Expression of Cyclin E Renders Cyclin D-CDK4 Dispensable for Inactivation of the Retinoblastoma Tumor Suppressor Protein, Activation of E2F, and G₁-S Phase Progression*

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The activation of CDK2-cyclin E in late G₁ phase has been shown to play a critical role in retinoblastoma protein (pRb) inactivation and G₁-S phase progression of the cell cycle. The phosphatidylinositol 3-OH-kinase inhibitor LY294002 has been shown to block cyclin D1 accumulation, CDK4 activity and, thus, G₁ progression in α-thrombin-stimulated IIC9 cells (Chinese hamster embryonic fibroblasts). Our previous results show that expression of cyclin E rescues S phase progression in α-thrombin-stimulated IIC9 cells treated with LY294002, arguing that cyclin E renders CDK4 activity dispensable for G₁ progression. In this work we investigate the ability of α-thrombin-induced CDK2-cyclin E activity to inactivate pRb in the absence of prior CDK4-cyclin D1 activity. We report that in the absence of CDK4-cyclin D1 activity, CDK2-cyclin E phosphorylates pRb in vivo on at least one residue and abolishes pRb binding to E2F response elements. We also find that expression of cyclin E rescues E2F activation and cyclin A expression in cyclin D kinase-inhibited, α-thrombin-stimulated cells. Furthermore, the rescue of E2F activity, cyclin A expression, and DNA synthesis by expression of E can be blocked by the expression of either CDK2(D145N) or RbΔCDK, a constitutively active mutant of pRb. However, restoring four known cyclin E-CDK2 phosphorylation sites to RbΔCDK renders it susceptible to inactivation in late G₁, as assayed by E2F activation, cyclin A expression, and S phase progression. These data indicate that CDK2-cyclin E, without prior CDK4-cyclin D activity, can phosphorylate and inactivate pRb, activate E2F, and induce DNA synthesis.

The mammalian cell cycle is controlled by two important families of proteins, the cyclins and the cyclin-dependent kinases (CDKs)
1 (for reviews, see Refs. 1 and 2). Progression through the cell cycle is governed by the kinase activities of specific CDKs, which are regulated by association with the regulatory cyclin subunits. The sequential activation of early G₁ CDK activity, CDK4 or CDK6 together with cyclin D1, D2, or D3, and the late G₁ CDK activity, CDK2 together with cyclin E1 or E2, is believed to be required for progression through G₁ and into S phase. Because IIC9 cells contain CDK4, but not CDK6, and cyclin D1, but not D2 or D3, CDK4-cyclin D1 activation in early G₁ is required for the expression of cyclin E, CDK2 activity, and G₁-S phase progression (3–5).

In most cell types inactivation of the retinoblastoma (pRb) protein is essential for passage through G₁ and transition of cells into S phase (2, 6–9). pRb regulates this progression by its association with the E2F family of transcription factors (10–13). In quiescent cells (G₀ phase) pRb is unphosphorylated; in early- to mid-G₁ pRb is hypophosphorylated by the D-type CDKs (14, 15). This hypophosphorylated form of pRb, which binds to and inhibits E2F transcription factors, has been shown in vivo to be phosphorylated on 13 of 16 potential CDK phosphorylation sites, suggesting that hypophosphorylated pRb may consist of multiple phospho-isoforms (16–18). The hypophosphorylation of pRb in early G₁ stimulates the release of HDAC1 and the recruitment of SWI/SNF family members to the pRb-containing chromatin remodeling complexes, thus allowing the expression of cyclin E (19–21). In late G₁ and S phase, pRb is hyperphosphorylated by CDK2-cyclin E and, later, by CDK2-cyclin A (22–26). The hyperphosphorylated form of pRb is inactivated because it loses affinity for and, therefore, fails to inhibit the E2F transcription factors (7, 23, 27). Numerous proteins that are essential for growth, such as the cyclins E and A, and proteins essential for DNA replication, such as DNA polymerase α, thymidine kinase, dihydrofolate reductase, and histone H2A are controlled at least in part by E2F-responsive promoters (28–35). pRb inhibits these promoters by either directly blocking the activation domain of E2F or by acting as a member of a repression complex (21, 36–41). Therefore, the major role of the G₁ CDKs, CDK4/6-cyclin D and CDK2-cyclin E in controlling G₁-S phase progression, is the inactivation of pRb.

