Cyclin-dependent Kinase 2 Nucleocytoplasmic Translocation Is Regulated by Extracellular Regulated Kinase*

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Activation of cyclin-dependent kinase 2 (CDK2)-cyclin E in the late G1 phase of the cell cycle is important for transit into S phase. In Chinese hamster embryonic fibroblasts (IIC9) phosphatidylinositol 3-kinase and ERK regulate α-thrombin-induced G1 transit by their effects on cyclin D1 protein accumulation (Phillips-Mason, P. J., Raben, D. M., and Baldassare, J. J. (2000) J. Biol. Chem. 275, 18046–18053). Here, we show that ERK also affects CDK2-cyclin E activation by regulating the subcellular localization of CDK2. Ectopic expression of cyclin E rescues the inhibition of α-thrombin-induced activation of CDK2-cyclin E and transit into S phase brought about by treatment of IIC9 cells with LY29004, a selective inhibitor of mitogen stimulation of phosphatidylinositol 3-kinase activity. However, cyclin E expression is ineffectual in rescuing these effects when ERK activation is blocked by treatment with PD98059, a selective inhibitor of MEK activation of ERK. Investigation into the mechanistic reasons for this difference found the following. 1) Although treatment with LY29004 inhibits α-thrombin-stimulated nuclear localization, ectopic expression of cyclin E rescues CDK2 translocation. 2) In contrast to treatment with LY29004, ectopic expression of cyclin E fails to restore α-thrombin-stimulated nuclear CDK2 translocation in IIC9 cells treated with PD98059. 3) CDK2-cyclin E complexes are not affected by treatment with either inhibitor. These data indicate that, in addition to its effects on cyclin D1 expression, ERK activity is an important controller of the translocation of CDK2 into the nucleus where it is activated.

Cyclin-dependent kinase 2 (CDK2) is a member of a family of serine/threonine kinases essential for ordered progression through the cell cycle (2). Activation of CDK2 requires assembly of CDK2 with its regulatory subunit cyclin E. The synthesis of cyclin E occurs late in G1, and is controlled by CDK4,6-cyclin D activity, which increases in early G1 (3–5). CDK4,6-cyclin D phosphorylates retinoblastoma (pRb) protein partially inactivating its negative regulatory functions, allowing for cyclin E expression and the activation of CDK2-cyclin E. The ordered activation of these CDKs results in the sequential phosphorylation of pRb (6–8), the activation of the E2F family of transcription factors (9, 10), and controlled progression through the G1 phase of the cell cycle.

Mitogen stimulation of quiescent cells is essential for cyclin D expression and activation of CDK4,6-cyclin D (11–13). In late G1 cells become refractory to the removal of extracellular growth factor (11–13). Advancement through the restriction point requires sequential phosphorylation of pRb by CDK4-cyclin D and then by CDK2-cyclin E (11–13). Phosphorylation of pRb by CDK2-cyclin E results in full inactivation of pRb (11–13). Although the only known substrate for CDK4-cyclin D is pRb, CDK2-cyclin E affects G1 progression by phosphorylating substrates other than pRb. For example, in cells in which pRb is inactivated by expression of SV40 large T antigen, inactivation of CDK2-cyclin E still results in G1 arrest (14). However, inactivation of CDK4-cyclin D is without effect. In addition, although mice in which the coding sequences of the cyclin D1 gene are deleted show several developmental abnormalities, including hypoplastic retinas, replacement of the missing cyclin D1 coding sequences with cyclin E rescues the wild type phenotypes (15). In these mice expression of cyclin E is under control of the cyclin D1 promoter and, therefore, independent of regulation by Rb phosphorylation. Furthermore, pRb lysates from the developing retinas of the cyclin E “knockin” mice show only 20% of the levels of Rb phosphorylation when compared with wild type (15). These data indicate that ectopic expression of cyclin E can rescue the wild type phenotype without the need for full inactivation of pRb (15). Furthermore, passage of pRb−/− cells through G1 still requires CDK2-cyclin E activation suggesting CDK2-cyclin E phosphorylates targets other than pRb, and these are essential for progression through G1 into S (14).

