**Stimulation of the Raf/MEK/ERK Cascade Is Necessary and Sufficient for Activation and Thr-160 Phosphorylation of a Nuclear-targeted CDK2**

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* The abbreviations used are: CDK, cyclin-dependent kinase; FCS, fetal calf serum; ERK, extracellular signal-regulated kinase; CAK, CDK-activating kinase; NLS, nuclear localization signal; pRb, retinoblastoma protein; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.

In eukaryotic cells, growth factors activate signaling pathways that stimulate cells to divide. The sequential activation of the cyclin-dependent kinases (CDKs) controls the orderly progression of cell cycle events (1, 2). As the name implies, CDKs require the binding of a cyclin subunit for full activation (3, 4). The first CDK to become active in G1 is CDK4/6, which requires the binding of a cyclin subunit for full activation (3, 4). The activation of CDK2 requires the removal of two inhibitory phosphates (Thr-14 and Tyr-15) and the addition of one activating phosphate (Thr-160) by a nuclear localized CAK-activating kinase, which is thought to be constitutively active. Surprisingly, nuclear localized CDK2-NLS and CDK2-NLS(A14,F15), which lacks the inhibitory phosphorylation sites, require serum to become active, despite complexing with expressed cyclin E. We show that inhibition of mitogen-mediated ERK activation by treatment with U0126, a selective MEK inhibitor, or expression of dominant-negative ERK markedly reduces the phosphorylation of Thr-160 and enzymatic activity of both CDK2-NLS constructs. Consistent with a role for ERK in Thr-160 phosphorylation, expression of constitutively active Raf-1 induces Thr-160 phosphorylation of CDK2-NLS in serum-arrested cells, an effect that is blocked by treatment with U0126. Taken together, these data show a new role for ERK in G1 cell cycle progression: In addition to its role in stimulating cyclin D1 expression and nuclear translocation of CDK2, ERK regulates Thr-160 phosphorylation of CDK2-cyclin E.

In vitro and in vivo studies indicate that the major and perhaps only function of cyclin D-CDK4/6 is to regulate the accumulation of the next activating cyclin, cyclin E (14, 15). Cyclin E is required for the late G1 activation of CDK2 (16, 17). CDK2-cyclin E has many reported substrates and is critical for regulating cell cycle checkpoints necessary for progression into S phase (18–22). In addition to cyclin E accumulation, activation of CDK2 requires additional modifications to become active. Recently, CDK2-cyclin E translocation to the nucleus has been shown to be necessary for enzymatic activation (23–25). This is, presumably, because of post-translational modifications that occur only in the nucleus and are required for CDK2 activity.

One such nuclear modification is phosphorylation on Thr-160. The phosphorylation site, located on the “T-loop” is highly conserved among all CDKs and is essential for proper alignment of the kinase domain (26, 27). Thr-160 phosphorylation is thought to be carried out by a CAK-activating kinase (CAK) activity. The identity of CAK is reported to be the heterotrimeric complex consisting of p40Mds2 (CDK7), cyclin H, and menage-a-trois (MAT1), which is believed to be constitutively active and nuclear (28–31). Another post-translational modification that is required for CDK2 activity is the removal of inhibitory phosphorylations on Thr-14 and Tyr-15 (32). These phosphates are added to CDK2 by the cytosolic mixed-lineage kinase Wee-1. They are removed by cdc25A phosphatase (32, 34). The regulation and localization of cdc25A is not well understood (35).

Our laboratory recently found a second role for ERK in G1 progression. In that study (23), we show that U0126, a MEK inhibitor, blocks nuclear translocation and activation of CDK2-cyclin E, indicating a role for ERK kinase in CDK2-cyclin E nuclear translocation. However, these studies did not address whether other mitogen-mediated modifications are critical for nuclear CDK2-cyclin E activation. To address this question, we targeted CDK2-cyclin E to the nucleus, examined the activation of CDK2-cyclin E complexes, and found a novel role for ERK in the activation of nuclear targeted CDK2-cyclin E (CDK2-NLS).