Although the role of CDK4-cyclin D in the inactivation of pRb is well established, the role of CDK2-cyclin E in the inactivation of pRb is less clear. CDK2-cyclin E, in the absence of prior phosphorylation by CDK4-cyclin D, is able to phosphorylate pRb in vitro (16), and overexpression of cyclin E or A can overcome pRb-mediated suppression of proliferation (42). Furthermore, Ezhevsky et al. (18) show that CDK2-cyclin E activity phosphorylates pRb in vivo. In agreement with these data, Lundberg and Weinberg (43) also find that CDK2-cyclin E activity was necessary for phosphorylation-induced inactivation of pRb. However, these authors and others suggest that phosphorylation of pRb by CDK2-cyclin E requires pRb to be
hypophosphorylated, and thus, the inactivation of pRb involves sequential phosphorylation by cyclin D-CDK4/6 and cyclin E-CDK2 (24, 44). Conversely, the report of a cyclin E → D1 “knockin” mouse offers an argument against the strict requirement for sequential phosphorylation of pRb by the G1 CDK-cyclin complexes (45). These mice, in which the coding sequence of the cyclin D1 gene is replaced with the coding sequence for cyclin E, reveal that cyclin E expression, which results in a 20% change in the phosphorylation state of pRb, rescues the phenotypic deficiencies found in the cyclin D1-ablated mouse (46). Although many tissues are unaffected by the loss of cyclin D1, compensatory functions of cyclins D2 and D3, several tissues, including retinal and breast tissue are severely deficient in growth, presumably because D2 and D3 are not expressed in these tissues and could not compensate. Implicit in the discovery that cyclin E expression under the cyclin D1 promoter reverses the cyclin D1−/− phenotypes is the understanding that, in the rescued tissues, pRb is inactivated by cyclin E kinase activity without prior hypophosphorylation by cyclin D kinase, with the caveat that the genes for cyclin D2 and D3 are still present in these animals.

Previously, we reported that inhibition of phosphatidylinositol 3-OH kinase by LY294002 inhibits cyclin D1 accumulation, CDK4-cyclin D1 activity, and passage through G1 into S phase in IIC9 cells (3). Surprisingly, expression of cyclin E in the presence of LY294002 rescues cyclin E-CDK2 activity and G1 progression (47). Under these conditions, cyclin D kinase activation is inhibited, whereas cyclin E-kinase activity is rescued. Therefore, this offers a unique opportunity to question the necessity of prior phosphorylation of Rb by cyclin D-dependent kinases by examining whether CDK2-cyclin E activity alone can phosphorylate and inactivate pRb. Here we show that expression of cyclin E recovers CDK2, but not CDK4 activation in LY294002-treated cells. Under these conditions, in which CDK2 can be activated without prior CDK4 activation, we report that the phosphorylation of pRb on Ser-795, the release of pRb from E2F response elements, the activation of E2F, and G1-S phase progression all occur normally. Furthermore, we report that the cyclin E-mediated rescue of E2F activity can be prevented by co-expression of CDK2(D145N) or Rb(ΔRbκ). Finally, we restore four CDK2 phosphorylation sites on Rb(ΔRbκ) and show that expression of this construct, Rb(ΔRbκ)K, does not block E2F activation or DNA synthesis. Taken together, these data indicate that, in IIC9 cells, prior phosphorylation of pRb by cyclin D-CDK4/6 is not necessary for the phosphorylation of pRb by cyclin E-CDK2 and the subsequent activation of E2F and entry into S phase of the cell cycle.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—IIC9 cells, a subclone of Chinese hamster embryo fibroblasts (48, 49), were maintained as previously described (50). Quiescent cells were established by washing subconfluent (80%) cells once with phosphate-buffered saline followed by a 48-h incubation with αMEM (minimum essential medium) containing 2 mM l-glutamine (BioWhittaker) supplemented with 100 units/ml penicillin and 100 mg/ml of streptomycin (basal medium). For transient transfections IIC9 cells were grown to subconfluence (80%). The cells were transfected as previously described (51). After 12 h the cells were serum-starved for 4 h before stimulation. >80% transfection efficiency was determined by co-transfection of green fluorescent protein. At the indicated times total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol (QuickChange™ kit, Stratagene). Restoration of the threonine or serine codons was confirmed by automated capillary sequencing following the manufacturer’s protocol (Beckman-Coulter).