Activation of CDK2 is regulated by dephosphorylation of Thr14 and Tyr15 by cdc25A (16) and phosphorylation of Thr160 by CDK-activating enzyme (CAK) (17, 18). Ectopic expression of cdc25A shortens the time of G1 by dephosphorylating CDK2-cyclin E (19). Because both cdc25A and CAK are localized to the nucleus, translocation of CDK2-cyclin E to the nucleus is important for interaction of the CDK2-cyclin E complex with these regulatory factors. The mechanism responsible for CDK2 translocation from the cytoplasm to the nucleus is still poorly understood. α-Thrombin, a potent mitogen of IIC9 cells, is a serine protease that activates a G-protein-coupled receptor known as PAR-1 (protease-activated receptor) (20). Receptor activation and subsequent dissociation of the α-GTP subunit from the βγ subunits results in coordinate stimulation of several mitogenic signaling pathways, cell cycle re-entry, and progression through the cell cycle (21). Recent data from our laboratory (1) demonstrate a role for ERK and phosphorylated p38 MAPKK (PI) 3-kinase activities in cell growth. Interestingly, both ERK and PI 3-kinase regulate the expression of cyclin D1 and activation of CDK4-cyclin D1 activity in Chinese hamster embryonic fibroblasts (IIC9).
In this study we show ERK activity regulates CDK2-cyclin E activity independent of its effect on cyclin D1 expression and CDK4-cyclin D1 activity. Both ERK and PI 3-kinase are essential for cyclin E expression by their effects on the expression of cyclin D1 and CDK4-cyclin D1 activation (1). Although transient expression of cyclin E rescues α-thrombin-induced CDK2-cyclin E activity and G1 progression in IIC9 cells treated with LY294002, an inhibitor of PI 3-kinase activation, transient expression of cyclin E is without effect in IIC9 cells treated with PD98059, an inhibitor of MEK activation of ERK. We also provide evidence that the ectopically expressed cyclin E associates with cyclin D in cells treated with PD98059, but the complex does not translocate into the nucleus. These data clearly demonstrate that in addition to its ability to control cyclin D1 expression, ERK also regulates CDK2-cyclin E nucleo-cytoplasmic translocation.

MATERIALS AND METHODS

Cell Culture and Reagents—IIC9 cells were maintained in Dulbecco’s modified Eagle’s medium 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 of streptomycin (all from Sigma). Growth-arrested IIC9 cells were established by washing subconfluent (80%) cells once with phosphate-buffered saline (PBS) followed by a 48-h incubation with α-MEM medium containing 2 mM l-glutamine (BioWhittaker) supplemented with 100 units/ml penicillin and 100 mg/ml of streptomycin (basal medium). Growth-arrested IIC9 cells were stimulated with 1 unit/ml of human α-thrombin and incubated for the indicated times at 37 °C in 5% CO2, PD98059 (New England Biolabs, Beverly, MA) was used at 15 μM. LY294002 (Calbiochem) was used at 10 μM.

Transient Transfections—IIC9 cells were grown to subconfluence (80%) in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose and 2 mM l-glutamine (BioWhittaker) supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 mg/ml of streptomycin (Sigma). The cells were transfected with a solution containing 5 μl PlusTM, 4 μlLipofectAMINE™ (Life Technologies, Inc.), and 2 μg/ml DNA in Opti-MEM medium (Life Technologies, Inc.) following the manufacturer’s protocol. 5 h post-transfection, Dulbecco’s modified Eagle’s medium supplemented with calf serum (final serum concentration of 1% (v/v)), 100 units/ml penicillin, and 100 mg/ml of streptomycin were added. After 12 h the cells were treated with cold ethanol at -20 °C. The cells were washed twice with PBS and then blocked in 1 ml of blocking buffer (0.8 g of fatty acid-free bovine serum albumin (Sigma) in 100 μl of PBS) for 2 h at room temperature. Polyclonal CDK2 antibody (Santa Cruz Biotechnology) or monoclonal cyclin D1 antibody (Santa Cruz Biotechnology) was added at 1:50 dilution in blocking buffer for 45 min at room temperature. After washing three times with PBS, the secondary antibody, Texas Red-linked anti-monomonal IgG (Amersham Pharmacia Biotech), was added at 1:50 dilution in blocking buffer for 45 min at room temperature. The immunoreactive bands were visualized using a fluorescence microscope.

