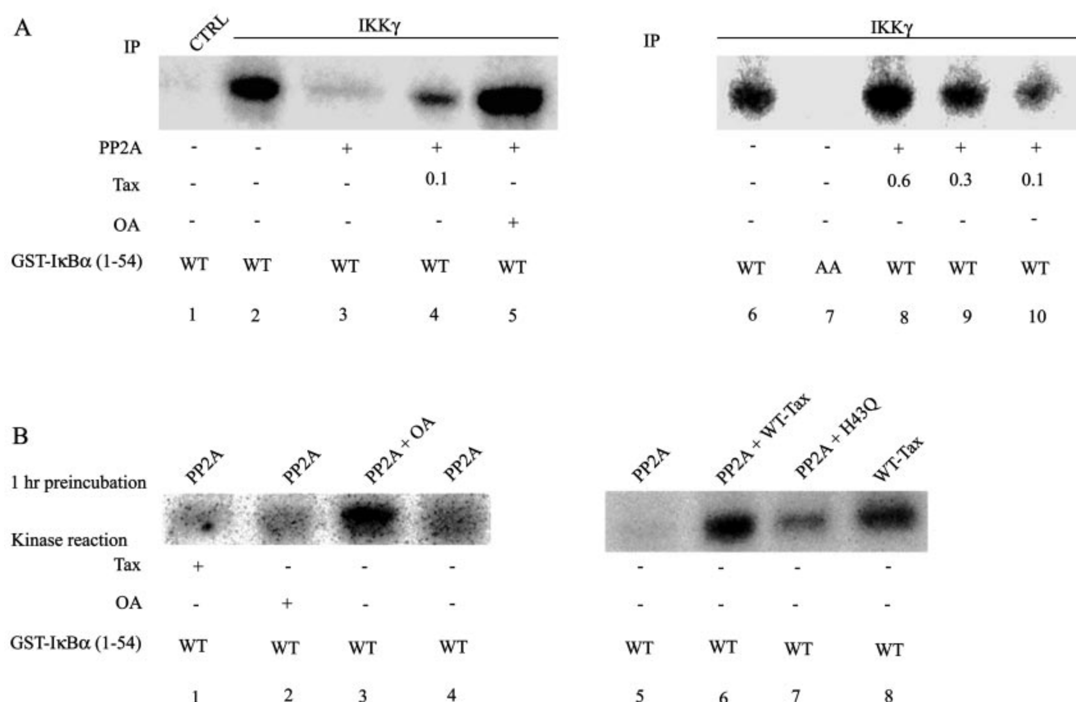

FIG. 4. **Tax prevents PP2A from inactivating IKK *in vitro*.** *A*, MT4 cell extracts were prepared as described under "Experimental Procedures" and immunoprecipitated with nonspecific mouse sera (*CTRL*, *lane 1*) or IKKγ antibody (*lanes 2–10*). IKK immune complex kinase assays contained 1 μg of recombinant GST fusion containing amino acid residues 1–54 of IκBα (GST- IκBα-(1–54), denoted as wild type (*WT*)) or its phosphorylation-defective mutant (with S32A–S36A amino acid substitutions, denoted as *AA*), and 10 μM [γ-^{32}P]ATP in a final volume of 30 μl. When applicable, 5 ng of PP2A were added (*lanes 3–5* and *8–10*). *Lanes 4*, *8*, *9*, and *10* contained 0.1, 0.6, 0.3, and 0.1 μg of Tax, respectively. *Lane 5* contained OA at a concentration of 10 nM. The reactions were carried out at 25 °C for 30 min and terminated by the addition of 6 μl of 6 × SDS gel-loading buffer, and the phosphorylation level of IκBα was evaluated by SDS, 12% PAGE followed by autoradiography. *B*, the IKK kinase reactions were carried out as in *A* except that the IKK immunoprecipitates were incubated with PP2A for 1 h at 25 °C for 1 h (*lanes 1–7*) with or without OA, GST-Tax, or GST-H43Q. In *lane 2*, OA was added together with the substrates after the PP2A preincubation. In *lane 8*, no PP2A was added. One of three repeats of the experiment is shown. *IP*, immunoprecipitates.