Cyclin-dependent Kinase Activity Assays—CDK4-cyclin D1 and CDK2-cyclin E assays were performed as previously described (3, 47, 50, 52). Briefly, after transformation and serum starvation cells were stimulated with 1 unit/ml α-thrombin for 8 h (for CDK4) or 17 h (for CDK2). Lysates (100 μg) were immunoprecipitated with monoclonal antibodies to cyclin D1 or polyclonal antibodies to CDK4 or CDK2, as indicated. Immunoprecipitates were analyzed for the ability to phosphorylate GST-Rb (for CDK4 or cyclin D1) or histone H1 (for CDK2) in vitro. Phosphorylation was quantified using a PhosphorImager™ (Molecular Dynamics). Data are presented as fold-activation over basal level.

Western Blot Analysis—Asynchronously growing IIC9 cells or human HL60 cells were washed twice with cold phosphate-buffered saline and lysed in cold lysis buffer (52). Lysates were sonicated briefly, and insoluble material was pelleted by microcentrifugation at 14,000 rpm at 4 °C for 2 min. 40 μg of protein lysate was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Boston, MA) as recommended by the manufacturer. Membranes were probed individually with polyclonal antibodies to CDK2, cyclin D1, cyclin D2, or cyclin D3 (all from Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by enhancer chemiluminescence (ECL) detection (Amersham Biosciences) as recommended by the manufacturer.

Growth-arrested IIC9 cells were incubated in the presence or absence of 1 unit/ml α-thrombin for 19 h before pretreatment in the presence or absence of 10 μM LY294002 for 30 min. Cells were then washed twice with cold phosphate-buffered saline and lysed in cold lysis buffer (52). Lysates were prepared for Western analysis as described and probed with polyclonal antibodies to phosphorylated Ser-780- or Ser-795-pRb (Cell Signaling Technology, Beverly, MA) or cyclin A (Santa Cruz Biotechnology, Santa Cruz, CA).

 Luciferase Reporter Assay—IIC9 cells were transiently transfected with 0.5 μg/ml pGL3-TATA-E1α-Luc, 200 ng/ml β-galactosidase, and 1.5–2.0 μg/ml cyclin E, CDK2(D145N), Rb(ΔRbκ), or Rb(ΔRbκ)K as indicated in figure legends. Basal cells were stimulated with 1 unit/ml α-thrombin where indicated either in the presence or absence of 30 μM LY294002. Lysate protein was prepared for luciferase activity assay as recommended by the manufacturer (Promega, Madison, WI), and 10 μl of room temperature luciferase assay buffer reagent (Promega) and placed in an OpticompII luminometer (MCM Instruments, Baltimore, MD). Light produced was measured and normalized to transfection efficiency by dividing relative light units by optical density units obtained from β-galactosidase activity. β-Galactosidase activity was measured as previously described (53).

Northern Blot Analysis—Quiescent IIC9 cells were incubated in the presence or absence of 1 unit/ml α-thrombin for indicated lengths of time after preincubation in the presence or absence of 10 μM LY294002. At the indicated times total RNA was isolated using TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. RNA (20 μg) was resolved in a 2% agarose-formaldehyde gel. After electrophoresis formaldehyde was removed from the gel by washing in 0.5% ammonium acetate. RNA was then transferred to a Hybond N nylon membrane. (Amersham Biosciences) using a Turboblotter™ system (Schleicher & Schuell) and cross-linked to the membrane using an ultraviolet cross-linker (Amersham Biosciences) as recommended by the manufacturer.

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Rb(ΔRbκ)K was generated from Rb(ΔRbκ)E by site-directed mutagenesis, restoring Thr-373, Thr-412, Ser-795, and Thr-821 individually following the manufacturer’s protocol (QuickChange™ kit, Stratagene). Restoration of the threonine or serine codons was confirmed by automated capillary sequencing following the manufacturer’s protocol (Beckman-Coulter).