Western Blot Analysis—Growth-arrested IIC9 cells were incubated in the absence or presence of 1 units/ml α-thrombin for 17 h after preincubation in the absence or presence of 10 μM LY294002 or 15 μM PD98059 for 30 min. Subsequent to activation, the cells were fixed in a 3.7% formalin (Sigma) solution for 10 min at room temperature followed by a 6-min incubation in ice-cold methanol at -20 °C. The cells were washed twice with cold PBS and lysed in cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 μM β-glycerophosphate, and 10 μg/ml pepstatin). The lysates were sonicated briefly, and insoluble material was pelleted by microfugation at 14,000 × g at 4 °C for 4 min. Protein concentrations were determined using Coomassie™ Plus (Pierce) as recommended by the manufacturer. Protein lysates (15-20 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) as recommended by the manufacturer. Membranes were probed with polyclonal antibodies to cyclin D1, CDK2, CDK4 (Santa Cruz Biotechnology, Santa Cruz, CA), and cyclin E (Upstate Biotechnology, Lake Placid, NY). Immunoreactive bands were visualized by ECL detection (Amersham Pharmacia Biotech) as recommended by the manufacturer.

CDK2-Cyclin E Assay—Growth-arrested IIC9 cells were incubated in the absence or presence of 1 units/ml α-thrombin in the presence or presence of 10 μM LY294002 or 15 μM PD98059 for 30 min. After 17 h, cells were washed twice with cold PBS and lysed in cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM PMSF, 2 μM sodium vanadate, 20 mM sodium fluoride, 50 μM β-glycerophosphate, 10 μM aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). The lysates were sonicated briefly, and insoluble material was pelleted by microfugation at 14,000 × g at 4 °C for 4 min. Protein concentrations were determined using Coomassie™ Plus (Pierce) as recommended by the manufacturer. Protein lysates (150 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) as recommended by the manufacturer. Membranes were probed with polyclonal antibodies to cyclin D1, CDK2, CDK4, and cyclin E (Upstate Biotechnology, Lake Placid, NY). Immunoreactive bands were visualized by ECL detection (Amersham Pharmacia Biotech) as recommended by the manufacturer.
A

![Graph](image)

**FIG. 1.** Treatment with PD98059 and LY294002 inhibits cyclin E protein accumulation and CDK2-cyclin E activity. Growth-arrested IIC9 cells were stimulated with 1 units/ml α-thrombin for 0, 4, 8, 12, 17, or 24 h. At the indicated times cells lysates were prepared by scraping into cold lysis buffer (see "Materials and Methods"). A, an aliquot from each time point was analyzed for cyclin E protein accumulation and CDK2-cyclin E activity. Lysate proteins (25–35 μg) were separated by SDS-polyacrylamide gel electrophoresis (9.75%) and immunoblotted with a polyclonal cyclin E antibody, or CDK2-cyclin E complexes were immunoprecipitated from lysates containing equal amounts of protein with a polyclonal CDK2 antibody, and CDK2-cyclin E activity was assayed for their ability to phosphophorylate histone H1 in *vitro* as described under "Materials and Methods." B, growth-arrested IIC9 cells were stimulated with 1 units/ml α-thrombin for 17 h in the absence or presence of 10 μM LY294002 or 15 μM PD98059. Cyclin E levels and CDK2-cyclin E activities were assayed as described above. The data are representative of three independent experiments.

mm EGTA, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, 1 mM PMSF, 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. Immune complexes were resuspended in cold lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Millipore Corp.) as recommended by the manufacturer. Membranes were probed with polyclonal antibodies to CDK2 or CDK4 (Santa Cruz Biotechnology). Immunoreactive bands were visualized by ECL detection (Amersham Pharmacia Biotech) as recommended by the manufacturer.

