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Inducible Expression of Cyclin D1 in T-47D Human Breast Cancer Cells Is Sufficient for Cdk2 Activation and pRB Hyperphosphorylation

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Abstract The sequential transcriptional activation of cyclins, the regulatory subunits of cell cycle specific kinases, regulates progress through the cell cycle. In mitogen-stimulated cells cyclin D1 induction in early G_1 is followed by induction of cyclin E, activation of the cyclin-dependent kinase Cdk2, and hyperphosphorylation of the retinoblastoma gene product (pRB) in mid-to-late G_1 phase. T-47D breast cancer cells expressing cyclin D1 under the control of a metal-responsive metallothionein promoter were used to determine whether Cdk2 activation and pRB hyperphosphorylation are consequences of cyclin D1 induction. A 4–5-fold increase in cyclin D1 protein abundance was followed by approximately 2-fold increases in cyclin E protein abundance and Cdk2 activity and by hyperphosphorylation of pRB. These responses were apparent ~3 h after the increase in cyclin D1 protein, and ~3 h prior to the entry of cyclin D1-stimulated cells into S phase 12 h after zinc treatment. Cyclin D1 immunoprecipitates contained Cdk4 but no detectable Cdk2 and displayed pRb but not histone H1 kinase activity. Cdk2 activation was therefore likely to be due to increased abundance of cyclin E/Cdk2 complexes rather than formation of active cyclin D1/Cdk2 complexes. The sequence of events following zinc induction of cyclin D1 thus mimicked that following mitogen induction of cyclin D1. These data show that cyclin D1 induction is sufficient for Cdk2 activation and pRB hyperphosphorylation in T-47D human breast cancer cells, providing evidence that cyclin D1 induction is a critical event in G_1 phase progression.

Key words: cyclin D1 function, CDK activity, pRB phosphorylation, G_1 phase, cell cycle control

A series of increasingly well-defined events accompanies progress through the G_1 phase of the cell cycle. The activation of cyclin/cyclin dependent kinase (CDK) complexes is a key element in the control of these events and cyclins D1, D2, D3, and E are rate-limiting for progress through G_1 phase [Ando et al., 1993; Jiang et al., 1993; Ohtsubo and Roberts, 1993; Quelle et al., 1993; Musgrove et al., 1994; Resnitzky et al., 1994; Wimmel et al., 1994]. These cyclins are induced following mitogen stimulation in a sequence which is largely independent of the mitogen used or the cell type examined. Thus, cyclin D1 abundance typically increases in early G_1 phase while cyclin E abundance increases as cells approach the G_1-S phase boundary [Matsushime et al., 1991; Dulic et al., 1992; Koff et al., 1992; Motokura et al., 1992; Won et al., 1992; Musgrove et al., 1993]. Although the D-type cyclins interact with multiple CDKs [Xiong et al., 1992; Bates et al., 1994; Meyerson and Harlow, 1994], Cdk4 is the predominant partner for cyclin D1 in several cell types, including macrophages, fibroblasts, and mammary epithelial cells [Xiong et al., 1992; Matsushime et al., 1994; Tam et al., 1994]. Induction of cyclin D1 upon mitogen stimulation of rodent macrophages or fibroblasts is followed by activation of Cdk4 in mid-G_1 phase [Matsushime et al., 1994]. Similarly, induction of cyclin E is accompanied by activation of Cdk2 late in G_1 phase [Dulic et al., 1992; Koff et al., 1992; Rosenblatt et al., 1992]. These events are necessary for entry into S phase, since microinjection of anti-cyclin D1, anti-cyclin E, or anti-Cdk2 antibodies or expression of dominant-negative Cdk2 mutants ar-

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rests cells in G_1 phase [Baldin et al., 1993; Quelle et al., 1993; Tsai et al., 1993; van den Heuvel and Harlow, 1993; Ohtsubo et al., 1995].