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were slightly adapted from established method (12, 39). For nuclear fractionation IIC9 cells were quickly scraped in 3 ml/100 mm2 pH 7.5, containing 1 μM NaCl, 1 mM EDTA, 4 μg/ml. The cells were homogenized 20 times in a Potter-Elvehjem homogenizer and spun at 500 × g for 7 min. The nuclear pellet was suspended in Buffer A and homogenized 15 times in a Dounce homogenizer with a pestle, layered over 45% sucrose in Buffer A, and centrifuged at 1660 × g for 15
min. The nuclei were washed once and resuspended in 10 mM Tris/HCl, pH 7.5, containing 10 mM NaCl, 1 mM MgCl₂, and 10% sucrose and sonicated to lyse the nuclei. Nuclear proteins (4 μg) were immunoprecipitated by incubating with 1 μg of anti-RB monoclonal antibody (Calbiochem). Immunoprecipitated proteins were recovered on protein G-Sepharose beads and dissociated by treatment with deoxycholate (12, 39). EMSAs were performed as described previously using pRb immunoprecipitates and an end-labelled double-stranded DNA fragment (1.5–2 × 10⁴ cpm/assay) containing a single E2F consensus binding site derived from the dihydrofolate reductase promoter (sc-2507; Santa Cruz Biotechnology), termed E2FRE (12, 39). For competition studies the DNA binding assays also included 10 ng of unlabeled E2FRE, mutant E2FRE double-stranded oligonucleotide with a CG to AT substitution at the E2F binding motif (E2FREmut, sc-2508) as a nonspecific competitor. To identify proteins in complex with the E2F consensus site extracts were preincubated with rabbit polyclonal antibodies (0.5 μg each) to E2F1 (sc-193 X) and E2F4 (sc-1083 X) from Santa Cruz Biotechnology before EMSA.

Thymidine Incorporation Assay—Performed as previously described (55).

RESULTS

Expression of Cyclin E Rescues CDK2, but Not CDK4 Activity in LY294002-treated cells—Phosphatidylinositol 3-OH kinase activity is essential for the accumulation of cyclin D protein and CDK4-cyclin D activity (54). In most cell types, including IIC9 cells (3), activation of CDK4-cyclin D in early G₁ results in expression of cyclin E and the activation of CDK2-cyclin E in late G₁. To determine whether ectopic expression of cyclin E rescues CDK2 activity in the absence of CDK4 activity we performed in vitro kinase assays with histone H₁ or recombinant pRb, respectively, as the substrate (Fig. 1). As expected, the activity of CDK4 and CDK2 was negligible in quiescent cells, and α-thrombin stimulated a marked activation of both CDK4 (Fig. 1A) and CDK2 (Fig. 1B). However, pretreatment with LY294002 prevents the activation of both CDK4 and CDK2. These data are consistent with the prevailing notion that CDK4-cyclin D activity is essential for pRb phosphorylation, and the subsequent expression of cyclin E and CDK2-cyclin E activation. Interestingly, ectopic expression of cyclin E rescues the α-thrombin-induced activation of CDK2 (Fig. 1B) but not CDK4 (Fig. 1A). Because IIC9 cells do not express CDK6 or cyclins D2 or D3 (Fig. 1C), these conditions represent a G₁ cell in which CDK2 is activated without the prior activation of D-type CDK activity.

In addition to providing an ideal system for studying the ability of CDK2-cyclin E alone to phosphorlyate and inactivate pRb, these data argue that cyclin D-CDK4/6 activity is dispensable for CDK2 activity and G₁ progression in the event of unscheduled cyclin E expression (Ref. 47 and see Fig. 7). According to the current model, prior activation of cyclin D-CDK complexes has two roles in the activation of cyclin E-CDK2 activation. First, the hypophosphorylation of pRb is required for the expression of cyclin E. This role is easily bypassed in our system by transfection of cyclin E. An additional function of cyclin D in the activation of cyclin E is that the formation of cyclin D-CDK complexes is thought to titrate and sequester CDK inhibitors, such as p21CIP1/WAF1 and p27KIP1, away from CDK2 complexes. Transfection of cyclin E also bypasses this second function of cyclin D complexes because cyclin E protein levels are ectopically maintained at levels above the threshold required for formation of active cyclin E-CDK2 complexes and subsequent destruction of p27KIP1 by ubiquitin-mediated proteolysis, as indicated by the observed activation of CDK2 in Fig. 1B. In any event we generated conditions in which CDK2 complexes can be activated without prior activation of CDK4 complexes, and it is these conditions that we utilized to address the inactivation of pRb by cyclin E-CDK2 alone.