**RESULTS AND DISCUSSION**

*Both ERK and PI 3-kinase Activities Are Required for CDK2-Cyclin E Activity*—In IIC9 cells ERK and PI 3-kinase activities are essential for α-thrombin-induced cyclin D1 expression, CDK4-cyclin D kinase activity, and cell growth (1). Because pRb, which is the only known substrate of CDK4-cyclin D, binds to and represses the E2F family of transcription factors (9), and the cyclin E gene promoter is under E2F regulation, we expect that cyclin E expression and CDK2-cyclin E activity will be blocked in cells treated with either PD98059, an inhibitor of MEK activation of ERK, or LY294002, a selective inhibitor of PI 3-kinase (Fig. 1). We initially examined by Western blot analysis the time course of cyclin E protein expression (Fig. 1A). Cyclin E protein increases ~8 h after α-thrombin addition reaching maximal protein levels after 24 h (Fig. 1A). Consistent with the observed increases in cyclin E protein, CDK2-cyclin E activity does not increase until 10 to 12 h (Fig. 1A). We next examined the effects of the inhibition of ERK and PI 3-kinase activities on cyclin E expression and CDK2-cyclin E activity. As found previously (1), pretreatment with either PD98059 or LY294002 dramatically prevents α-thrombin-mediated cyclin D1 expression and CDK4-cyclin D1 activity (data not shown). Consistent with the prevailing notion that the phosphorylation of pRb by CDK4-cyclin D is essential for cyclin E expression, pretreatment with PD98059 or LY294002 blocks α-thrombin-stimulated cyclin E expression (Fig. 1B). As expected, treatment with either inhibitor prevents α-thrombin-induced CDK2-cyclin E stimulation (Fig. 1B).

**Ectopic Expression of Cyclin E Rescues the Inhibition of CDK2-Cyclin E Activity by Inhibitors of PI 3-kinase but Not ERK**—Because the synthesis of cyclin E is essential for CDK2-cyclin E activity, we reasoned that ectopic expression of cyclin E would rescue the inhibition of CDK2-cyclin E activity that is seen with treatment with either LY294002 or PD98059 (Fig. 1B). Consistent with this hypothesis, replacing the cyclin D1 gene with cyclin E gene restores the wild type phenotype in animals deficient in cyclin D1 expression (15). We next examined whether the lack of CDK2-cyclin E activity is rescued by ectopic cyclin E expression and, therefore, is dependent on the decrease in cyclin E protein levels that we observed in the presence of either LY294002 or PD98059. In IIC9 cells transient expression of cyclin E results in marked increases in the amounts of cyclin E protein in the presence or absence of either PD98059 or LY294002 (Fig. 2). Ectopic expression of cyclin E rescues the LY294002-induced decrease in CDK2-cyclin E activity (Fig. 2) as determined by *in vitro* histone phosphorylation. Surprisingly, expression of cyclin E does not rescue the inhibition of CDK2-cyclin E activity by PD98059 (Fig. 2), although the levels of cyclin E are similar to those in cells treated with LY294002 (Fig. 2). These data suggest that ERK activity has an additional role in regulating CDK2-cyclin E activity, in addition to controlling cyclin E expression via its effect on cyclin D1 levels (22–25).

**Expression of Cyclin E Rescues Growth in IIC9 Cells in Which α-Thrombin-induced PI 3-kinase but Not ERK Activation Is Blocked**—Ectopic expression of cyclin E rescues CDK2-cyclin E activity in cells in which cyclin D expression and CDK4-cyclin D1 activity are repressed. To address the effects of the activation of CDK2-cyclin E in the absence of CDK4-cyclin D1 activity on cell cycle progression, we examined the ability of expression of cyclin E to rescue cell cycle re-entry and G1 progression in cells treated with the PI 3-kinase or ERK inhibitors (Fig. 3). As previously shown (1), treatment of IIC9 cells with either PD98059 or LY294002 blocks growth as determined by [3H]thymidine incorporation (Fig. 3). Although ectopic expression of cyclin E rescues α-thrombin-stimulated growth in IIC9 cells treated with LY294002, it is ineffective in cells in which ERK activity is blocked by pretreatment with PD98059 (Fig. 3). These data are consistent with the observation that in
of [3H]thymidine. Cells were washed, and DNA was precipitated as described for CDK2-cyclin E activity. Lysate proteins (15–35 μg) were separated by SDS-polyacrylamide gel electrophoresis (9.75% or 12.75%) and immunoblotted with polyclonal cyclin E or CDK2 antibodies. CDK2-cyclin E complexes were immunoprecipitated from lysates containing equal protein with a polyclonal CDK2 antibody and assayed for CDK2-cyclin E activity as described under Fig. 1. The data are representative of three independent experiments.

