

## Activation of Cyclin-dependent Kinase 2 by Full Length and Low Molecular Weight Forms of Cyclin E in Breast Cancer Cells\*

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**Cyclin E, a positive regulator of the cell cycle, controls the transition of cells from G<sub>1</sub> to S phase. Deregulation of the G<sub>1</sub>-S checkpoint contributes to uncontrolled cell division, a hallmark of cancer. We have reported previously that cyclin E is overexpressed in breast cancer and such overexpression is usually accompanied by the appearance of low molecular weight isoforms of cyclin E protein, which are not present in normal cells. Furthermore, we have shown that the expression of cyclin E low molecular weight isoforms can be used as a reliable prognostic marker for breast cancer to predict patient outcome. In this study we examined the role of cyclin E in directly activating cyclin-dependent kinase (CDK) 2. For this purpose, a series of N-terminal deleted forms of cyclin E corresponding to the low molecular weight forms detected only in cancer cells were translated *in vitro* and mixed with cell extracts. These tumor-specific N-terminal deleted forms of cyclin E are able to activate CDK2. Addition of cyclin E into both normal and tumor cell extracts was shown to increase the levels of CDK2 activity, along with an increase in the amount of phosphorylated CDK2. The increase in CDK2 activity was because of cyclin E binding to endogenous CDK2 in complex with endogenous cyclin E, cyclin A, or unbound CDK2. The increase in CDK2 phosphorylation was through a pathway involving cyclin-activating kinase, but addition of cyclin E to an extract containing unphosphorylated CDK2 can still lead to increase in CDK2 activity. Our data suggest that the ability of high levels of full-length and low molecular weight forms of cyclin E to activate CDK2 may be one mechanism that leads to the constitutive activation of cyclin E-CDK2 complexes leading to G<sub>1</sub>/S deregulation and tumor progression.**

Cyclin E has an important role in the regulation of the restriction point transition in the cell cycle. In normal cells, cyclin E expression occurs during a brief window of time from late G<sub>1</sub> into early S phase, with a peak expression level near the restriction point (1). Kinase activity of cyclin E-CDK2<sup>1</sup> complexes are also at maximum levels near the restriction point

(2, 3). Cyclin E activation of CDK2 is an essential step in cell cycle progression through the restriction point. There are several examples that demonstrate the importance of cyclin E-CDK2 complexes in transition from G<sub>1</sub> to S phase. For example, functional knockout of cyclin E by injection of anti-cyclin E antibodies into fibroblast cells causes cell arrest in G<sub>1</sub> of the cell cycle (4, 5). Conversely, the overexpression of cyclin E protein results in a decrease in the length of G<sub>1</sub>, along with a decreased cell size (6, 7). Similarly, expression of a dominant negative CDK2, which is deficient in its ability to phosphorylate substrates, in U2OS osteosarcoma, Saos-2 osteosarcoma, C33A cervical carcinoma, or T98G glioblastoma cells, causes G<sub>1</sub> phase arrest (8).

Cyclin E binding is not the only step required for CDK2 activation. The activity of CDK2 is also dependent upon its phosphorylation state at the amino acid Thr-160 (9); mutation of the Thr-160 site abolishes all CDK2 kinase activity *in vitro*, pinpointing it as a site of activating phosphorylation (9). Mutation of the Tyr-15 or Thr-14 sites increases kinase activity, suggesting that these amino acids are the site of inhibitory phosphorylation (9). Phosphorylation at Thr-160 is performed by cyclin-activating kinase (CAK), a complex composed of cyclin H and CDK7 (10). CAK was first identified as a factor present in cell extracts that could activate purified CDC2 *in vitro* (11, 12). Purification of the CAK complex led to the identification of its two subunits, cyclin H and CDK7 (13, 14). CDK7 is the catalytic subunit of CAK, which phosphorylates CDK substrates (13, 15). CDK7 does not contain catalytic activity alone; it requires binding with cyclin H to become active (4, 14, 15). *In vitro* synthesized cyclin H-CDK7 complexes were shown to phosphorylate both CDK2 and CDC2 (14). Negative regulation of CDK phosphorylation at Thr-160 is mediated by the CDK-associated phosphatase (KAP) (17). KAP has been shown to dephosphorylate Thr-160 of CDK2 *in vitro*, by direct association with the CDK (17). The dephosphorylation event is blocked by cyclin binding, but KAP is still able to associate with the cyclin-CDK complex as demonstrated by immunoprecipitation (17). Inhibitory phosphorylation of Thr-14 and Tyr-15 of CDK2 is a function of Wee1, mammalian homologue of the yeast wee1 (18). Mammalian cells also have a homologue to yeast CDC25, which is a phosphatase that removes the inhibitory phosphorylation at Thr-14 and Tyr-15 (18). Together, the phosphorylation state of CDK2 at three separate amino acids under the control of CAK (Thr-160 activating phosphorylation), KAP (Thr-160 inhibitory dephosphorylation), Wee1 (Tyr-15, Thr-14 inhibitory phosphorylation), and CDC25 (Tyr-15, Thr-14 activating dephosphorylation), give the cell additional levels of control over CDK2 activity and cell cycle progression, beyond cyclin binding. However, cyclins are the major positive regulators of the cell cycle and determinants of CDK activation within the cell. Furthermore, many cyclins have been shown to

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<sup>1</sup> The abbreviations used are: CDK, cyclin-dependent kinase; CAK, cyclin-activating kinase; KAP, CDK-associated phosphatase; LMW, low molecular weight; ID, immunodepleted.

have a strong involvement in tumor development, including cyclins E, D, and A (19, 20).

The role of cyclin E as a CDK2 activator in control of restriction point transition in the cell cycle (21) makes cyclin E an excellent candidate as a protein involved in tumor development. Cyclin E deregulations are commonly seen in tumor cell lines, as well as tumor tissues from breast cancer patients (20, 22). Cell cycle regulation of cyclin E expression is commonly lost in tumor cells, leading to constitutive cyclin E expression throughout the cell cycle (23–25). Tumor cells commonly overexpress cyclin E protein (26), which is accompanied by the appearance of low molecular weight (LMW) isoforms, which are not present in normal cells (26–28). Our previous studies suggest that these LMW forms are generated in breast tumor cells by post-translational modification of the full-length cyclin E by the elastase class of serine proteases (26, 29). In addition to the full-length protein at 50-kDa, we have identified five LMW forms that are generated mainly by proteolysis at two domains in cyclin E, which results in two pairs of closely migrating doublets (EL-2/EL-3 and EL-5/EL-6). These isoforms, when in complex with CDK2, are biochemically hyperactive, as evidenced by their enhanced ability to phosphorylate histone H1 and GST-Rb (29) compared with full-length cyclin E-CDK2 complex. When transfected into normal mammary epithelial cells, the LMW forms have significant mitogenic effect, readily inducing cells to enter the cell cycle (26, 29, 30). Clinically, we have also shown that the presence of the LMW forms in breast cancer patients is associated with increasing grade and stage (27), as well as a significantly worse prognosis (31, 32).

The question we address in this work is whether overexpression of cyclin E in tumor cells has the ability to directly activate CDK2. The strategy we use is to mix *in vitro* translated cyclin E (full-length or the LMW forms) with tumor cell extracts and assess the role of the exogenous cyclin E in activating endogenous CDK2. Here we provide evidence that the addition of cyclin E (both full-length and LMW) results in the activation of CDK2 in both normal or tumor cell extracts.

#### MATERIALS AND METHODS

**Vectors and Cloning**—The pCDNA3.1 vector alone or vector containing FLAG-tagged cyclin EL, E, T1, T2, or T3 was used to produce cyclin E protein products *in vitro*. The generation of all vectors was described previously (26).

**Preparation of Cell Extracts**—Cell lysates were prepared by resuspension in protease phosphatase buffer (25  $\mu$ g/ml leupeptin, 25  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml pepstatin, 1 mM benzamide, 10  $\mu$ g/ml soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.5 mM sodium orthovanadate) in Solution A (27 mM Hepes, 10 mM glucose, 3.3 mM KCl, 13 mM NaCl, 0.5 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.6), to prevent protein degradation. Cell lysates were homogenized by sonication followed by centrifugation at  $100,000 \times g$ . The soluble supernatant fraction was removed, and protein concentrations were determined using the Bradford assay (Bio-Rad).

**Transcription/Translation and Mixing**—Synthesis of the five FLAG-tagged cyclin E constructs was examined using a coupled *in vitro* rabbit reticulocyte transcription/translation (TnT) kit (Promega). pCDNA3.1 plasmid containing the cyclin E inserts (EL, cyclin E, T1, T2, or T3) or no insert was added to the reaction mix at 0.02  $\mu$ g/ $\mu$ l with 1 mM amino acid mixture for 90 min at 30 °C. TnT samples were then used in mixing experiments. For mixing, 2  $\mu$ l (~3 ng) of *in vitro* synthesized cyclin EL, cyclin E, T1, T2, or T3, or pCDNA3.1 with no insert was added to tumor MDA-MB-157 or normal 76N cell extracts. Either 300 or 37.5  $\mu$ g of cell extract was used per mixing, as indicated per experiment. Mixing reactions were performed at 4 °C overnight.