NH_2-terminal 100-amino acid residues of Tax (Fig. 3*A*).[2] This region is distinct from the domain responsible for IKKγ binding previously localized to approximately amino acid residues 100–150 previously (Fig. 3*A*) (40). Two Tax mutants, H43Q and K85N, shown to be defective in binding PP2A earlier,[2] and another mutant, H41Q (41), were examined for their interactions with PP2A and IKKγ. As reported previously and confirmed here, H43Q together with H41Q transactivate HTLV-I LTR reporter at a level comparable with that of the wild-type Tax but are considerably attenuated in activating the E-selectin enhancer/promoter driven by two NF-κB binding motifs (Fig. 3*B*). K85N is a much more severe mutation that abrogated both LTR and NF-κB activation of Tax (Fig. 3*B*). As shown in Fig. 3*C*, all three mutants are indeed defective in PP2A binding. Notably, H41Q and H43Q bound IKKγ efficiently. Under the same conditions, K85N also showed reduced but detectable interaction with IKKγ. These results suggest that for IKK activation, Tax-IKKγ interaction is necessary but not sufficient. Most likely, the binding and inhibition of PP2A by Tax is also needed. We also examined two other Tax mutants, M22 (T130A,L131S) and M47 (L319R,L320S), which have been used widely in HTLV-I Tax studies (42). M22 is a mutant impaired in dimerization (43) and IKKγ binding (14, 16, 18). It is unable to activate NF-κB but can transactivate HTLV-I LTR (Fig. 3*B*). By contrast, M47 is defective in LTR activation (because of a defect in P/CAF binding) (42, 44) but shows considerable activity in promoting NF-κB activation (Fig. 3*B*). Consistent with the notion that Tax interacts with both IKK and PP2A to activate IKK and NF-κB, M22 showed little binding to either IKK or PP2A; by contrast, M47, like wild-type Tax, interacted with both IKK and PP2A (Fig. 3*D*). Together, these data support the functional importance of PP2A binding/inhibition by Tax in activating IKK and NF-κB. Finally, although we think distinct domains of Tax interact with PP2A and IKKγ, M22 and K85N mutants appeared to be impaired in both IKKγ binding and PP2A binding. This may be due to conformational alterations of Tax as a result of the amino acid substitutions. Indeed, K85N also failed to trans-activate HTLV-I LTR (Fig. 4*B*), and M22 has been shown previously to be impaired in dimerization (43).

Tax Prevents I-κB Kinase Down-regulation by PP2A—To test the idea that Tax inhibits IKK-associated PP2A and by so doing maintains IKK in an active state, we performed *in vitro* IKK assays using IKK immunoprecipitated from MT-4 cell extracts and a GST fusion containing amino acid residues 1–54 of IκBα, GST-IκBα-(1–54), as a substrate (8). The form of IKK derived from MT-4 cells is activated (by Tax). As expected, it phosphorylated GST-IκBα-(1–54) efficiently but not the AA mutant in which the Ser-32 and Ser-36 of IκBα-(1–54) were substituted by alanine residues (compare Fig. 4*A*, *lanes 6* and *7*). Consistent with the notion that IKK activity is negatively regulated by PP2A, phosphorylation of GST-IκBα-(1–54) *in vitro* became significantly reduced in the presence of exogenously added PP2A (compare Fig. 4*A*, *lanes 2* and *3*). The PP2A inhibitor, okadaic acid (*OA*), effectively blocked this effect of PP2A (Fig. 4*A*, *lane 5*). Likewise, Tax also dampened the decrease in IKK activity caused by PP2A (Fig. 4*A*, *lane 4*) in a dose-dependent manner (Fig. 4*A*, *lanes 8–10*). To show that IKK is inactivated by PP2A directly, we treated the IKK immunoprecipitate with PP2A in the presence or absence of OA for 1 h. The PP2A-pretreated IKK was then incubated with the reaction substrates GST-IκBα-(1–54) and [γ-^{32}P]ATP for 30 min. As expected, IKK pre-incubated with PP2A lost its kinase activity, as reflected by a significant reduction in ^{32}P incorporation into