Growth-arrested IIC9 cells were stimulated with 1 units/ml α-thrombin for 17 h in the absence or presence of 10 μM LY294002 or 15 μM PD98059. Where indicated, cells were transiently transfected with cyclin E as described under “Materials and Methods.” Cells were harvested by scraping into cold lysis buffer (see “Materials and Methods”). Lysates were assayed for cyclin E and CDK2 protein expression. Lysates were also assayed for CDK2-cyclin E activity. Lysate proteins (15–35 μg) were separated by SDS-polyacrylamide gel electrophoresis (9.75% or 12.75%) and immunoblotted with polyclonal CDK2 antibody and assayed for CDK2-cyclin E activity as described under Fig. 1. The data are representative of three independent experiments.

mice in which the coding sequences of the cyclin E gene replace those of cyclin D1, the mice appear to be normal (15). Furthermore, these results suggest that ERK activation controls CDK2-cyclin E activity independent of its effects in regulating cyclin D1 expression and CDK4-cyclin D1 activation. CDK2 Nucleocytoplasmic Translocation Is Dependent upon ERK Activity—Subcellular compartmentalization of protein complexes is an underlying regulatory process that affects many cellular events. The subcellular distribution of CDKs and their regulatory cyclins is an important factor for regulating their activities and their ability to control cell cycle progression. The nucleocytoplasmic redistribution of CDK2 is essential for activation, and indeed both the regulatory CK required for Thr160 phosphorylation and cdc25A, the phosphatase responsible for dephosphorylation of Thr14 and Tyr15, are constitutively located in the nucleus (26, 27). To determine whether the inability of ectopically expressed cyclin E to rescue α-thrombin-induced CDK2-cyclin E activation in the presence of PD98059 is a result of the failure of CDK2 to translocate to the nucleus, we examined the effects of both PD98059 and LY294002 on CDK2 redistribution in α-thrombin-stimulated IIC cells transiently transfected with cyclin E (Fig. 4). In serum-starved IIC9 cells CDK2 is detected in the cytoplasm (Fig. 4A). We next asked whether translocation of significant amounts of CDK2 is seen in cells stimulated with α-thrombin for 17 h. We selected 17 h, because significant CDK2-cyclin E activity is found at this time (Fig. 1A). After 17 h significant amounts of CDK2 are found in the nucleus (Fig. 4B). In IIC9 cells stimulated with α-thrombin but pretreated with either PD98059 or LY294002, CDK2 is localized exclusively to the cytoplasm (Fig. 4, C and E). In agreement with the ability of ectopic expression of cyclin E to rescue CDK2-cyclin E activity in IIC9 cells treated with LY294002, ectopic expression of cyclin E induces significant nuclear localization of CDK2 in α-thrombin-stimulated cells treated with LY294002 (Fig. 4D). However, ectopic cyclin E expression is ineffective in rescuing CDK2 translocation in cells preincubated with PD98059 (Fig. 4F). Thus, ERK promotes CDK2-cyclin E activation by two mechanisms, the regulation of CDK4-cyclin D1 activity, which in turn controls the synthesis of cyclin E, and the translocation of the CDK2-cyclin E complex into the nucleus.