**Experimental Procedures**

Expression Constructs—Human HU4 cyclin E fragment expression plasmid was generously provided by James Roberts (16). CDK2-NLS was generated by subcloning human CDK2 cDNA (plus HA tag) into the Strategene One-Shot vector pOMV/myc/nuc according to manufacturer’s protocol. CDK2-NLS(A14,F15) was generated by Strategene One-Shot™ site-directed mutagenesis; mutations were confirmed by se-
quencing (Beckman-Coulter). ERK1(K71R) was generated by site-directed mutagenesis from ERK1 cDNA generously provided by Melanie Cobb (36). Constitutively active Raf-1 was generously provided by Thomas Sturgill (37). E2F1 expression plasmid was generously provided by Jason Weber. Rb actin expression plasmid was generously provided by J. Wade Harper (38).

**Cell Culture and Reagents—**IIC9 cells (Chinese Hamster Embryonic Fibroblasts) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose and 2 mM L-glutamine (BioWhittaker, Walkersville, MD) supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (all from Sigma). Growth-arrested IIC9 cells were established by washing subconfluent (80%) or transfected cells once with phosphate-buffered saline (PBS) followed by 18- to 20-h incubation with a c-Myc containing monoclonal (9E10) c-Myc tag antibody (Upstate Biotechnology) or 10 μg/ml pepstatin). Lysates were sonicated briefly, and the insoluble material was pelleted by microcentrifugation at 14,000 × g at 4 °C for 2 min. Protein concentrations were determined using Coomassie Plus (Pierce, Rockford, IL) as recommended by the manufacturer. Cell lysates (50 μg of protein) were incubated with 1 μg of monoclonal c-Myc tag antibody (Upstate Biotechnology, Lake Placid, New York) or 1 μg of CDK4 antibody (Santa Cruz Biotechnology) at 4 °C with gentle rocking overnight. Immune complexes were then immunoprecipitated with 2-h incubation with protein G-agarose (Sigma) at 4 °C with gentle rocking. The immune complexes were pelleted by microcentrifugation at 6,000 × g and washed three times with cold lysis buffer and two times with cold PBS supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 20 mM sodium fluoride, 50 μM β-glycerophosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). Immunocomplexes were resuspended in 1× PBS in Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were dried and CDK2–cyclin E when only CDK2-NLS is expressed (Fig. 2A, lane 2). As a specificity control we immunoprecipitated CDK4 and examined with antibodies directed against the c-Myc tag. c-Myc tagged-CDK2 immunocomplexes from basal cell lysates do not contain cyclin E only when CDK2-NLS is expressed (Fig. 2A, lane 2). Cyclin E does not co-immunoprecipitate with CDK2 when CDK2-NLS and cyclin E are co-expressed in basal cells (Fig. 2A, lane 3). As a specificity control we immunoprecipitated CDK4 and examined whether the immunocomplexes contain CDK2 or cyclin E (Fig. 2A, lane 4). These data indicate that CDK2-NLS does not require serum to form a complex with ectopically expressed cyclin E.

We next asked whether nuclear-targeted CDK2 requires mi-
CDK2-NLS localizes to the nucleus independent of serum stimulation. A, CDK2-NLS and CDK2-NLS(A160) expression plasmids were constructed as described under “Experimental Procedures.” B and C, IIC9 cells were transfected with either CDK2-NLS or CDK2-NLS(A160) as described under “Experimental Procedures.” After 2 days of serum depletion, cells were incubated in the presence or absence of FCS for 17 h.

Cells were then fixed, probed with c-Myc tag antibodies, and visualized using a fluorescent microscope, as described. DAPI staining was used as a nuclear marker. Data are representative of at least three independent experiments.

To examine this, we transfected CDK2-NLS into IIC9 cells, together with cyclin E, and measured in vitro kinase activity from serum-arrested and serum-stimulated cells. Surprisingly, in serum-arrested cells, CDK2-NLS shows negligible kinase activity (Fig. 2B, lane 3). However, addition of serum induces an approximate 17-fold activation of CDK2-NLS (Fig. 2B, lane 4), indicating that CDK2-NLS requires mitogenic signaling to become enzymatically active. For our activity control, we also constructed CDK2-NLS(A160) (Fig. 1A), which contains an alanine substituted for Thr-160 (Fig. 2B, lanes 1 and 2). This construct is also found exclusively in the nucleus, independent of serum (Fig. 1C). These data show that targeting CDK2-NLS-cyclin E complexes to the nucleus is not sufficient for catalytic activation.