The amount of protein produced by the TnT reaction was calculated as follows for a 50-kDa protein with 10 methionine (Met) residues per protein (Promega). 2  $\mu$ l of 10mCi/ml [ $^{35}\text{S}$ ]Met with a specific activity of 1200Ci/mmol was added to the reaction (2  $\mu$ l)/(10  $\mu$ Ci/ $\mu$ l)/(1200 Ci) = 16.7 pmol of [ $^{35}\text{S}$ ]Met. 135 pmol of cold Met was also added to a 50- $\mu$ l TnT reaction, giving a concentration of 151.7 pmol of total Met. 10% incorporation Met yields 15 pmol of Met incorporated into 10 Met sites per protein. (50,000 pg)/(10 pmol Met)  $\times$  (15 pmol Met incorporated)

yields 75 ng of protein per 50  $\mu$ l of TnT reaction (Promega). This is an estimation of the amount of protein produced by the TnT reaction, independent of the number of Met residues per protein.

**Immunoprecipitation and Kinase Assays**—For immunoprecipitations and H1 kinase assays, 300 or 37.5  $\mu$ g of cell extract was used per immunoprecipitation with either polyclonal antibody to FLAG (Santa Cruz Biotechnology, Inc.) or CDK2 (29). 7.5  $\mu$ l of crude rabbit polyclonal CDK2 serum was added to either 300 or 37.5  $\mu$ g as indicated for each experiment. For the FLAG immunoprecipitation, 0.01  $\mu$ g of FLAG polyclonal antibody was added to 300  $\mu$ g for immunoprecipitation. The antibody binding reaction was carried out in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40). The protein/antibody mixture was incubated with protein A-Sepharose for 1 h and then washed four times with lysis buffer and four times with kinase buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin). Immunoprecipitates were then analyzed by either Western blot analysis or H1 kinase assay. For Western blot analysis, protein A-Sepharose beads from immunoprecipitation were boiled in SDS, and proteins were separated on a 10% SDS-PAGE gel as described previously (26). Western blotting was performed with monoclonal anti-CDK2 (1  $\mu$ g/ml), anti-cyclin E (0.1  $\mu$ g/ml), anti-p21 (1  $\mu$ g/ml), anti-p27 (1  $\mu$ g/ml), anti-cyclin A (1  $\mu$ g/ml), or anti-CDK7 (1  $\mu$ g/ml) antibodies (Santa Cruz Biotechnology, Inc.). For the kinase assay, immunoprecipitates were incubated with kinase buffer (5  $\mu$ g of histone H1, 60  $\mu$ M ATP,  $\mu$ Ci of [ $^{32}\text{P}$ ]dATP, 50 mM Tris-HCl, pH 7.5, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 70 mM NaCl, 0.1 mg/ml bovine serum albumin) in a final volume of 50  $\mu$ l at 37 °C for 30 min. H1 kinase reactions were analyzed on a 10% SDS-PAGE gel. The gel was then stained, de-stained, dried, and exposed to x-ray film. For quantification, the histone H1 bands were excised, and radioactivity was measured by Cerenkov counting on a Beckman LS-9000 Scintillation Counter.

**Immunodepletion**—For these analyses the antibodies were first conjugated to beads before being used for immunoprecipitation. Non-affinity-purified anti-cyclin E rabbit serum polyclonal (0.33  $\mu$ l per 1  $\mu$ l of beads), affinity-purified cyclin A rabbit serum polyclonal (0.03  $\mu$ l per 1  $\mu$ l of beads), or CDK7 (0.17  $\mu$ g/ $\mu$ l) polyclonal antibodies were incubated with protein A-Sepharose beads for 1 h to conjugate the antibody to the beads. Beads were washed three times with lysis buffer and added to 1 mg of cell extract. Antibody-bead conjugates or beads alone were incubated with cell extracts for 1 h at 4 °C for antibody-antigen binding to take place, removed, and replaced with fresh beads for a total of three rounds of depletion. Antibody-bead conjugates were washed four times with lysis buffer and four times with kinase buffer to remove nonspecific protein interactions. Protein concentrations of immunodepleted cell extracts were then determined using the Bio-Rad Bradford protein assay. Immunodepleted cell extracts were then used in mixing experiments with cyclin EL and T3. 3 ng of *in vitro* synthesized cyclin EL and T3 was mixed with 37.5  $\mu$ g of depleted cell extracts, immunoprecipitated with anti-CDK2 antibodies, and subjected to Western blot analysis with anti-cyclin E, anti-cyclin A, anti-CDK2, anti-CDK7 antibodies, or histone H1 kinase assay.

**Expression of Wild-type and Mutant CDK2 in Insect Cells**—Recombinant virus expressing wild-type CDK2 was generated using the BaculoGold vector system (PharMingen) as described previously (29). Virus expressing hemagglutinin-tagged T160A mutant CDK2 was obtained as a gift from the laboratory of J. Wade Harper (Baylor College of Medicine, Houston, TX). Sf9 insect cells were infected with virus at a multiplicity of infection number of 1 and harvested at 65 h post-infection. Protein extracts were subjected to Western blotting, immunoprecipitation, and H1 kinase analysis as described above. The Thr-160 phospho-specific CDK2 antibody was obtained from Cell Signaling Technology (Beverly, MA).

#### RESULTS

To examine the ability of the LMW forms of cyclin E (*i.e.* cyclin EL1–6) to activate CDK2, a series of five epitope-tagged (*i.e.* FLAG), full-length, or N-terminal truncated cyclin E cDNAs that correspond to different LMW forms were generated (Fig. 1A). These forms, described previously (26), include cyclin EL (EL1), cyclin E, T1 (EL2/3), T2 (EL5/6), and T3. Cyclin EL, T1, and T2 correspond exactly to the forms of cyclin E found endogenously in tumor cells, which we refer to as EL-1 (51 kDa), EL2/3 (44 kDa), and EL5/6 (40 kDa), respectively (29). Cyclin E refers to the 45-kDa form originally cloned via yeast complementation assays (1, 33). We recently provided evidence that this form, an alternatively spliced variant of cyclin EL,