Expression of Cyclin E Rescues LY294002- and p16Ink4a, inhibited E2F Activation—Ectopic cyclin E expression restores DNA synthesis in LY294002-treated cells (47) presumably because E2F activation occurs normally in these cells and ushers cells into S phase. To examine E2F activity under these condi-
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Fig. 2. CDK2-cyclin E activity drives E2F activation in the absence of CDK4-cyclin D activity. A, IIC9 cells were transiently transfected with pGL3-TATA-6xE2F-Luc, which is a construct containing six E2F response elements in tandem driving the expression of the firefly luciferase open reading frame (38, 55). An assay of relative luciferase activity from pGL3-TATA-6xE2F-transfected cells revealed that in 17 h α-thrombin stimulated a ~6-fold increase in E2F activity over that of quiescent cells (Fig. 2A). Not surprisingly, LY294002 abolished this stimulation presumably by inhibiting CDK activation and, thus, pRb phosphorylation and subsequent E2F activation. Expression of cyclin E, however, completely reversed this inhibition, as the level of E2F activity is similar to that of uninhibited cells (Fig. 2A). This argues that E2F activation is normal in these cells despite CDK4-cyclin D inhibition.

In addition to the luciferase reporter of E2F activity we also examined the steady-state mRNA levels of cyclin A, a gene that is known to contain an E2F response element and is transcriptionally regulated by E2F1 (56). Cyclin A mRNA levels reached a maximum after cells entered S phase, and by Northern blotting we observe a -9-fold induction of cyclin mRNA (normalized for GAPDH) over basal levels after 17 h of α-thrombin stimulation (Fig. 2B). Interestingly, both cyclin A mRNA accumulation (Fig. 2B) and E2F reporter activation (Fig. 2A) display G1 time-course kinetics suggestively similar to CDK2-cyclin E activation (47, 50, 57, 58). Also in concurrence with the data from the E2F reporter, we find that the α-thrombin-stimulated induction of cyclin A message levels can be largely blocked by preincubation with LY294002 (Fig. 2C). However, as also seen with the E2F reporter, this attenuation can be reversed by ectopic expression of cyclin E.

To ensure that these observations regarding mRNA levels translate to steady-state protein expression, we also assessed cyclin A protein levels by Western blotting. In accordance with our observations of mRNA levels, we find that cyclin E expression completely restores the LY29402-attenuated, α-thrombin-stimulated induction of cyclin A protein expression (Fig. 2D). Although treatment of IIC9 cells with LY29402 attenuates the activity of CDK4-cyclin D1 to the basal level of detection (Fig. 1A), we utilized a potent inhibitor of D-type CDKs, p16^INK4a (59), to ensure complete inhibition of cyclin D1-CDK4. As expected, p16 expression prevents α-thrombin-stimulated cyclin A protein induction (Fig. 2D). However, cyclin E expression restored cyclin A accumulation in p16-expressing, LY294002-treated cells. These data taken together demonstrate that cyclin E expression renders cyclin D-CDK4 activity dispensable in the initiation of E2F activity.