A possible explanation of the serum dependence of CDK2-NLS-cyclin E activation is the presence of known CDK2 inhibitor proteins (such as members of the CIP/KIP family) in the CDK2-NLS/cyclin E complex in basal, but not stimulated cells (40–42). The CDK2 inhibitor p27Kip1 accumulates in serum-arrested cells, but re-addition of serum induces a reduction in steady-state protein levels (Fig. 3, lanes 1 and 2) (43, 44). However, we were unable to find p27Kip1 in the CDK2-NLS/cyclin E c-Myc tag immunocomplexes in basal or stimulated cells (Fig. 3, lanes 3 and 4). Similarly, we were also unable to detect p21CIP1/WAF1 in complex with CDK2-NLS/cyclin E (data not shown). Therefore, it is unlikely that either p27 or p21 blocks CDK2-NLS activation in serum-arrested cells.

CDK2-NLS Becomes Active Earlier in G1 than Endogenous CDK2—Because cyclin E accumulation, CDK2 nuclear translocation, and the subsequent post-translational modifications, which are required for activation, occur late in G1 (23, 44), endogenous CDK2-cyclin E becomes active late in G1. We expect CDK2-NLS-cyclin E to become active more rapidly than endogenous CDK2, because it localizes to the nucleus in serum-arrested cells. Consistent with this expectation, serum induces a marked increase in CDK2-NLS-cyclin E activity in 3 h, with full activation in only 5 h of serum stimulation (Fig. 4B). In accordance with earlier reports, endogenous CDK2 does not become fully active until 12 h of serum stimulation (Fig. 4A). These data are consistent with the notion that endogenous CDK2 is activated in the nucleus. Furthermore, because nuclear translocation of endogenous CDK2-cyclin E occurs 12 h after serum addition (23) and CDK2-NLS-cyclin E is activated after 5 h (Fig. 4B, lane 4), the results suggest that the enzymatic activities required for the post-translational modifications of CDK2 are present hours before nuclear translocation.

CDK2-NLS(A14,F15) Requires Serum to Become Active—In addition to association with cyclin E, CDK2 requires post-translational modifications in order to become activated. Inhibitory phosphorylations on Thr-14 and Tyr-15, which are added by the cytosolic mixed-lineage kinase Wee-1 and removed by the mixed phosphatase cdc25A, must be removed for CDK2 to become active (34). Little is known about the regulation of cdc25A. A possible explanation of the serum independence of CDK2-NLS requires serum to become active is that cdc25A requires serum to de-phosphorylate Thr-14 and Tyr-15. To examine this, we constructed CDK2-NLS(A14,F15), which contains an alanine and phenylalanine substitution for Thr-160 and Tyr-15, respectively (Fig. 5A). This construct also localizes exclusively to the nucleus (data not shown). Because this construct cannot be phosphorylated at these sites, cdc25A phosphatase activity is not required for activation. Intriguingly, when co-transfected with cyclin E, CDK2-NLS(A14,F15) does not have catalytic activity in serum-arrested cells, but displays 17-fold stimulation with the addition of serum (Fig. 5B). Furthermore, the time course of activation of CDK2-NLS(A14,F15) mirrors that of CDK2-NLS, becoming fully active in 5 h (data not shown). This argues that the dephosphorylation of Thr-14 and Tyr-15 is not the serum-dependent step required for CDK2-NLS activation.