GST-IκBα-(1–54) (Fig. 4*B*, *lanes 1*, *2*, and *4*). IKK inactivated by PP2A pretreatment lost its activity irreversibly; the addition of OA or Tax after the PP2A preincubation could not reverse the inhibitory effect of PP2A (Fig. 4*B*, compare *lanes 1* and *2*). By contrast, in the reaction where PP2A is inhibited by the simultaneous addition of OA (Fig. 4*B*, *lane 3*) or GST-Tax (Fig. 4*B*, *lanes 6*) during the pretreatment, IKK retained significant activity. The addition of PP2A binding-defective GST-H43Q, however, could not effectively prevent IKK inactivation by PP2A (Fig. 4*B*, *lane 7*). Finally, the addition of GST-Tax in the absence of PP2A did not affect IKK kinase activity significantly (Fig. 4*B*, *lane 8*), consistent with the notion that Tax does not activate IKK directly. In aggregates, these data support the idea that IKK activation by Tax is most likely mediated by its binding and inhibition of PP2A.

DISCUSSION

In this study, we have demonstrated that the IKKγ regulatory subunit, PP2A, and Tax form a stable multiprotein complex in HTLV-I-transformed MT-4 cells and *in vitro*. Furthermore, we show that Tax mutants defective in PP2A binding failed to activate NF-κB despite wild-type levels of binding to IKK, indicating that interaction with both IKKγ and PP2A is necessary for Tax to activate IKK-NF-κB. Finally, evidence is provided to indicate that Tax prevents the active form of IKK isolated from MT-4 cells from inactivation by PP2A *in vitro*. Together, these results provide a mechanistic explanation for the constitutive activation of IKK by Tax; IKK-associated PP2A is responsible for rapid deactivation of IKK; in the context of the IKK·PP2A·Tax complex, PP2A is inhibited by Tax, causing constitutive phosphorylation/activation of IKK; the activated IKK, in turn, promotes phosphorylation and degradation of I-κB, and potent NF-κB activation.

Although interaction between IKK and PP2A can be detected in Tax-null Jurkat cells, their interaction appears weaker than that seen in Tax-positive MT-4 cells (Fig. 1). This is most likely due to the tripartite IKK-PP2A-Tax interaction and is supported by the increased stability of IKK and PP2A interaction *in vitro* when Tax is present (Fig. 2). Whether PP2A may also be recruited specifically to the activated form of IKK is not clear. Although our results indicate that Tax can prevent active IKK from dephosphorylation and inactivation by PP2A, they do not rule out the possibility that Tax may also prevent phospho-I-κBα from dephosphorylation by PP2A. Further experiments are needed to determine whether the latter mechanism contributes to the potent NF-κB activation brought on by Tax.

Two Tax mutants, H41Q and H43Q, are of particular interest. As previously reported by Semmes and Jeang (41) and confirmed here, H41Q and H43Q transactivate HTLV-I LTR reporter at a level comparable with that of the wild-type Tax but is considerably impaired in activating the E-selectin enhancer/promoter, whose activity is driven by two NF-κB binding motifs, suggesting that these two amino acid substitutions impact on a very specific protein-protein interaction critical for NF-κB activation. Our data indicate that the specific defect of H43Q and H41Q lies in an inability to interact with and, most likely, a failure to inhibit PP2A. We have also examined two other Tax mutants, M22 and M47. M22, a mutant that fails to activate NF-κB but can transactivate via HTLV-I LTR, is defective in both IKK and PP2A binding. As expected, M47, a mutant that cannot transactivate LTR because of a defect in P/CAF binding but continues to promote NF-κB activation binds both IKK and PP2A. These data add to the IKK interaction with M47 and the lack thereof with M22 previously reported and support the notion that for NF-κB activation, Tax needs to interact with both IKK and PP2A.

The functional diversity of PP2A is mediated by the regulatory subunits that control its subcellular localization and substrate specificity. Our data indicate that IKKγ binds both PP2A and Tax. Yeast 2-hybrid analysis had shown that Tax and PP2A C-subunit interact directly.[2] Whether other PP2A or IKK subunits also participate in the tripartite interaction remains to be determined. Finally, IKKγΔC, which is deleted for the COOH-terminal Tax binding region, interact with PP2A like full-length IKKγ, indicating that Tax and PP2A binds to distinct domains of IKKγ.