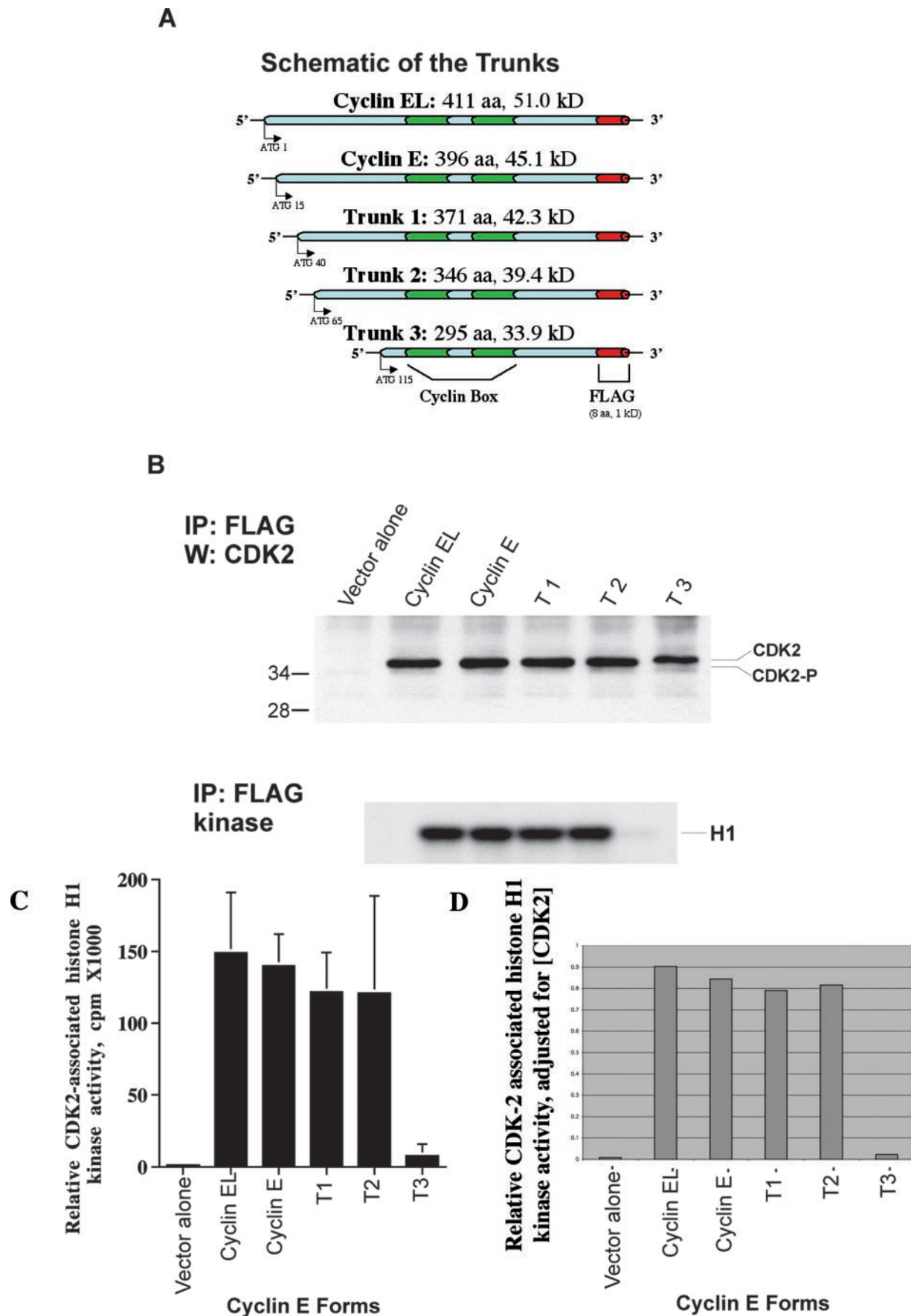


FIG. 1. **Cyclin E LMW forms bind to and activate CDK2.** 2  $\mu$ l of each cyclin E form synthesized using an *in vitro* rabbit reticulocyte transcription translation kit (TNT) was mixed with 300  $\mu$ g of tumor cell extract at 4 °C overnight. As a negative control, pCDNA3.1 vector with no insert was used in the TNT reaction and mixed with cell extracts. Mixes were then immunoprecipitated with anti-FLAG antibodies and subjected to CDK2 Western blot analysis (B) and H1 kinase assay (C). Kinase activity is normalized by [CDK2] in D. Panel A depicts the schematic diagram of the constructs used.



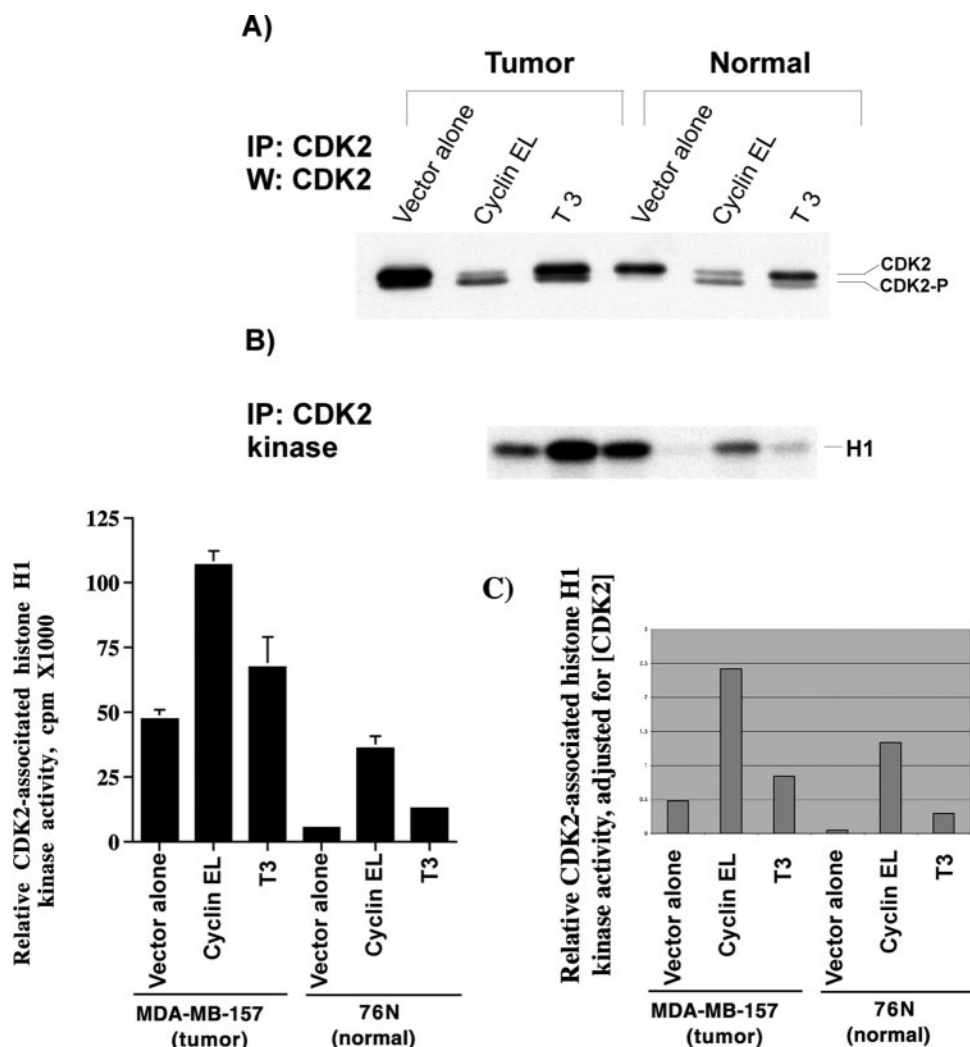


FIG. 2. Cyclin E can activate CDK2 from either normal or tumor cell extracts. 2  $\mu$ l of cyclin EL or T3 was mixed with 37.5  $\mu$ g of tumor or normal cell extract at 4 °C overnight. Mixes were then immunoprecipitated with anti-CDK2 antibodies and subjected to CDK2 Western blot analysis (A) and H1 kinase assay (B). Kinase activity has been normalized by [CDK2] in C.

does not give rise to any major protein products in human cell lines (29). *In vitro* expression of the cyclin EL, T1, T2, and T3 span the size range of LMW forms seen in tumor cells from 52 kDa to 37 kDa as we have already described (26). To determine whether N-terminal deleted cyclin E forms are able to bind to and activate CDK2 *in vitro*, the *in vitro* translated products of the five cyclin E forms were each mixed separately with MDA-MB-157 tumor cell extract. The cell extract mixture was then immunoprecipitated with anti-FLAG antibodies and subjected to both CDK2 immunoblotting (Fig. 1B) and H1 kinase assay (Fig. 1C). The results revealed that all five cyclin E forms were able to co-immunoprecipitate with CDK2, suggesting each was able to bind to CDK2 (Fig. 1B). However only EL, E, T1, and T2 were able to activate CDK2, as shown by their ability to phosphorylate histone H1, whereas T3, despite binding with CDK2, was unable to cause activation (Fig. 1C). Additionally, T3 can only bind to the inactive, slower migrating form of CDK2 (Fig. 1C). The identity of the active, phosphorylated, faster-migrating band of CDK2 has been determined previously (9). Specifically, the authors show that the activating phosphorylation of CDK2 results in the appearance of the faster migrating CDK2 band. They also indicated that the phosphorylated residues in CDK2 can be identified based on their predicted mobilities. Additionally the authors, through metabolically labeling of cells transfected with T160A form of CDK2 were able to unequivocally label the faster migrating band as the Thr-160 form

of CDK2. The results with T3 are consistent with previous reports indicating cyclin binding to CDK2 is an independent event from substrate recognition and phosphorylation (34).

The results obtained in Fig. 1 provide the opportunity to explore the *in vitro* biochemical consequences of expression of both full-length cyclin E (EL), which can activate CDK2, and an N-terminal deleted cyclin E (T3), which cannot activate CDK2, using normal and tumor cell extracts. The kinase assay for Fig. 1 was performed with anti-FLAG antibody, to separate the kinase activity of the *in vitro* translated cyclin E from the endogenous cyclins E and A, which also complex with CDK2. To compare the effects of cyclin EL and T3 on the overall kinase activity in normal and tumor cells, an antibody to CDK2 was employed to capture the immune complexes (Fig. 2). For these experiments 3 ng of *in vitro* translated cyclin EL and T3 were mixed with 37.5  $\mu$ g of tumor and normal cell extract, immunoprecipitated with anti-CDK2 antibodies, and subjected to Western blot analysis (Fig. 2A) and H1 kinase assay (Fig. 2B). The level of phosphorylated CDK2 captured is increased by the addition of cyclin EL (Fig. 2A, Tumor, Cyclin EL; Normal, Cyclin EL), and to a lesser extent by the addition of T3 (Fig. 2A, Tumor, T3; Normal, T3) in both normal and tumor extracts. Tumor cells have a higher level of endogenous phosphorylated CDK2 than normal cells before addition of any cyclin E (Fig. 2A, Tumor, Vector alone; Normal, Vector alone). Addition of cyclin EL causes an increase in CDK2 activity from about

45,000 to 110,000 cpm in tumor extracts (Fig. 2B, *Tumor, Cyclin EL*) and from about 6,000 to 35,000 cpm in normal cell extracts (Fig. 2B, *Normal, Cyclin EL*). Addition of T3, on the other hand, resulted in less of an increase in CDK2 activity, up to 70,000 cpm in tumor cell extracts (Fig. 2B, *Tumor, T3*) and up to 12,000 cpm in normal extracts (Fig. 2B, *Normal, T3*), showing only a 2-fold induction of CDK2 activity over basal levels. Hence, binding to non-phosphorylated CDK2 also causes an increase in CDK2 activity, albeit at a lower level, which may explain the low levels of CDK2 kinase activity seen with T3 despite the fact that T3 does not complex to phosphorylated CDK2. These results suggest that when exogenous cyclin E protein, in the form of *in vitro* synthesized cyclin EL, was added to the cell extracts, two events occurred: (i) the amount of phosphorylated (active) CDK2 was higher than in normal and tumor cells without exogenous cyclin E, and (ii) the overall CDK2 activity increased 3–6-fold over basal levels in normal and tumor cells. In both cases tumor cells have a higher level of basal CDK2 levels and activity than normal cells. These two results raise questions as to what is causing the increase in phosphorylated CDK2 captured by immunoprecipitation and what is causing the increase in CDK2 activity. There are several possible sources for the increase in CDK2 activity following addition of cyclin E into cell extracts. These potential sources are (i) an intrinsic activity in the TnT rabbit reticulocyte lysate, (ii) binding of exogenous cyclin E to free monomeric CDK2, (iii) binding of exogenous cyclin E to CDK2 competing off endogenous cyclin A or cyclin E or, (iv) increased stability of active CDK2 because of bound exogenous cyclin E.