CDK2-Cyclin E Complex Formation Is Independent of PI 3-kinase and ERK—The association of CDK2 with the regulatory subunit, cyclin E, is a rate-limiting step in the formation of active CDK2-cyclin E complexes (28, 29), indicating that formation of these complexes with cyclin E in late G1 are essential for activation. At present little is known concerning the formation of these complexes with cyclin E in vivo. A possible explanation for the ability of expression of cyclin E to rescue CDK2-cyclin E activity in cells in which PI 3-kinase activity is blocked, but is ineffective when ERK activation is prevented, is that ERK plays an important role in complex formation in vivo. We investigated this possibility by determining whether endogenous CDK2 associates with ectopically expressed cyclin E (Fig. 5). Cyclin E immunoprecipitates from α-thrombin-stimulated IIC9 cells overexpressing cyclin E contain CDK2 as detected by Western blot analysis (Fig. 5, lane 2). To ensure the selectivity of the association of ectopically expressed cyclin E with CDK2, we examined the ability of overexpressed cyclin E to bring...
inhibitors do not affect the association of CDK2 with cyclin E. Late G1 enables cells to progress into S phase and replicate cellular DNA (14, 30, 31). Because cyclin E expression depends on CDK4-cyclin D1 activity, CDK2-cyclin E activity is downstream of CDK4-cyclin D1 (1). Accumulation of cyclin D1 is controlled by both ERK (23–25) and PI 3-kinase (1, 32, 33); therefore both signaling pathways are important for mitogen activation of cyclin D1 expression and CDK4-cyclin D1 activity. Furthermore, these pathways have similar effects on pRb inactivation, cyclin E expression, and CDK2-cyclin E activation. Because of their similar effects on cyclin E expression, we expected that ectopic expression of cyclin E would rescue the inhibition of α-thrombin-induced CDK2-cyclin E activity observed with pretreatment with either inhibitor. However, whereas the ectopic expression of cyclin E rescues the effects of the inhibition of PI 3-kinase on growth and CDK2-cyclin E activity, it is ineffectual in rescuing the inhibition of CDK2-cyclin E seen when ERK activation is blocked. These data suggest an additional role for ERK in the regulation of translocation of CDK2-cyclin E into the nucleus where it is activated by dephosphorylation by cdc25A and phosphorylation by CAK (34).

The Role of ERK in CDK2-Cyclin E Nucleocytoplasmic Translocation—It is likely that the effect of ERK on CDK2-cyclin E translocation to the nucleus is not solely a direct effect as has recently been proposed (35). These investigators put forward that nuclear translocation of CDK2 is associated with complexes containing active ERK. In this scheme CDK2-cyclin E associates with active ERK and is carried into the nucleus along with ERK, which acts as a nuclear transport factor. In IIC9 cells CDK2 translocation occurs several hours after the addition of α-thrombin. However, mitogens including α-thrombin (36, 37) activate ERK and induce ERK translocation to the nucleus within 10 to 15 min. Importantly, in cells in which cyclin E is ectopically expressed prior to α-thrombin addition, the earliest we are able to detect CDK2 translocation is ~12 h after α-thrombin stimulation (data not shown). This suggests that CDK2 translocation is dependent on some other function of ERK, possibly its effect on Elk-1-dependent transcriptional activation. Therefore, we propose it likely that ERK activation results indirectly in the translocation of CDK2-cyclin E. Although the mechanism of CDK2 nuclear translocation awaits further investigation, the data in this paper clearly show that there is a role for ERK activity, in addition to regulation of cyclin E expression.

FIG. 4. CDK2 nucleocytoplasmic translocation is dependent on ERK. Growth-arrested IIC9 cells were stimulated with 1 unit/ml α-thrombin (B–F) for 17 h in the absence of (B) or presence of 10 μM LY294002 (C and D) or 15 μM PD98059 (E and F). Where indicated, cells were transiently transfected with cyclin E (D and F). Cells were fixed in a 3.7% Formalin solution (see “Materials and Methods”), and cellular DNA was visualized with a fluorescent microscope. The data are representative of three independent experiments.

FIG. 5. CDK2-cyclin E complex formation is independent of PI 3-kinase and ERK. Growth-arrested IIC9 cells were stimulated with 1 unit/ml α-thrombin for 17 h following preincubation in the absence or presence of 10 μM LY294002 or 15 μM PD98059. Where indicated, cells were transiently transfected with cyclin E or cyclin D. Cells were harvested by scraping into cold lysis buffer (see “Materials and Methods”). Cyclin E or cyclin D were immunoprecipitated from lysates containing equal protein with a polyclonal antibody to cyclin E or monoclonal cyclin D antibody, respectively. Proteins from each immunoprecipitate were separated by SDS-polyacrylamide gel electrophoresis (12.75%) and immunoblotted with a polyclonal antibody to CDK2, CDK4, phosphorylated CDK2, and cyclin D (data not shown). CDK2-cyclin E complex formation is independent of PI 3-kinase and ERK. Therefore, both signaling pathways are important for mitogen activation of cyclin D1 expression and CDK4-cyclin D1 activity.

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Regulation of CDK2-Cyclin E Nucleocytoplasmic Translocation

22409

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