Our recent data indicate that Tax binds the catalytic subunit of PP2A and acts as a noncompetitive inhibitor of PP2A. In reactions where ^{32}P-labeled glycogen phosphorylase *a* is used as a substrate, the k_i (inhibitory constant) of Tax for PP2A is estimated to be ~300 nM. This contrasts with the most common inhibitor of PP2A, okadaic acid, a compound isolated from marine plankton, which has an IC_{50} of 1 nM or lower for PP2A. How can the weaker interaction/inhibition of PP2A by Tax explain the potent activation of IKK and NF-κB? We think the interaction between Tax and IKK is particularly relevant. Even though Tax is a weaker inhibitor for PP2A, the interaction between Tax and IKK would locate Tax in the immediate environment where its interaction with PP2A would be the most effective. In essence, through Tax-IKK interaction, the effective concentration of Tax around IKK is high, thus allowing strong inhibition of PP2A to occur. Indeed, we think Tax does not target all forms of PP2A. Rather, via additional protein-protein interactions such as binding to IKK, it interacts and inhibits sub-populations of PP2A involved in regulating specific signaling pathways. The fact that ~2–4% of PP2Ac co-immunoprecipitated with Tax[2] is consistent with this notion. Finally, it should be pointed out that IKK down-regulation involves not only phosphatases such as PP2A. The highly active IKK phosphorylated in the activation loop undergoes autophosphorylation at multiple sites in the COOH termini (9), which down-modulates its activity. Although it is not clear which form(s) of IKK and which phosphoamino acid residue(s) is the target of PP2A, based on the okadaic acid results and Tax-related studies it may be inferred that the phosphoserine residues in the activation loop are most likely the targets. In summary, the data reported here provide a novel, mechanistic explanation for the potent activation of IKK/NF-κB-signaling pathway by Tax.

Acknowledgments—We thank Drs. M. Di Donato and M. Karin for the plasmids for GST-IκBα-(1–54) and it mutant, GST-IκBα-(1–54)AA and other members of the Giam lab for helpful discussions and critical reading of the manuscript.

REFERENCES

1. Beg, A. A., and Baldwin, A. S. J. (1993) *Genes Dev.* **7,** 2064–2070
2. Liou, H. C., and Baltimore, D. (1993) *Curr. Opin. Cell Biol.* **5,** 477–487
3. Siebenlist, U., Franzoso, G., and Brown, K. (1994) *Annu. Rev. Cell Biol.* **10,** 405–455
4. Neri, A., Chang, C. C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A. T., Chaganti, R. S., and Dalla Favera, R. (1991) *Cell* **67,** 1075–1087
5. Gilmore, T. D. (1992) *Cancer Surv.* **15,** 69–87
6. Beg, A. A., Sha, W. C., Bronson, R. T., and Baltimore, D. (1995) *Genes Dev.* **9,** 2736–2746
7. Klement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., Chen, C. H., Rosen, C. A., and Stewart, C. L. (1996) *Mol. Cell. Biol.* **16,** 2341–2349
8. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554
9. Karin, M., and Ben Neriah, Y. L. (2000) *Annu. Rev. Immunol.* **18,** 621–663
10. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93,** 1231–1240
11. Sun, S. C., Elwood, J., Beraud, C., and Greene, W. C. (1994) *Mol. Cell. Biol.* **14,** 7377–7384
12. Good, L., and Sun, S. C. (1996) *J. Virol.* **70,** 2730–2735
13. Sun, S. C., and Ballard, D. W. (1999) *Oncogene* **18,** 6948–6958
14. Xiao, G., and Sun, S. C. (2000) *Oncogene* **19,** 5198–5203
15. Chu, Z. L., Di Donato, J. A., Hawiger, J., and Ballard, D. W. (1998) *J. Biol. Chem.* **273,** 15891–15894
16. Chu, Z. L., Shin, Y. A., Yang, J. M., Di Donato, J. A., and Ballard, D. W. L. H. (1999) *J. Biol. Chem.* **274,** 15297–15300
17. Kuo, Y. L., Tang, Y., Harrod, R., Cai, P., and Giam, C. Z. (2000) *AIDS Res.*