GW8510 or Co-expression of CDK2(D145N) Eliminates the Ability of Cyclin E to Rescue E2F Activity—Because cyclin E is not known to bind to or activate other CDKs, the ability of cells were incubated in the absence or presence of 10 μM LY294002 or 7.5 μM GW8510 followed by stimulation with α-thrombin for 17 h. Lysates were assayed for luciferase and β-galactosidase activity (B-gal), and relative light units (RLU) were divided by units of optical density, respectively, for quantification and normalization. B, quiescent IIC9 cells were stimulated with α-thrombin for indicated lengths of time. RNA was isolated and analyzed for cyclin A and GAPDH mRNA and quantified as described under “Materials and Methods”; cyclin A arbitrary units were divided by GAPDH arbitrary units for normalization. Error bars represent S.D. (n = 3). C, D, IIC9 cells were transiently transfected with cyclin E and/or p16^INK4a where indicated followed by 48–60 h of serum deprivation. Cells were incubated in the presence or absence of 10 μM LY29004 or 7.5 μM GW8510 for 30 min, then stimulated with 1 unit/ml α-thrombin for 17 h (C) or 19 h (D). C, RNA was isolated and analyzed for cyclin A mRNA and quantified, normalizing for GAPDH mRNA for normalization. D, protein lysates were analyzed for cyclin A protein levels by Western blotting, as described under “Materials and Methods.” All data are representative of at least three independent experiments.
ectopic cyclin E expression to rescue E2F activity in LY294002-treated cells is presumably through the activation of CDK2. To test this we made use of a dominant-negative (dnCDK2) construct, CDK2(D145N) (60). We find that co-expression of dnCDK2 prevented the rescue of E2F activity by cyclin E in LY294002-treated cells, as measured by luciferase and β-galactosidase (B-gal) activity, and relative light units (RLU) were divided by units of optical density, respectively, for quantification and normalization. B, IIC9 cells were transiently transfected with cyclin C and/or CDK2(D145N) where indicated followed by serum deprivation for 48–60 h. Quiescent cells were incubated in the presence or absence of 10 μM LY294002 for 30 min, then stimulated with 1 unit/ml α-thrombin for 17 h. Lysates were analyzed for cyclin A and GAPDH mRNA and quantified; cyclin A arbitrary units were divided by GAPDH arbitrary units for normalization. All data are representative of three independent experiments.

Our evidence of E2F activation under conditions of CDK4 inhibition substantiates the assertion that phosphorylation of pRb by CDK2-cyclin E alone can trigger pRb inactivation (42, 45). To more directly test this supposition we next conducted an EMSA according to an established protocol utilizing the E2F response element as the probe (12, 39). Fig. 4, lane 2, shows electromobility shift of the probe when incubated with pRb immunoprecipitates from quiescent nuclei. Specificity was ensured by failure of pRb to shift a mutated probe (not shown). These data together with a time-course of E2F activation that closely mirrors CDK2 activation (Fig. 2A, first through fourth lanes; Fig. 3A) (50, 57, 58, 62) strongly support the notion of a function for CDK2 in the activation of E2F during G1 progression. We cannot rule out the possibility that, as may be suggested by recent reports, an additional pRb kinase activity exists (see “Discussion”). However, the kinase activity in our system requires phosphatidylinositol 3-OH kinase for activation, is activated by cyclin E expression, and is sensitive to inhibition by GW8510 and dnCDK2, compelling evidence that CDK2 is the pRb kinase responsible for E2F activation in this study.

CDK2-cyclin E Alone Can Phosphorylate and Inactivate pRb in Vivo—The implication of our observation that cells in which α-thrombin stimulates E2F activation and G1 progression despite CDK4 inhibition is that CDK2-cyclin E is able to phosphorylate and inactivate pRb without prior phosphorylation by CDK4-cyclin D1. However, the possibility remains that the activation of E2F observed under these conditions is somehow independent of pRb phosphorylation. To investigate the phosphorylation of pRb in cells lacking CDK4 activation, we made use of two phospho-specific pRb antibodies. One recognizes only pRb that is phosphorylated on position Ser-795, which can be phosphorylated by either CDK4 or CDK2, and the other recognizes pRb that is phosphorylated on position Ser-780, which is only phosphorylated by CDK4 and not CDK2 (7, 16, 18, 43). Not surprisingly, very little phosphorylation of these residues is detected in lysates from serum-starved cells (Fig. 4A) as pRb is thought to be unphosphorylated in quiescence (17, 18, 24, 43, 44). The minimal phosphorylation seen in our blots is most likely because of a small population of cells that escape serum starvation. In contrast, robust phosphorylation of pRb is observed with both phospho-specific antibodies after 17 h of α-thrombin stimulation. As expected, preincubation with LY294002 diminishes phosphorylation at both sites. Although having no effect on the LY294002-inhibited phosphorylation of Ser-780, dramatically, ectopic cyclin E expression completely restores the level of phosphorylation of Ser-795 to uninhibited levels. Thus, a CDK4-specific phosphorylation site remains unphosphorylated, whereas a site that can be phosphorylated by CDK2-cyclin E is indeed phosphorylated. Although far from a complete analysis of pRb phosphorylation, this observation demonstrates that at least a subset of potential CDK2 phosphorylation sites on pRb is phosphorylated without prior hypophosphorylation of pRb by CDK4.