The first and most logical source of increased kinase activity examined was a potential intrinsic activity within the TnT reaction, which was used in each mix. The TnT reaction mix used to synthesize cyclin E *in vitro* contains rabbit reticulocyte lysate. Rabbit reticulocyte lysate, being a cell extract itself, would contain cell cycle proteins that could potentially contribute to histone phosphorylation. To address this possibility, rabbit reticulocyte lysate (TnT) alone or mixed with cyclin EL or T3 was immunoprecipitated with CDK2, and subjected to H1 kinase assay (Fig. 3). 300  $\mu$ g of tumor cell extract alone (in the presence of TnT mix) or mixed with cyclin EL TnT or T3 TnT was also immunoprecipitated with CDK2 and subjected to H1 kinase assay in the same experiment to compare side by side contribution of TnT mix to CDK2 activity from cell extracts (Fig. 3). TnT alone or mixed with T3 showed only background activity at about 1,000 cpm (Fig. 3). TnT mixed with cyclin EL showed a slight increase over background levels. However, an increase in CDK2 activity from 57,000 (tumor extracts alone plus TnT mix) to 135,000 cpm occurred from addition of cyclin EL TnT to tumor extracts. Addition of T3 TnT to extracts caused an increase from 57,000 to 82,000 cpm. The difference in activity caused by addition of cyclin EL to cell extracts is 120,000 cpm higher than the activity in TnT mixed with cyclin EL (Fig. 3, *TnT Cyclin EL*), suggesting that the activity in the TnT cannot account for the increase in CDK2 activity.

Having ruled out the effect of TnT being solely responsible for the increase in CDK2 activity, we next examined the effects of endogenous cyclin E/A on the increase in CDK2 activity. Endogenous CDK2 can exist in at least three complex states: bound to cyclin A, bound to cyclin E, or free/unbound. Exogenous cyclin E added to cell extracts could potentially bind to CDK2 from any of the three complex states, such as binding to CDK2 that has been sequestered away from cyclin A. The strategy used to determine the effects of cyclin A on the increase in activity was to remove cyclin A from extracts by immunodepletion and then examine CDK2 activity after mixing with cyclin EL. Cyclin A was immunodepleted (ID) by three

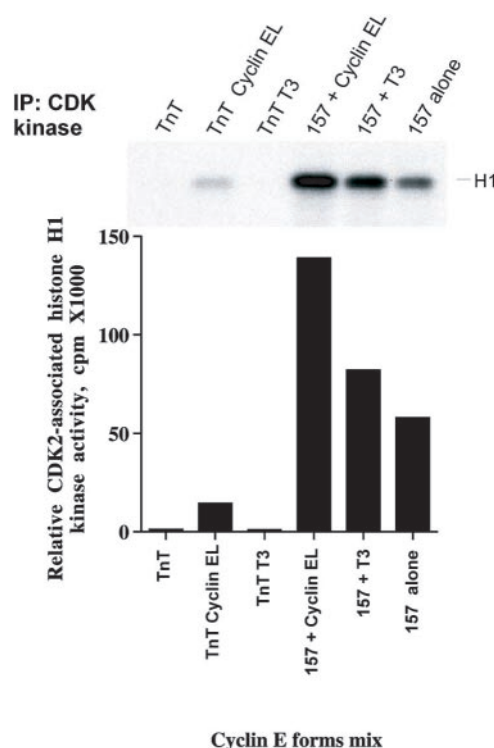
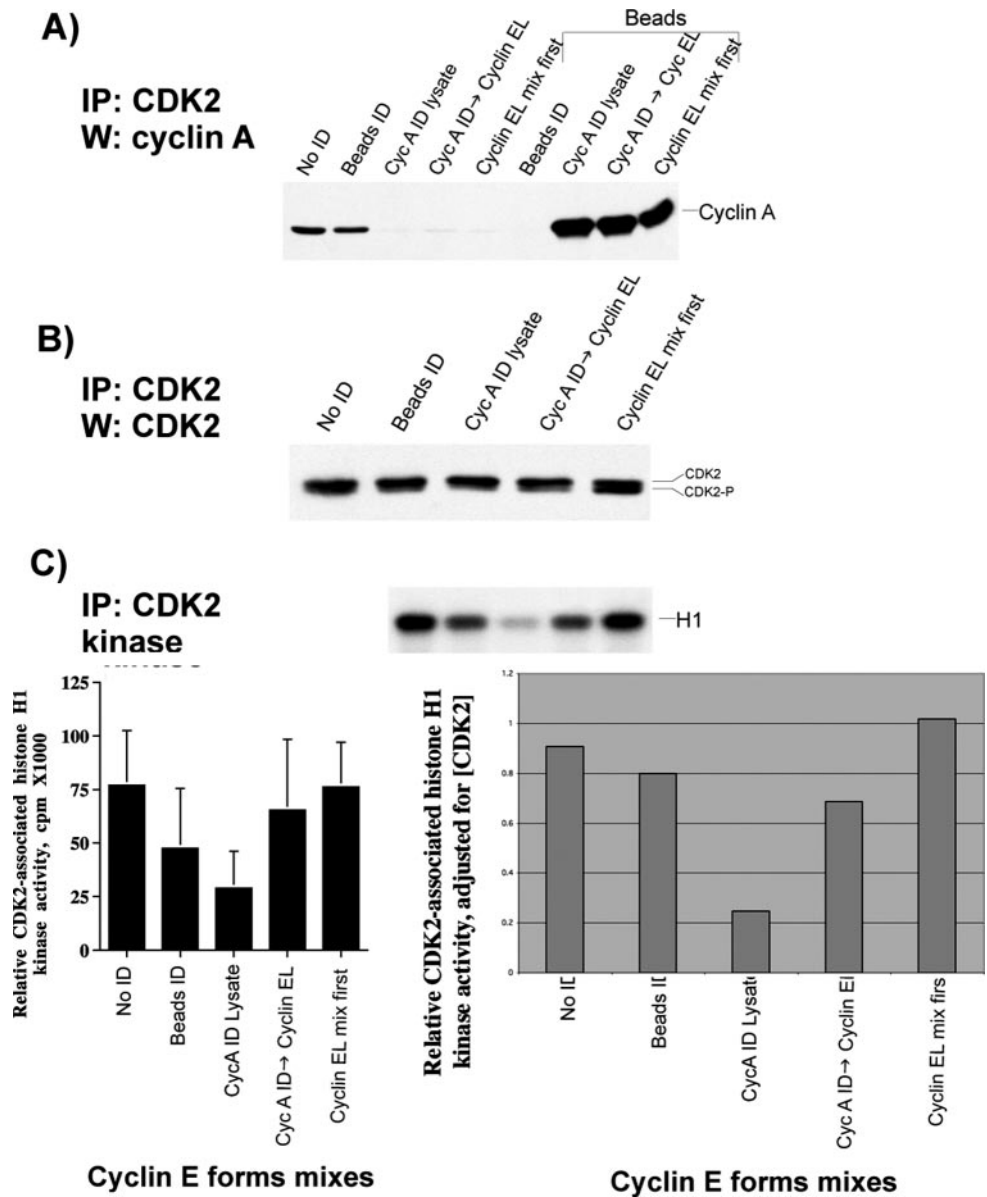


FIG. 3. **H1 kinase activity of TnT reaction mix.** TnT reaction mix alone, TnT reaction mix containing cyclin EL or T3 alone, or cyclin EL or T3 mixed with MDA-MB-157 (*i.e.* 157) cell extracts were incubated overnight at 4 °C. Mixes were immunoprecipitated with anti-CDK2 antibodies and subjected to H1 kinase assay.

rounds of cyclin A immunoprecipitation from cell extracts, and the ID extracts were mixed with full-length cyclin E (cyclin EL). Cyclin A ID/cyclin EL mixes were then immunoprecipitated with anti-CDK2 antibodies, subjected to Western blot analysis with anti-CDK2 antibodies, and H1 kinase assay (Fig. 4). To ensure that most of the cyclin A was ID, the cyclin A-depleted cell extracts, along with the control extracts immunodepleted with protein-A beads alone, were examined by Western blot analysis with anti-cyclin A antibodies (Fig. 4A). A slight amount of cyclin A was lost during the control ID using beads alone (Fig. 4A, *no ID* compared with *ID beads alone*), which was probably because of nonspecific protein binding. No cyclin A was detected in the cell extracts following depletion (Fig. 4A, *cycA ID TnT, Cyclin EL, or mix first*). The cyclin A that was removed from the cell extracts can be detected from the cyclin A antibody beads used in the ID (Fig. 4A, *cycA ID beads*). These results show that the cyclin A ID removed almost all cyclin A from the cell extracts and that the protein A beads alone remove only a small amount of cyclin A in the control extracts.