CDK2-NLS Requires Serum to Be Phosphorylated on Thr-160—Because CDK2-NLS(A14,F15) requires serum to become active, we reasoned that the serum-dependent step for CDK2-NLS-cyclin E activation is the phosphorylation on Thr-160. We tested this by examining Thr-160 phosphorylation of CDK-NLS at various times after re-addition of serum, using an antibody specific for Thr-160-phosphorylated CDK2. By immunoprecipitating equal amounts of CDK2-NLS (Fig. 6, upper), we ob-
served that serum induces the phosphorylation of Thr-160 in 3 h, with CDK2-NLS becoming maximally phosphorylated in 5 h (Fig. 6, lower). The Thr-160 phosphorylation of CDK2-NLS(A14,F15) is similarly induced by serum (Fig. 7B). These results indicate that, although CDK2-NLS localizes to the nucleus, serum is required for the phosphorylation of Thr-160, and therefore, activity. This suggests that CAK activity is growth factor-dependent and required for CDK2-NLS activity.
Serum stimulates the Thr-160 phosphorylation of CDK2-NLS in 5 hours. IIC9 cells were transfected with cyclin E and c-Myc-tagged CDK2-NLS, growth-arrested for 24 h, and stimulated with 10% FCS for indicated lengths of time. Lysates were collected by scraping into cold lysis buffer. Protein lysate (300 μg) was immunoprecipitated with 3 μg of c-Myc tag antibody. Immunocomplexes were split into two samples and resolved by 12% SDS-PAGE and Western blotting for CDK2 or phospho-CDK2(T160). Data are representative of three independent experiments.

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The MEK Inhibitor U0126 Inhibits CDK2-NLS and CDK2-NLS(A14,F15) Activity and Thr-160 Phosphorylation—In an effort to elucidate the specific signaling mechanisms that are required for CDK2-NLS activation, we made use of selective inhibitors of phosphatidylinositol 3-kinase and the ERK cascade, two pathways that are critical for cell-cycle progression. Previously, our laboratory demonstrated that inhibition of CDK2-cyclin E activity by a phosphatidylinositol 3-kinase inhibitor, but not an ERK cascade inhibitor, can be overcome by transient transfection of cyclin E in stimulated cells (23). Pre-treatment of cells with the MEK inhibitor U0126, which prevents the MEK activation of ERK, inhibited the activation of CDK2-NLS in a concentration-dependent manner, fully inhibiting Thr-160 phosphorylation. Because we found the phosphorylation of CDK2-NLS on Thr-160 to be the serum-dependent step in activation, and because MEK activity is critical for activation, we reasoned that ERK regulates the phosphorylation of CDK2 on Thr-160. Not surprisingly, we found that U0126 inhibits Thr-160 phosphorylation of CDK2-NLS(A14,F15) in serum-stimulated cells, whereas LY29004 is ineffective (Fig. 7B). The same result was found with CDK2-NLS (Fig. 8A, lanes 1–4). These data indicate a critical role for ERK in the phosphorylation of CDK2 on Thr-160. Furthermore, the time course of Thr-160 phosphorylation of CDK2-NLS (Fig. 6) and CDK2-NLS(A14,F15) (Fig. 7B, lanes 1–4) were concomitant with the time course of enzymatic activation (Fig. 4B).

The Raf-MEK-ERK Cascade Is Necessary and Sufficient for Thr-160 Phosphorylation of CDK2-NLS—If ERK activity is required for the phosphorylation of CDK2-NLS on Thr-160,
transfection of a dominant-negative ERK construct should prevent serum-stimulated Thr-160 phosphorylation. As expected, in serum-stimulated cells, expression of ERK(R71K) diminishes Thr-160 phosphorylation of CDK2-NLS (Fig. 8A, lane 5) to levels below that of basal (Fig. 8A, lane 1), demonstrating that ERK activity is necessary for this phosphorylation. To further demonstrate the role of the ERK cascade in Thr-160 phosphorylation, we expressed a constitutively active Raf-1 construct in basal cells. Raf-1 is the upstream activator (mitogen-activated protein kinase kinase kinase) of the ERK cascade, and therefore, constitutively active Raf-1 stimulates the ERK cascade independent of growth factors (46-48). Intriguingly, expression of constitutively active Raf-1 induced robust Thr-160 phosphorylation of CDK2-NLS in basal cells (Fig. 8B, lane 4), indicating that stimulation of the RAF/MEK/ERK pathway is sufficient for Thr-160 phosphorylation. Moreover, this effect was almost completely blocked by treatment with the MEK inhibitor (Fig. 8B, lane 5). Taken together, these data (Figs. 7 and 8) strongly indicate a crucial role for the ERK pathway in regulating CDK2 phosphorylation on Thr-160.