Our interpretation is supported by the observation that antibodies to E2F1 (lane 4) and E2F4 (lane 5) super-shift the pRb-retarded band. Failure of saturating amounts of these antibodies to induce complete super-shift is consistent with the
notion that in quiescent cells pRb exists in multiple complexes (7, 63). Antibodies to E2F1 and E2F4 were chosen for study because they are thought to be binding partners of pRb in quiescent cells (7, 64–66). When coalesced with our observations regarding E2F activation, these data regarding pRb phosphorylation and E2F-responsive promoter binding make a strong case that hypophosphorylation by cyclin D-dependent kinase, once thought to be prerequisite, is actually dispensable for the phosphorylation and inactivation of pRb by cyclin E-dependent kinase under the condition of constitutive cyclin E expression.

Expression of Rb<sup>ΔCDK</sup> Blocks the Activation of E2F by CDK2-Cyclin E—Given our data regarding activation of E2F and release of pRb from E2F transcription complexes in the absence of cyclin D1-CDK4 activity, we reasoned that expression of Rb<sup>ΔCDK</sup>, a hyper-repressive mutant of pRb, would block cyclin E-dependent rescue of E2F activation in our system. Rb<sup>ΔCDK</sup> has 14 of the 16 known CDK phosphorylation sites mutated to alanine and, therefore, functions as a constitutively active cell cycle repressor immune to the inactivating effects of the G1 cyclin-CDKs (67). This construct, when transfected into IIC9 cells, prevents the α-thrombin-induced activation of the E2F-luciferase reporter plasmid (not shown). Interestingly, expression of Rb<sup>ΔCDK</sup> further prevents the ectopic cyclin E-mediated rescue of E2F activation in LY294002-treated cells (Fig. 5A), whereas expression of wild-type pRb is ineffective in blocking E2F activation, presumably because wild-type pRb is subject to inactivation by the CDKs as cells progress through G1 (Fig. 6A and data not shown). In accordance with this result, Rb<sup>ΔCDK</sup> significantly attenuates the cyclin E rescue of cyclin A induc-
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Unscheduled Cyclin E Expression Renders Cyclin D-CDK Activity Dispensable for G1 Progression—CDK2-cyclin E activity has been long thought to be essential for the progression from G1 to S phase of the cell cycle (2, 69). In cells expressing pRb, hyperphosphorylation of pRb by the G1 CDKs, which plays a role in the transition from G1 to S phase, is not able to occur because of the presence of pRb. However, cyclin E expression rescues S phase entry by relieving the constitutive repression of E2F activity mediated by pRb. This rescue is dependent on the ability of cyclin E to activate CDKs. Specifically, cyclin E activity is required to inactivate pRb, allowing for E2F-mediated transcription of S-phase genes and the initiation of DNA synthesis.

**DISCUSSION**

Unscheduled Cyclin E Expression Renders Cyclin D-CDK Activity Dispensable for G1 Progression—CDK2-cyclin E activity has been long thought to be essential for the progression from G1 to S phase of the cell cycle (2, 69). In cells expressing pRb, hyperphosphorylation of pRb by the G1 CDKs, which...
Cells were then pulsed with 0.5 μM H9262 incubated in the presence or absence of 10 μM GW8510 30 min before stimulation with 1 unit/ml thrombin for 17 h. The observation that cyclin E-null MEFs cannot be stimulated to proliferate following serum starvation suggests a crucial role of cyclin E in cell cycle re-entry through the inactivation of pRb.