Cyclin EL protein was added to the extracts before and after depletion with cyclin A antibodies to compare CDK2 levels and CDK2 kinase levels with and without the presence of cyclin A-CDK2 complexes. CDK2 protein that remained after depletion of cyclin A, and its associated kinase activity, can be seen in Fig. 4, B and C. Any CDK2 that remains after cyclin A depletion is mostly either in complex with endogenous cyclin E or unbound. A moderate decrease in CDK2 kinase activity from 75,000 to 50,000 cpm occurred following the control immunodepletion with beads alone (Fig. 4C, *no ID, beads alone ID*), which is probably a result of the CDK2 lost in the control ID (Fig. 4B, *no ID, beads alone ID*). Depletion of cyclin A caused a decrease in kinase activity to 30,000 cpm in the cell extracts without addition of any exogenous cyclin E (Fig. 4C, *cycA ID TnT*). However, when cyclin EL was added to extracts depleted



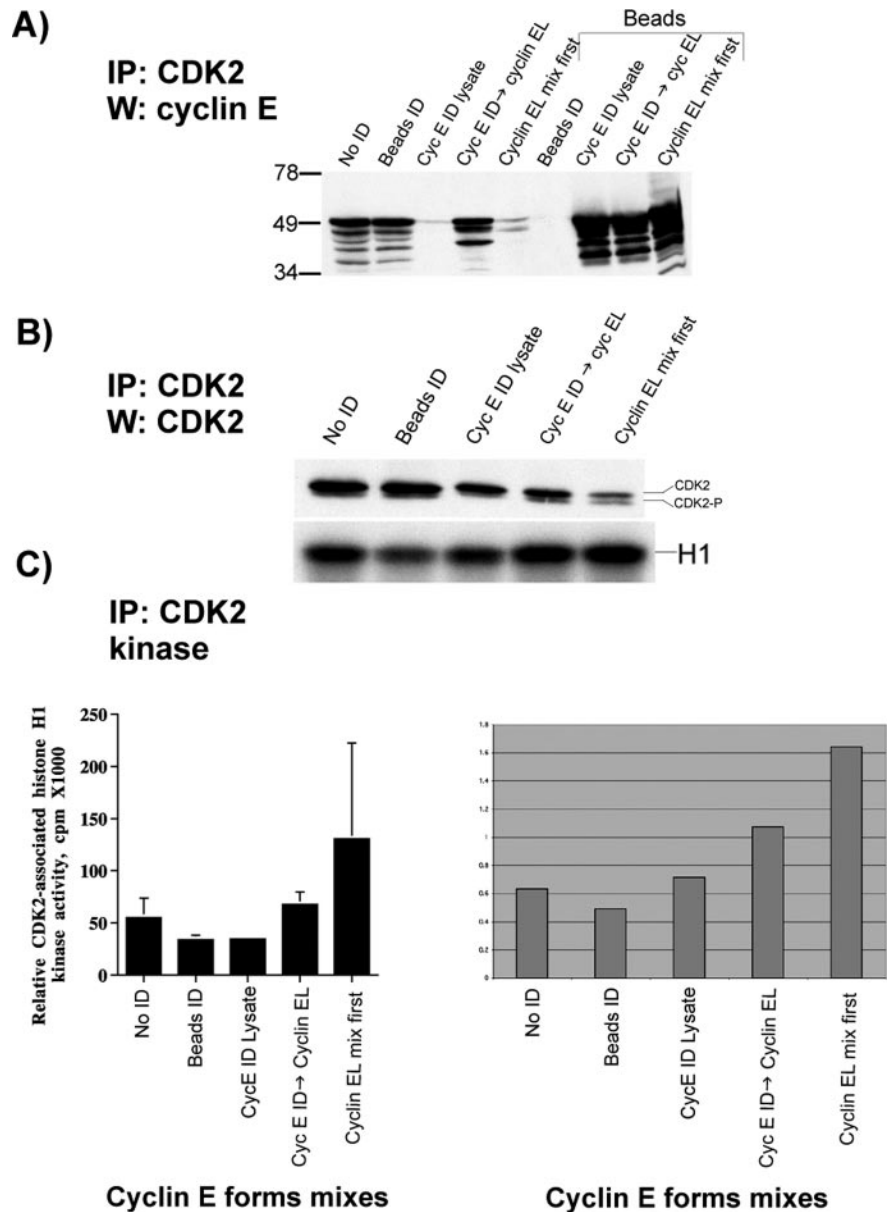
**FIG. 4. Activation of CDK2 by exogenous cyclin E is, in part, because of displacement of endogenous cyclin A from CDK2.** Cyclin EL was mixed before and after immunodepleting tumor cell extracts of cyclin A overnight at 4 °C. Three rounds of immunodepletion were carried out for each mix using anti-cyclin A polyclonal antibodies. As a control, the immunodepletion was carried out using protein A beads alone. Mixes were then subjected to cyclin A (A) or CDK2 (B) Western blot analysis or immunoprecipitated with anti-CDK2 antibodies and subjected to H1 kinase assay (C). *No ID*, extracts without immunodepletion; *Beads ID*, extracts immunodepleted with beads only; *Cyc A ID lysate*, the supernatant of extracts after being immunodepleted; *Cyc A ID with cyclin EL*, cyclin A immunodepleted extracts were mixed with TNT cyclin EL; *Cyclin EL mix first*, extracts were first mixed with TNT cyclin EL and then immunodepleted for cyclin A. The right figure in panel C represents kinase activity normalized by [CDK2].

of cyclin A, the CDK2 activity increased to 65,000 cpm (Fig. 4C, *cycA ID TNT* and *Cyclin EL*). Addition of cyclin EL to extracts prior to being depleted of cyclin A resulted in a further increase of CDK2 activity up to 75,000 cpm (Fig. 4C, *cycA ID TNT, mix first*). Comparing the CDK2 kinase levels of cell extracts mixed with cyclin EL before and after cyclin A depletion demonstrates that the kinase activity that is because of cyclin EL competing cyclin A for CDK2 binding is about 10,000 cpm. The proportion of CDK2 activity because of CDK2-cyclin A complexes represents only a fraction of CDK2 bound to exogenous cyclin E. Because addition of cyclin EL to extracts depleted of cyclin A/CDK2 can still cause an increase in CDK2 activity (Fig. 4C, *no ID* compared with *cycA ID Cyclin EL*), it suggests that the increase CDK2 activity is from other sources as well.

To determine how much of the increase in CDK2 activation is because of the competition with endogenous cyclin E to binding

to CDK2, cyclin E was immunodepleted from cell extracts, and the ID extracts were mixed with *in vitro* translated cyclin EL (Fig. 5). Mixes were then immunoprecipitated with anti-CDK2 antibodies, subjected to Western blot analysis with anti-CDK2 antibodies, and H1 kinase assay (Fig. 5). As in the cyclin A immunodepletion (Fig. 4), cyclin EL was added to extracts before and after endogenous cyclin E was depleted, as a way to compare CDK2 protein levels and kinase activity with and without the presence of cyclin E-CDK2 complexes.

Only a small portion of cyclin E was lost during the control ID using beads alone without cyclin E antibody (Fig. 5A, *no ID* compared with *ID beads alone*). Nearly all of the endogenous cyclin E was successfully depleted (Fig. 5A, *cycE ID*), and *cycE ID cyclin EL* shows the cyclin EL cyclin E added back after depletion. When cyclin EL was added before depletion, a small amount of cyclin E protein remained after the ID, probably



**FIG. 5. Activation of CDK2 by exogenous cyclin E is, in part, because of displacement of endogenous cyclin E from CDK2.** Cyclin EL was mixed before and after immunodepleting tumor cell extracts of cyclin E overnight at 4 °C. Three rounds of immunodepletion was carried out for each mix using anti cyclin E polyclonal antibodies. As a control, the immunodepletion was carried out using protein A beads alone. Mixes were then subjected to cyclin E (A) or CDK2 (B) Western blot analysis or immunoprecipitated with anti-CDK2 antibodies and subjected to H1 kinase assay (C). *No ID*, extracts without immunodepletion; *Beads ID*, extracts immunodepleted with beads only; *Cyc E ID lysate*, the supernatant of extracts after being immunodepleted; *Cyc E ID → cyclin EL*, cyclin E immunodepleted extracts were mixed with TNT cyclin EL; *Cyclin EL mix first*, extracts were first mixed with TNT cyclin EL and then immunodepleted for cyclin E. The right figure in panel C represents kinase activity normalized by [CDK2].