Hyperphosphorylation of the retinoblastoma gene product, pRB, is a late G_1 event which is also thought to be necessary for entry into S phase. This protein undergoes cell cycle dependent phosphorylation: during G_1 phase the hypophosphorylated form predominates, but hyperphosphorylated forms appear in mid-to-late G_1 phase and predominate through the remainder of the cell cycle [Ewen, 1994; Riley et al., 1994]. Although several CDKs, including Cdk2 and Cdk4, phosphorylate pRB in vitro at sites corresponding to those phosphorylated in vivo [Lees et al., 1991; Lin et al., 1991; Akiyama et al., 1992; Kato et al., 1993], cells lacking functional pRB are no longer dependent on cyclin D1 function even when they contain active cyclin D1/Cdk4 complexes [Baldin et al., 1993; Lukas et al., 1994, 1995]. In contrast, cyclin E is essential independent of pRB status [Ohtsubo et al., 1995]. This evidence indicates a close link between cyclin D1/Cdk4 and pRB function, arguing that pRB is a key substrate for this kinase in vivo.

Despite increased understanding of the sequence of events leading to progress through G_1 phase and subsequent initiation of DNA replication, the relationship between the elements of the sequence has yet to be established. Thus, it is not clear whether the activation of cyclin D1/Cdk4 and activation of cyclin E/Cdk2 are dependent events, so that cyclin E/Cdk2 activation results from prior activation of cyclin D1/Cdk4, or whether these events lie on independent pathways. Some recent data suggest that Cdk4 activation lies upstream of Cdk2 activation. For example, in mink lung epithelial cells released from contact inhibition, TGFβ prevents activation of Cdk2 and cell cycle progression [Ewen et al., 1993b]. This is likely to be due to suppression of Cdk4 induction, since in cells constitutively expressing transfected Cdk4, Cdk2 is activated even in the presence of TGFβ [Ewen et al., 1993b]. Other studies have shown that cyclin D/Cdk4 complexes assembled in vitro allow activation of Cdk2 in extracts from TGFβ-inhibited cells by sequestering the Cdk2 inhibitor $p27^{Kip1}$, providing further evidence for a pathway linking Cdk4 to Cdk2 activation [Polyak et al., 1994a].

Alterations in cyclin expression have been documented in a number of human cancers and are likely to play a role in the loss of growth control associated with oncogenesis. Cyclin D1 is frequently overexpressed in breast cancer cells compared with normal mammary epithelium [Buckley et al., 1993; Bartkova et al., 1994; Gillett et al., 1994] and cyclin D1 overexpression targeted to the mammary epithelium in transgenic mice led to the development of mammary adenocarcinomas [Wang et al., 1994]. In T-47D human breast cancer cells proliferating at submaximal rates in defined serum-free medium [Musgrove et al., 1991], cyclin D1 abundance governs the initiation of cell cycle progression as well as the rate of transit through G_1 phase [Musgrove et al., 1994], suggesting that overexpression of cyclin D1 in breast epithelium could contribute to autonomous growth. This might occur by mechanisms including amplification of pre-existing cell cycle control mechanisms, or aberrations in normal cell cycle control, for example, formation of abnormal cyclin/Cdk complexes or inappropriate expression of other cell cycle control genes. There is evidence for the latter mechanisms in fibroblasts, since transformation is associated with dissociation of some proteins found in cyclin/Cdk complexes in untransformed fibroblasts [Xiong et al., 1993b] and the expression of genes including cyclin A is increased upon overexpression of cyclin D1 in rodent fibroblasts [Jiang et al., 1993]. However, these issues have not been widely examined in other cell types. We have therefore used T-47D breast cancer cells transfected with cyclin D1 under the control of an inducible promoter to examine the consequences of increased cyclin D1 expression in epithelial cells typical of breast carcinomas in which cyclin D1 overexpression commonly occurs. These experiments show that events which follow cyclin D1 induction during G_1 progression after mitogen stimulation, i.e., Cdk2 activation and pRB hyperphosphorylation, can be initiated by the induction of cyclin D1 alone.