The Role for ERK in Regulating Thr-160 Phosphorylation of CDK2-NLS Is Not through E2F/pRb—The phosphorylation of the retinoblastoma protein (pRb) by CDK4 and CDK2 relieves the pRb-mediated repression of the E2F transcription factors, thus promoting G1 progression (49-51). Because pRb represses E2F-mediated transcription and cyclin E is downstream of E2F, we anticipate that the activation of CDK2-NLS-cyclin E is independent of pRb. Rb<sup>ACDK</sup> is a mutant pRb that cannot be phosphorylated by CDKs and does not release from E2F on serum stimulation (38). Expression of Rb<sup>ACDK</sup> has been shown to inhibit E2F-driven reporters, cyclin A expression, and cell-cycle progression. However, expression of Rb<sup>ACDK</sup> does not diminish Thr-160 phosphorylation of CDK2-NLS (Fig. 9, lane 4), arguing against a role for pRb in the phosphorylation of CDK2-NLS on Thr-160. Consistent with this, overexpression of E2F1 in basal cells does not induce Thr-160 phosphorylation of CDK2-NLS (Fig. 9, lane 3). These data, together with the rapid time course, argue that the role for ERK in inducing Thr-160 phosphorylation of CDK2-NLS is through a target other than pRb/E2F.

Regulation of CDK2 Phosphorylation on Thr-160 Represents a Third Role for ERK in G<sub>1</sub> Progression—The enzymatic activation of the first CDK to become active in G<sub>1</sub> progression, CDK4-cyclin D, has been studied in detail. However, much less is known about the regulation of CDK2-cyclin E, an activity crucial for progression into S phase. The role for ERK in promoting the accumulation of cyclin D1 in early G<sub>1</sub> is well established. In demonstrating the importance of ERK in regulating CDK2 nuclear translocation, a second role for ERK in G<sub>1</sub> progression was found (23). In the present study, using a CDK2 that localized to the nucleus independent of mitogen, and over-expressing cyclin E, we found yet another role for ERK in G<sub>1</sub> progression, i.e., Thr-160 phosphorylation and activation of CDK2-cyclin E. Thus, ERK activity is important throughout G<sub>1</sub>; regulating cyclin D1 expression, CDK2-cyclin E nuclear translocation, and the phosphorylation and activation of CDK2-cyclin E (Fig. 10).

In an interesting study, Chiariello et al. (52) used ERK cascade inhibitors and showed that CDK2 failed to become phosphorylated on Thr-160 upon serum stimulation. However, the role for ERK in CDK2 nuclear translocation was not known, the authors were most likely observing a failure of CDK2 to translocate to the nucleus, where Thr-160 phosphorylation is reported to take place. The use of nuclear-targeted CDK2 afforded us the ability to examine Thr-160 phosphorylation independent of nuclear translocation.

The activity thought to be responsible for this phosphorylation is termed CAK, the identity of CAK is reported to be the CDK7-cyclin H-MAT1 complex (28, 31, 53). The mechanism by which ERK regulates the activity of CAK, if at all, remains unknown. In IIC9 cells, CDK7 immunocomplexes appear to have full CDK7-cyclin H-MAT1 activity in basal, stimulated, and U0126-treated cells, demonstrating that ERK is not regulating CDK7-cyclin H-MAT1 activation. Some studies (54, 55) have speculated that growth factors may alter CDK7 substrate specificity, suggesting that ERK may regulate the ability of CDK7 to recognize CDK2-cyclin E. Another explanation is the subcellular localization of CDK7, for example it may be associated with the transcription factor TFIIH complex in serum-arrested cells and become dissociated in late G<sub>1</sub>. A final explanation is that ERK regulates a CAK activity different than CDK7-cyclin H-MAT1. Interestingly, a CAK activity that is not CDK7-cyclin H-MAT1 has been reported in yeast and human cells (33, 39, 56). As CAK activity is studied in more detail, the ERK substrate that mediates CAK regulation may become clear.

<sup>2</sup> S. M. Keenan, N. H. Lents, and J. J. Baldassare, unpublished results.

<sup>3</sup> N. H. Lents and J. J. Baldassare, unpublished results.