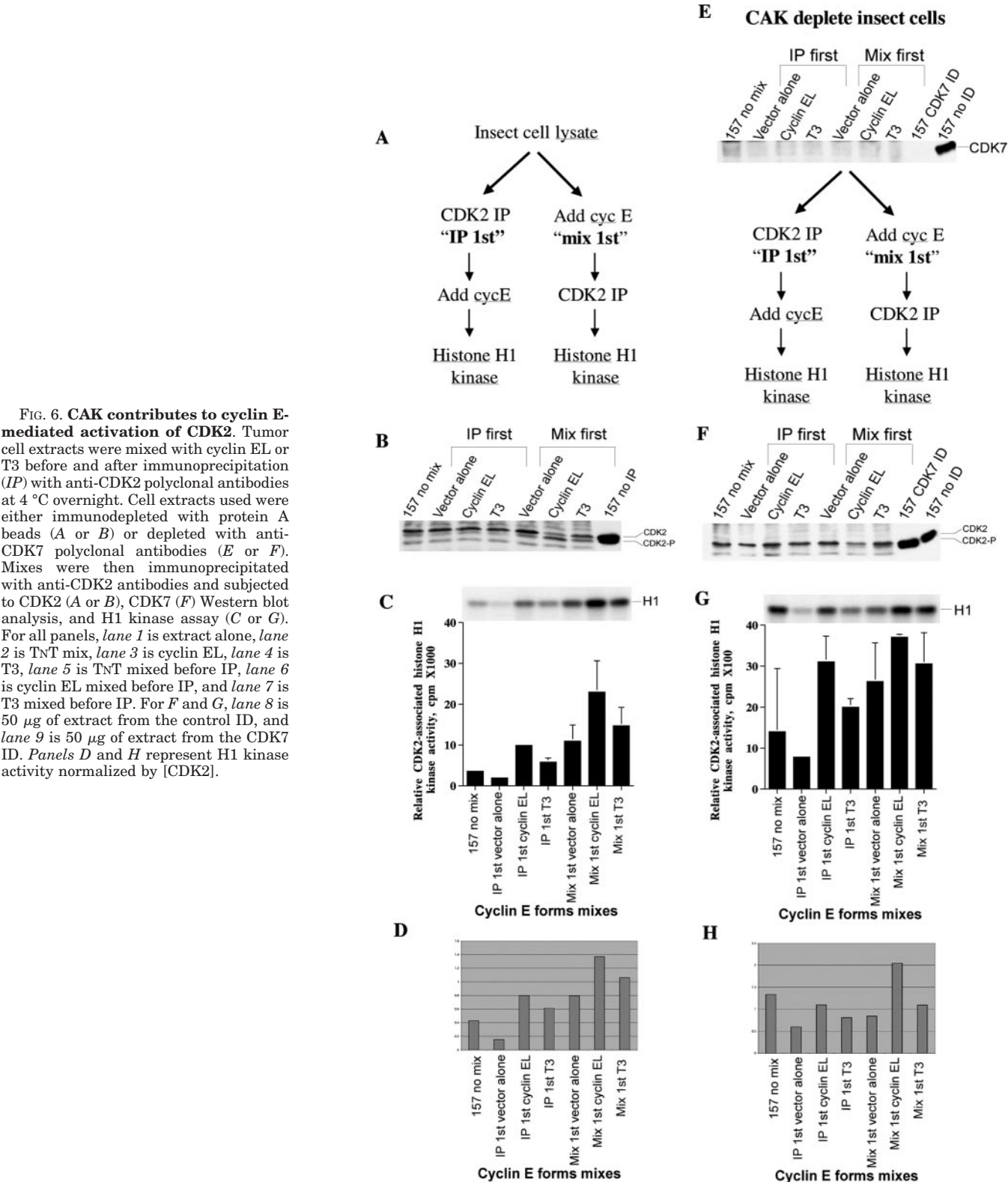
because the high levels of cyclin E exceeded the ability of the antibodies to deplete all cyclin E. The cyclin E that was removed from the cell extracts can be seen from the cyclin E antibody beads used in the ID (Fig. 5A, *cycE beads ID*). The small amount of cyclin E non-specifically removed from the cell extracts by the control ID with beads alone can be seen in Fig. 5A, *beads alone ID*. These results show that the cyclin E ID removed almost all endogenous cyclin E from the cell extracts and that the protein A beads alone removed only a small amount of cyclin E in the control extracts.

CDK2 protein that remained after depletion of cyclin E and its associated kinase activity can be seen in Fig. 5B. There was a decrease in CDK2 levels in the cyclin E-depleted extracts (Fig. 5B, *cycE ID*), which was because of the removal of cyclin E-CDK2 complexes from the extracts. A small decrease of CDK2 kinase activity from 55,000 to 35,000 cpm occurred following the control immunodepletion with beads alone, which again is probably a result of the small amount of CDK2 lost in the control ID (Fig. 5C, *no ID*, *beads alone ID*). Immunodepletion of cyclin E reduces CDK2 activity (compare *no ID* with *cycE ID*). However, addition of cyclin EL to cyclin E ID lysates increases CDK2 activity 2-fold (Fig. 5C, *cycE ID TNT* and

*Cyclin EL*). This is consistent with the cyclin A ID (Fig. 4), as well as kinase results from Fig. 2. However, addition of cyclin EL before cyclin E ID resulted in a further robust increase in kinase activity up to 130,000 cpm, which was 2-fold higher than addition of cyclin EL after ID (Fig. 5C, *mix first*). The addition of cyclin EL to cell extracts after cyclin E depletion still caused an increase in CDK2 activity, suggesting that cyclin EL binding to CDK2 from endogenous cyclin E is responsible for a portion of CDK2 activity. The addition of cyclin EL to cell extracts depleted of endogenous cyclin E caused an increase in kinase activity, suggesting that the CDK2 activity is from other sources as well. As seen from Fig. 4, cyclin A may contribute to the CDK2 activation. However, cyclin E can further up-regulate this activity.

Our results thus far suggest that the increase in CDK2 activity following addition of cyclin EL to cell extracts is in part because of binding of CDK2 from endogenous cyclin A and cyclin E and free CDK2. Furthermore, addition of cyclin EL to cell extracts has also been shown to cause the increase in phosphorylated (active) CDK2, suggesting that phosphorylated CDK2 may be contributing to the increase in CDK2 activity. The factor that phosphorylates CDK2 at Thr-160, causing a





**FIG. 6. CAK contributes to cyclin E-mediated activation of CDK2.** Tumor cell extracts were mixed with cyclin EL or T3 before and after immunoprecipitation (IP) with anti-CDK2 polyclonal antibodies at 4 °C overnight. Cell extracts used were either immunodepleted with protein A beads (A or B) or depleted with anti-CDK7 polyclonal antibodies (E or F). Mixes were then immunoprecipitated with anti-CDK2 antibodies and subjected to CDK2 (A or B), CDK7 (E or F) Western blot analysis, and H1 kinase assay (C or G). For all panels, lane 1 is extract alone, lane 2 is TnT mix, lane 3 is cyclin EL, lane 4 is T3, lane 5 is TnT mixed before IP, lane 6 is cyclin EL mixed before IP, and lane 7 is T3 mixed before IP. For F and G, lane 8 is 50 µg of extract from the control ID, and lane 9 is 50 µg of extract from the CDK7 ID. Panels D and H represent H1 kinase activity normalized by [CDK2].

faster migrating CDK2 form on a Western blot, is CAK (10, 14). It is plausible that CAK is the factor responsible for the increase in CDK2 phosphorylation observed when cyclin E is added to extracts. To examine this possibility, we first determined whether the protein/proteins causing CDK2 phosphorylation were present within the cyclin E-CDK2 complex. This was achieved by mixing of cyclin EL and T3 with tumor cell extracts before and after immunoprecipitation of tumor cell extract with anti-CDK2 antibodies. Because CAK has never been shown to

bind to cyclin-CDK complexes (data not shown), then it should not co-immunoprecipitate with CDK2. Immunoprecipitates were subjected to CDK2 Western blot analysis and H1 kinase assay (Fig. 6, A and B).

Addition of cyclin EL or T3 to cell extracts before immunoprecipitation causes the increase in phosphorylated CDK2 (Fig. 6A, mix first T1 or T3) as compared with extracts alone (Fig. 6A, mix first vector alone), which was seen previously in all previous figures. However, when cyclin EL or T3 was added

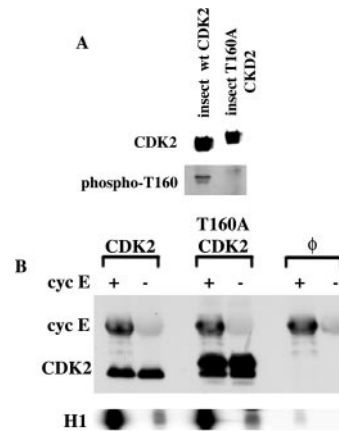


after CDK2 immunoprecipitation, the increase in phosphorylated CDK2 was not detected (Fig. 6A, *IP first, cyclin EL, or T3*). Because CDK2 phosphorylation occurs only in the cell extracts, and not in the immunoprecipitated complex, then it suggests that the factor causing the phosphorylation increase of CDK2 is not part of the cyclin EL-CDK2 complex. CAK has never been demonstrated to co-immunoprecipitate in cyclin EL-CDK complexes, which would explain the loss of CDK2 phosphorylation when mixing was performed after immunoprecipitation.