MATERIALS AND METHODS

Cell Culture

The selection of T-47D human breast cancer cells expressing cyclin D1 under the control of a metal-inducible metallothionein promoter has been previously described [Musgrove et al., 1994]. Briefly, a clonal cell line derived from T-47D and retaining the characteristics of the parent cell line, T-47D (7-2), was transfected with either pΔMT [Daly et al., 1991] or pΔMTcycD1 in combination with pSV2neo, and

G418-resistant colonies trypsinized and pooled to yield the T-47D ΔMT and T-47D ΔMTcycD1 cell lines. In parallel, individual G418-resistant colonies were selected using cloning cylinders, yielding clonal transfected cell lines (including T-47D ΔMT-1, T-47D ΔMTcycD1-1, and T-47D ΔMTcycD1-3). DNA fingerprinting using a *Pvu* II restriction fragment length polymorphism of the mucin gene confirmed that each cell line was a derivative of T-47D and Southern analysis demonstrated unique integration of exogenous cyclin D1 coding sequences in the clonal cell lines.

RPMI 1640 medium containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid) (20 mM), sodium bicarbonate (14 mM), L-glutamine (6 mM), and 20 μg/ml gentamycin was used throughout. Stock cultures were maintained in medium supplemented with 10 μg/ml human insulin (Actrapid, CSL-Novo, North Rocks, NSW, Australia) and 10% fetal calf serum. Serum-free medium was phenol red-free and supplemented with 300 nM human transferrin (Sigma Chemical Co., St. Louis, MO) and, where noted, 10 μg/ml insulin. Cells were cultured in serum-free medium following a previously described protocol [Musgrove et al., 1993].

RNA Isolation and Northern Analysis

Replicate 150 cm^2 flasks of cells were treated with 50 μM $ZnSO_4$ or vehicle and total RNA was extracted (using a guanidinium isothiocyanate-cesium chloride procedure) and blotted as previously described, using 20 μg total RNA/lane [Musgrove et al., 1991]. The membranes were hybridized overnight at 50°C in 50% (v/v) formamide, 2 × SSPE (0.3 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7.4), 1% (w/v) SDS, 0.5% (w/v) low fat skim milk (Diploma, St. Kilda, Vic, Australia), 10% (w/v) dextran sulphate (M_r 500,000), 200 μg/ml yeast RNA, 40 μg/ml polyadenylic acid (5'), 500 μg/ml salmon sperm DNA. Probes labelled with [α-^{32}P]dCTP (Amersham, Australia, North Ryde, NSW, Australia) specific activity ~3,000 Ci/mmol) to a specific activity of approximately 1 × 10^9 cpm/μg DNA using the Multiprime DNA labelling kit (Amersham) were added to the hybridisation mix to a final concentration of ≥10 ng/ml. The membranes were washed at a highest stringency of 0.2 × SSC (30 mM NaCl, 3 mM sodium citrate, pH 7.0), 1% SDS at 65°C and exposed to Kodak X-OMAT film at −70°C. Equivalent RNA loading was verified as previously described by hybridizing membranes with a [γ-^{32}P]ATP end-labelled oligonucleotide complementary to 18S rRNA [Musgrove et al., 1991].

Human cyclin and CDK cDNAs were generously provided by the following investigators: cyclin D1, Drs. Yue Xiong and David Beach, Cold Spring Harbor Laboratory, NY [Xiong et al., 1991]; cyclin E, Dr. Steven Reed, Scripps Research Institute, La Jolla, CA [Lew et al., 1991]; Cdk2: Drs. Jonathon Pines and Tony Hunter, Salk Institute, San Diego, CA; Cdk4 (PSK-J3): Dr. Steven Hanks, Vanderbilt University, Nashville, TN [Hanks, 1987]. A human histone H4 genomic clone was generously provided by Dr. Janet Stein, Dept. of Cell Biology, University of Massachusetts, Worcester, MA [Pauli *et al.*, 1987].