Hum. Retroviruses **16,** 1607–1612
18. Jin, D. Y., Giordano, V., Kibler, K. V., Nakano, H., and Jeang, K. T. L. H. (1999) *J. Biol. Chem.* **274,** 17402–17405
19. Evans, D. R., and Hemmings, B. A. (1998) *Nature* **394,** 23–24
20. Millward, T. A., Zolnierowicz, S., and Hemmings, B. A. (1999) *Trends Biochem. Sci.* **24,** 186–191
21. Janssens, V., and Goris, J. (2001) *Biochem. J.* **353,** 417–439
22. Ronne, H., Carlberg, M., Hu, G. Z., and Nehlin, J. O. (1991) *Mol. Cell. Biol.* **11,** 4876–4884
23. Mumby, M. C., and Walter, G. (1991) *Cell Regul.* **2,** 589–598
24. Kremmer, E., Ohst, K., Kiefer, J., Brewis, N., and Walter, G. (1997) *Mol. Cell. Biol.* **17,** 1692–1701
25. Groves, M. R., Hanlon, N., Turowski, P., Hemmings, B. A., and Barford, D. (1999) *Cell* **96,** 99–110
26. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883
27. Sun, S. C., Maggirwar, S. B., and Harhaj, E. (1995) *J. Biol. Chem.* **270,** 18347–18351
28. Adya, N., and Giam, C. Z. (1995) *J. Virol.* **69,** 1834–1841
29. Andjelkovic, N., Zolnierowicz, S., Van Hoof, C., Goris, J., and Hemmings, B. A. (1996) *EMBO J.* **15,** 7156–7167
30. Westphal, R. S., Coffee, R. L., Marotta, A., Pelech, S. L., and Wadzinski, B. E. (1999) *J. Biol. Chem.* **274,** 687–692
31. Abraham, D., Podar, K., Pacher, M., Kubicek, M., Welzel, N., Hemmings, B. A., Dilworth, S. M., Mischak, H., Kolch, W., and Baccarini, M. (2000) *J. Biol. Chem.* **275,** 22300–22304
32. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4438–4442
33. Seeling, J. M., Miller, J. R., Gil, R., Moon, R. T., White, R., and Virshup, D. M. (1999) *Science* **283,** 2089–2091
34. Westphal, R. S., Anderson, K. A., Means, A. R., and Wadzinski, B. E. (1998) *Science* **280,** 1258–1261
35. De, B., I, Derua, R., Janssens, V., Van Hoof, C., Waelkens, E., Merlevede, W., and Goris, J. (1999) *Biochemistry* **38,** 16539–16547
36. Tolstykh, T., Lee, J., Vafai, S., and Stock, J. B. (2000) *EMBO J.* **19,** 5682–5691
37. Evans, D. R., and Hemmings, B. A. (2000) *Mol. Gen. Genet.* **264,** 425–432
38. Ogris, E., Du, X., Nelson, K. C., Mak, E. K., Yu, X. X., Lane, W. S., and Pallas, D. C. (1999) *J. Biol. Chem.* **274,** 14382–14391
39. Wei, H., Ashby, D. G., Moreno, C. S., Ogris, E., Yeong, F. M., Corbett, A. H., and Pallas, D. C. (2001) *J. Biol. Chem.* **276,** 1570–1577
40. Xiao, G., Harhaj, E. W., and Sun, S. C. (2000) *J. Biol. Chem.* **275,** 34060–34067
41. Semmes, O. J., and Jeang, K. T. (1992) *J. Virol.* **66,** 7183–7192
42. Smith, M. R., and Greene, W. C. (1990) *Genes Dev.* **4,** 1875–1885
43. Tie, F., Adya, N., Greene, W. C., and Giam, C. Z. (1996) *J. Virol.* **70,** 8368–8374
44. Harrod, R., Kuo, Y. L., Tang, Y., Yao, Y., Vassilev, A., Nakatani, Y., and Giam, C. Z. (2000) *J. Biol. Chem.* **275,** 11852–11857
45. Zhao, L. J., and Giam, C. Z. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11445–11449