Our results further the understanding of the recognized role that deregulation of cyclin E plays in tumorigenesis. In addition to the observation that many cancer cell lines overexpress full-length cyclin E or an amino-truncated variant (70–73), deregulated cyclin E has been reported in primary tumors of many different cell types (74). The clinical impact of cyclin E expression appears most dramatic in the pathology of breast cancer. A recent report from Keyomarsi et al. (75) argues convincingly that cyclin E expression levels are the single most powerful clinical parameter in predicting long-term prognosis in breast cancer patients, with high levels of cyclin E having an even higher hazard ratio than the classic prognostic indicators cyclins D1 and D3, HER-2/neu, estrogen/progesterone receptor status, and lymph node metastases (75). Supplemeting that argument, Span et al. (76) recently reported that the expression level of cyclin E is a strong predictor of endocrine therapy failure in breast cancer patients, a result that builds upon mounting evidence that cyclin E is a critical target of endocrine signaling in promoting steroid-dendent tumor growth (77). Finally, 10 percent of mice that express a human cyclin E transgene develop carcinoma (78), and cyclin E knockout MEFs are strikingly resistant to transformation with oncogenic Ras (Ref. 79 and see next paragraph). The data reported here are consistent with the notion that the oncogenic potential of atypical cyclin E expression is due to inappropriate cell cycle re-entry through the inactivation of pRb.

Two recent controversial reports seemingly call into question the necessity of CDK2-cyclin E activity for cell cycle progression. The two reports that CDK2 knockout mice develop normally and, despite meiotic defects and sterility, are viable and healthy (80, 81) and that cyclin E1/E2 double knockouts can be born alive by placental rescue of cyclin E expression (79) suggest that CDK2-cyclin E activity is dispensable for cell-autonomous murine embryogenesis and cell proliferation. These findings, although intriguing and no doubt warrant re-examination of current cell cycle dogma, do not challenge the observations reported here. This work is particularly concerned with what unscheduled cyclin E expression can do in terms of pRb phosphorylation, E2F activation, and cell cycle progression. The observation that cyclin E-null MEFs cannot be stimulated to proliferate following serum starvation suggests a crucial role for cyclin E in cell cycle re-entry from quiescence, an event that may be critical during in vivo tumorigenesis (79). In any event, whether or not CDK2-cyclin E is the normal mediator of E2F activation awaits a more comprehensive study and is beyond the scope addressed in the present study. However, given our findings a strong case can be made for the ability of aberrant cyclin E expression to drive the inactivation of pRb and, thus, G1 progression, despite CDK4-cyclin D inhibition, a finding that augments the known role of cyclin E in tumor progression and prognosis.

Interestingly, another recent report has challenged previous interpretations regarding the role of pRb in G1 cell cycle control. By using “acute” conditional loss of pRb to cells in culture, a system more accurately representing the sporadic inactivation of RB alleles seen in the genesis of a tumor, Sage et al. (82) convincingly reaffirm that pRb is the critical mediator of the senescence program in cell culture-induced cell cycle exit in MEFs. By escaping the complications of developmental com-

**Fig. 7.** Cyclin E-CDK2 without prior cyclin D-CDK activity is sufficient for entry into S phase. IIC9 cells were transiently transfected with p16^INK4a, cyclin E, pcDNA3.1, pRb, Rb^+/−, and/or Rb^−/− where indicated followed by serum deprivation for 48–60 h. Cells were then pulsed with 0.5 μCi/ml [3H]thymidine for 4 h and assayed for trichloroacetic acid (TCA)-precipitable counts as described.
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penetration and plasticity, this report further highlights the tumor-promoting potential of pRb inactivation in quiescent somatic cells; that is, cells that cannot be recalibrated their senescence regulators for a light of its results, aberrant cyclin E expression could provide just the window of opportunity needed to initiate tumor formation in vivo.

Going further, it would be interesting to analyze the phosphorylation status of all of the CDK2-preferred sites on pRb in CDK2- or cyclin E knockout cells. Provocatively, in cyclin E1/ E2-null MEFs, pRb does appear to become phosphorylated sometime in late G1, suggesting the compensatory or perhaps redundant activity of a cyclin E-independent G1 kinase (79). Even more provocatively, pRb appears phosphorylated on Thr-821 in CDK2–/– MEFs, a thought to be phosphorylated by CDK2, suggesting that the crucial inactivation of pRb is somehow endogenously recovered in these cells (80).

Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80). Further complicating this issue is the fact that a mutant form of cyclin E can also be activated in CDK2–/– MEFs (80). Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80). Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80). Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80). Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80). Further complicating this issue is the fact that a mutant form of cyclin E that cannot bind to CDK2 transforms rat embryo fibroblasts (80).

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