Because addition of cyclin EL or T3 after immunoprecipitation did not cause an increase in CDK2 phosphorylation, it raises the question of whether the increase in CDK2 activity will still occur. Cyclin EL and T3 were mixed with tumor cell extracts before and after CDK2 immunoprecipitation and subjected to H1 kinase assay (Fig. 6B). When cyclin EL and T3 were mixed with extracts before immunoprecipitation, it resulted in an increase in activity to 22,000 cpm for cyclin EL and an increase in activity to 15,000 cpm for T3 (Fig. 6B, *mix first, cyclin EL or T3*) from an activity of 10,000 cpm when vector alone is used. When cyclin EL and T3 were mixed with extracts after immunoprecipitation, it still resulted in an activity increase in activity from 2,000 to 9,000 cpm for cyclin EL and up to 6,000 for T3 (Fig. 6B, *IP first, cyclin EL or T3*). Because cyclin EL and T3 were able to cause the increase in CDK2 activity without causing an increase in CDK2 phosphorylation, it suggests that cyclin E can still increase the kinase activity even when bound to unphosphorylated CDK2. The overall levels of CDK2 activity were 2–3-fold lower in the mixes that were immunoprecipitated first than when the mixing was done first (Fig. 6B, *IP first*). This could reflect the absence of CDK activating phosphorylation, or mixing with immunoprecipitates may simply result in lower kinase activity levels, because the mixing is performed away from the proteins native environment, the cell extract. These results suggest that cyclin EL and T3 are able to cause the increase in CDK2 activity by just binding to CDK2, independent of the increase in phosphorylated CDK2.

As explained earlier, we strongly suspected that CAK was causing the increase in phosphorylated CDK2 when adding cyclin E to cell extracts. To test this hypothesis, mixing was performed on tumor extracts depleted of CAK. Removal of CAK from cell extracts should prevent CDK2 phosphorylation from occurring, whether mixing was performed before or after immunoprecipitating. Cyclin EL and T3 were added to tumor cell extracts that were immunodepleted of CDK7 (the catalytic subunit of CAK) before and after immunoprecipitation with anti-CDK2 antibodies. Immunoprecipitates were then subjected to CDK7 (Fig. 6E) and CDK2 (Fig. 6F) Western blot analysis, CDK2/H1 kinase assay (Fig. 6G). To confirm that CDK7 was depleted from the extract, CDK7 Western blotting was performed on extracts before and after depletion (Fig. 6E). Fig. 6E, *no ID*, shows the presence of CDK7 in cell extracts before depletion, and CDK7 ID shows that CDK7 was not present in depleted extracts (Fig. 6E). No CDK7 was detected in the CDK2 immunoprecipitates, again confirming that CDK7 does not bind to cyclin-CDK2 complexes (Fig. 6E). This result shows that most CDK7 was successfully removed from cell extracts by immunodepletion.

When cyclin EL or T3 was mixed with extracts depleted of CDK7, no increase in the levels of phosphorylated CDK2 occurred, suggesting that indeed CAK was responsible for the CDK2 phosphorylation following addition of cyclin EL or T3 (Fig. 6F). However, despite the removal of CDK7 (CAK) from the cell extracts, addition of cyclin EL or T3 still caused an increase in CDK2 activity (Fig. 6G, *IP first or Mix first, cyclin EL or T3*). When mixing was performed before immunoprecipitation,



**FIG. 7. T160A mutant CDK2 displays kinase activity upon addition of cyclin E.** Sf9 insect cells infected with either wild-type or T160A mutant CDK2 were harvested, and protein was extracted as above. CDK2 was immunoprecipitated from the cell extracts. Western analysis with both anti-CDK2 and a Thr-160 phospho-specific antibody shows presence of CDK2 in both samples, but Thr-160-phosphorylated CDK2 only in wild type sample (A). Immunoprecipitated wild-type or mutant CDK2 extracts were assayed for kinase activity both with and without the presence of *in vitro* translated cyclin EL (B). Note that the mutant CDK2 is hemagglutinin-tagged, accounting for its retarded migration.

tation, the increase was from 2,600 to 3,700 cpm for cyclin EL and to 3,000 for T3. When mixing was done after immunoprecipitation, the increase was from 800 to 3,000 cpm for cyclin EL and to 2,000 for T3. The CDK2 activity levels are much lower than when performed on non-depleted extracts (Fig. 6, B and C), because the depletion process lowers overall CDK2 activity. The results of this experiment confirm that CAK is responsible for an increase in CDK2 phosphorylation that occurs when cyclin E protein is added to extracts. The increase in CDK2 phosphorylation by CAK, however, contributes to only a portion of the increase in CDK2 activity when cyclin E protein is added to extracts.

To further explore this observation, a mutant CDK2 protein was included into the Western and kinase analyses. T160A CDK2 lacks this activating site of phosphorylation. The mutant protein was purified using the Sf9 insect cell infection/harvesting protocol. For this experiment, CDK2, T160A CDK2, and mock-infected insect cell lysates were immunoprecipitated using anti-CDK2. For each sample, *in vitro* translated cyclin EL was either added or withheld, and kinase activity was assessed. As is seen in Fig. 7, CDK2 is present in both infected samples (T160A runs slower because of a hemagglutinin tag). Histone H1 kinase activity is seen in both the mutant and wild-type CDK2 samples and appears to be equivalent. No activity was seen in any sample lacking cyclin E. A Thr-160 phosphospecific Ab was used to confirm that the mutant CDK2 lacks phosphorylation at the active site. Despite this missing phosphate group, the mutant CDK2 was able to phosphorylate the histone H1 substrate after addition of cyclin EL. This further confirms that cyclin EL is able to activate CDK2, regardless of the Thr-160 phosphorylation status.

Collectively, our studies suggest that the increase in CDK2 activity from addition of cyclin E to cell extracts is a result of multiple factors including cyclin E binding to endogenous CDK2 from cyclin A, cyclin E, and any unbound CDK2, which is phosphorylated and active. A schematic of the proposed mechanism is shown in Fig. 8. Furthermore, we have demonstrated *in vitro* that cyclin E is able to confer kinase activity to CDK2 lacking activating phosphorylation. Hence, the limiting

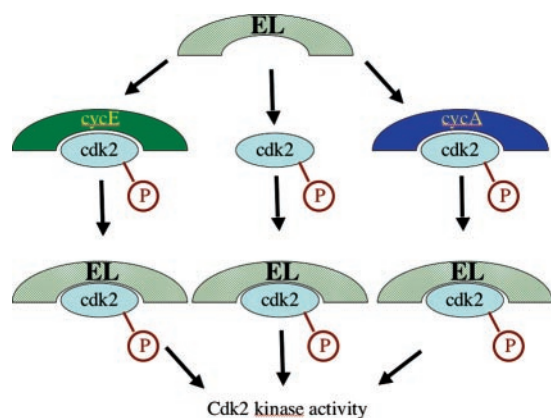


FIG. 8. Model of activation of CDK2 by exogenous cyclin E.

factor in tumor cells that could give rise to increased CDK2 activity is the amount of cyclin E.

#### DISCUSSION

Tumor cells overexpress cyclin E, which results in the constitutive expression and associated kinase activity of cyclin E throughout the tumor cell cycle (23). The tumor-specific overexpression of cyclin E raise the question as to the mechanism of its increase kinase activity. Addition of *in vitro* translated cyclin EL, representing the full-length cyclin E found in tumor and normal cells, to tumor cell extracts caused the increase of total CDK2 activity severalfold, along with an increase in phosphorylated (active) CDK2. The hypothesis we examined in this study was that the increase in CDK2 activity was because of the increase in phosphorylated CDK2, through a pathway involving CAK. Results, however, showed that the increase in CDK2 activity from addition of cyclin E to cell extracts is actually through binding to CDK2 from endogenous cyclin A (Fig. 4), CDK2 from endogenous cyclin E (Fig. 5), as well as unbound (but actively phosphorylated) CDK2. The increase in phosphorylated CDK2 was a result of CAK phosphorylation of CDK2. The increase in CDK2 activity observed after the addition of cyclin EL or T3 to cell extracts was found to be independent of the presence of CAK, however (Fig. 6). The differently migrating forms of CDK2 observed in Fig. 1 must be related to the activity of CDK2, as only the inactive (as shown by kinase assay) form bound to T3 migrates in a slower fashion. The samples were all treated identically except for the form of *in vitro* translated cyclin E added; therefore any difference seen can only be because of the added cyclin E forms. A likely possibility, given that cyclin E has been demonstrated to protect Thr-160-phosphorylated CDK2 from dephosphorylation by KAP phosphatase (17), is that this function may be impaired in the T3 truncated form. Therefore, either CDK2 is losing Thr-160 phosphorylation in the T3-bound sample, or the T3 form is unable to preferentially recognize and bind the Thr-160-phosphorylated CDK2. The results in Fig. 2 and Fig. 6A speak to this, as CDK2-immunoprecipitated and probed samples show both forms in all samples (*vector*, *EL*, or *T3*) but a prevalence of the active form only in the *EL* samples. This is corroborated by the increased histone H1 kinase activity seen the *EL*-supplied samples.

Full-length cyclin EL, E, T1, and T2 were all shown to bind to and activate CDK2. T3, however, was only shown to be in complex with unphosphorylated CDK2 and was not active *in vitro* when a FLAG antibody was used in the immunoprecipitation assays (Fig. 1). However, when CDK2 antibody was used to immunoprecipitate T3, its associated kinase activity was 1.5–2-fold higher than the activity associated with extracts alone. These studies suggests that although by itself T3-CDK2

complexes are not active, T3 can increase the overall levels of CDK2 phosphorylation and activity. The mechanism by which T3 can increase overall CDK2 activity was not thoroughly explored in this study. A possible explanation that we hypothesized was that T3 does preferentially bind non-phosphorylated CDK2. If T3 binds and sequesters all non-phosphorylated CDK2 from other cyclins, then it would provide an opportunity for all other cyclins to bind only active (phosphorylated) CDK2, thus increasing overall CDK2 activity.

To determine whether there is a normal *versus* tumor difference in CDK2 binding or CDK2 activity, cyclin EL or T3 TNT products were mixed in with either normal or tumor extracts (Fig. 2). Endogenous CDK2 activity levels were about 6-fold higher in tumor cells than normal cells, and activity after mixing with cyclin EL or T3 were about 3-fold higher in tumor cells than normal cells. Interestingly, when cyclin E protein is added to both normal and tumor extracts, it results in an increase in CDK2 activity 3–5-fold over the basal levels (Fig. 2). This increase in activity was proportionally similar between normal and tumor extracts, suggesting that both normal and tumor extracts have the potential to harness the CDK2 activation ability of an increased level of cyclin E. Not only was there an increase in activity, but addition of cyclin E also caused an increase in active (phosphorylated) CDK2 in both normal and tumor cells (Fig. 2).

If a normal cell were to overexpress cyclin E, then our results here suggest that it would increase its level of CDK2 activity and CDK2-activating phosphorylation, which may result in a growth advantage. We have observed exactly this outcome when we transfected normal mammary epithelial cells with the LMW forms of cyclin E (29). Normal cells receive a proportionally identical increase in CDK2 activity following addition of cyclin E into extracts. The increase in CDK2 activity resulting in cyclin E overexpression could potentially contribute to deregulations at the G<sub>1</sub>, S, and G<sub>2</sub> phase checkpoints, allowing cells to cycle uncontrollably, leading to tumor development.

An interesting result obtained was that adding cyclin E protein to normal or tumor cell extracts caused a large increase in CDK2 activity (Fig. 2). The increase in CDK2 activity may come from any of the following possibilities: an activity intrinsic to the TNT reaction mix, an increase in phosphorylated (activated) CDK2, binding to CDK2 from cyclin A, binding to CDK2 from endogenous cyclin E, or binding to free (but phosphorylated) CDK2. First, we examined the TNT reaction mix for activity alone or with the presence of cyclin E (Fig. 3). There was a small increase in H1 kinase activity when cyclin EL was present in the TNT reaction mix; however, not enough to account for severalfold increase seen when cyclin EL is added to cell extracts. Other sources of CDK2 activation after addition of cyclin EL are the binding of cyclin EL to endogenous CDK2, which can exist in complex with endogenous cyclins, or in a free monomeric form. We next examined what effect depleting cell extracts of endogenous cyclin A or cyclin E had on the increase in CDK2 activity (Figs. 4 and 5). If the increase in CDK2 activity is a result of cyclin EL binding to CDK2 from cyclin A or E, then depleting endogenous cyclin A or E would show a smaller increase in CDK2 activity when cyclin EL was added. Our results supported this premise and when either cyclin E or A was depleted from extracts, the level of activation was lower than when cyclin EL was added before depletion. These results demonstrated that a portion of the increase in CDK2 activity was a result of cyclin EL binding to CDK2 from endogenous cyclin A and E. In tumor cells that have a high level of cell cycle deregulated cyclin E, the binding of CDK2 away from cyclin A may offset cell cycle progression at late S, G<sub>2</sub>, and M phase where cyclin E is not supposed to be expressed. Along with



CDK2 from endogenous cyclin A and E, we had suspected an abundance of unbound monomeric CDK2 by the huge levels of cyclin E needed to saturate CDK2 during the competition with endogenous levels of cyclin E and A.

Addition of cyclin EL or T3 to cell extracts results in an increase in the levels of phosphorylated CDK2, along with an increase in the levels of CDK2 activity (Fig. 3). The two questions raised by this finding are what causes the increase in CDK2 phosphorylation, and is the increase in phosphorylated CDK2 contributing to the increase in CDK2 activity? We had strongly suspected CAK was responsible for the phosphorylation of CDK2, because CAK is known to phosphorylate CDK2 at Thr-160, which causes CDK2 to migrate slightly faster on a Western blot (35). The experiment in Fig. 6 was designed to simultaneously determine whether CAK was responsible for the increase in phosphorylated CDK2 and subsequent increase in CDK2 activity. Addition of cyclin EL to immunoprecipitates of cyclin-CDK2 complexes did not increase the levels of phosphorylated CDK2, suggesting that the factor phosphorylating CDK2 is not part of the cyclin-CDK2 complex (Fig. 6). When cyclin EL was mixed with cell extracts that have been immunodepleted of CDK7 (CAK), the increase of CDK2 phosphorylation did not occur, indicating that CAK is indeed the catalytic activity responsible for phosphorylating CDK2 (Fig. 6).

When cyclin EL or T3 were mixed with immunoprecipitates of CDK2 in CAK-depleted extracts, the increase in CDK2 activity occurred despite the lack of increased phosphorylation of CDK2 (Fig. 6). Furthermore, when cyclin EL and T3 were mixed with tumor cell extracts depleted of CDK7 (CAK), the increase in CDK2 activity was not comparable with when CAK is present. These results suggest that CAK is required for the increase in CDK2 phosphorylation following addition of cyclin E to cell extracts; however, the increase in CDK2 activity is not entirely dependent on the increase in phosphorylated CDK2 or the presence of CAK. This is further confirmed by the mutant CDK2 experiments (Fig. 7). The T160A CDK2 was able to phosphorylate substrate upon addition of cyclin EL, even though it lacks the activating site.

Because the N-terminal deleted forms of cyclin E were shown to bind to and activate CDK2, it provides evidence that endogenous N-terminal deleted forms of cyclin E are also capable of binding and activating CDK2 in tumor cells. LMW forms of cyclin E have been shown to have prognostic value for predicting the stage, severity, and outcome of breast cancer (31). The prognostic data combined with data from this study suggest that the LMW forms of cyclin E are able to activate CDK2 and that they may be involved in tumor development. Increased levels of cyclin E in tumor extracts resulted in the increase in phosphorylated CDK2 and increased CDK2 activity. Even T3, which by itself does not bind to activated CDK2, is able to increase overall CDK2 activity through a possible mechanism by which T3 sequesters all unphosphorylated CDK2. Because an addition of cyclin E to cell extracts causes an increase in the levels of CDK2 phosphorylation by CAK, it raises the possibility that cyclin E and CAK may function together in a feedback loop. Cyclin E/CDK2 may actually increase the activity of CAK possibly by phosphorylating CAK. Increased cyclin E levels in cells would increase CAK activity, which would further drive CDK2 and CDC2 activity by CAK phosphorylation.

In the mutant Thr-160 experiment (Fig. 7), it is shown that *in vitro* CDK2 is kinase active regardless of Thr-160 phosphorylation status. It has been shown that the predominant form of CDK2 bound to cyclin E in dividing cells is the Thr-160 monophosphorylated form. However, cyclin E was also found in

complex with unphosphorylated and Thr-160 + Tyr-15 dually phosphorylated CDK2 (16). In the samples for Fig. 7, where no choice of substrate is available to the cyclin EL, it may bind whatever target is abundantly available, be it the wild-type or mutant CDK2, and confer activity. In an intact cell system, the mutant CDK2 would likely be kinase-inactive, as cyclin E would preferentially bind the Thr-160-phosphorylated forms.

In summary we provide evidence that addition of *in vitro* synthesized cyclin E to cell extracts triggers a cascade of events leading to an increase in CDK2 activity. *In vivo*, when cells are transfected with cyclin E, the overexpression leads to a higher kinase activity compared with control, untransfected samples. Collectively these observations suggest that overexpression of cyclin E represents a key event in the biochemical activation of cell cycle, which could subsequently lead to uncontrolled cell proliferation.

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