Western Blot Analysis, Immunoprecipitation, and Histone H1 Kinase Assays

Cells were lysed as follows: cell monolayers were washed once in ice-cold PBS, then scraped into lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 200 μM sodium orthovanadate, 10 mM pyrophosphate, 100 mM NaF). The lysates were incubated on ice for 5 min and the cellular debris cleared by centrifugation (10,000*g*, 5 min). Equal amounts of total protein were separated by 10% SDS-PAGE or, for examination of pRB phosphorylation, 6% SDS-PAGE and transferred to nitrocellulose. Proteins were visualized using the ECL detection system (Amersham) after incubation (2–4 h at room temperature or overnight at 4°C) with the following primary antibodies: cyclin D1 (PRAD1), cyclin E (HE12), Cdk2 (M2), and Cdk4 (C-22) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; and pRB (14001A) from Pharmingen, San Diego, CA. Densitometric analysis of autoradiographs used a Bio-Rad (Richmond, CA) Video Densitometer, Model 620, and Bio-Rad 1-D Analyst software.

Cell lysates were immunoprecipitated using either an anti-Cdk2 antibody (M2, Santa Cruz Biotechnology, Inc.) or rabbit polyclonal anti-cyclin D1 serum (raised against a human cyclin D1-GST fusion protein) by incubation with antibody (2 h, 4°C) followed by precipitation using protein A-Sepharose beads (1 h, 4°C). The beads were then washed once with ice cold lysis buffer/1 mM DTT, twice with ice cold lysis buffer/1 M NaCl/1 mM DTT, again with ice cold

lysis buffer/1 mM DTT, and finally with ice cold 50 mM HEPES (pH 7.5), 1 mM DTT. The samples were then incubated at 90°C for 2 min before 12% SDS-PAGE. Proteins contained in the immunoprecipitates were visualised following incubation with primary antibody as described above, using either the ECL detection system or autoradiography following incubation with [^{125}I]protein A (specific activity, 30 mCi/mg) for 2 h at room temperature.

For histone H1 kinase assays, immunoprecipitates were prepared as described above except that two additional washes with ice cold 50 mM *HEPES* (pH 7.5), 1 mM DTT were performed. The kinase reaction was initiated by resuspending the beads in 30 μl kinase buffer (50 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, 200 μM ATP, 5 μCi [γ-^{32}P]ATP, 0.1 mM orthovanadate, 1 mM NaF, 15 mM β-glycerophosphate, 0.3 mM PMSF, 20 μg/ml leupeptin, 20 μg/ml aprotonin) containing 3 μg histone H1 as substrate. After incubation for 10 min at 30°C the reaction was terminated by the addition of 3 × SDS sample buffer (187 mM Tris-HCl, pH 6.8, 30% [v/v] glycerol, 6% SDS, 15% β-mercaptoethanol). The samples were then incubated at 90°C for 2 min, separated using 12% SDS-PAGE, and exposed to X-ray film. Relative intensities were quantitated by densitometric analysis as described above.

Cyclin D1-Associated pRB Kinase Assays

The substrate for the cyclin D1-associated kinase assay was a maltose binding protein-pRB fusion protein. A bacterial expression vector was constructed by cloning sequences encoding amino acids 379–928 of pRB [Kaelin et al., 1991] into pMAL (New England Biolabs, Beverly, MA). The pRB(379-928) fusion protein was induced by the addition of 0.4 mM isopropylthioglycoside to a culture of *Escherichia coli* transformed with the expression construct and incubating for 3 h at room temperature. The bacterial pellets were then lysed by sonication and the fusion protein purified by elution from an amylose resin column followed by dialysis against 20 mM Tris (pH 7.4), 1 mM DTT, 1 mM EDTA. The purity of the pRB(379-928) fusion protein was then assessed by polyacrylamide gel electrophoresis followed by Coomassie blue staining.

The kinase assay was based on the methods described by Matsushime et al., [1994], and required lysis conditions different to those described above for histone H1 kinase assays. T-47D cell monolayers were scraped into PBS, pelleted by centrifugation (15,000*g*, 5 min) and the pellets frozen in liquid nitrogen and then resuspended in 1 ml of ice-cold lysis buffer B (50 mM Hepes, pH 7.5, 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM PMSF). The lysate was placed on ice and vortexed vigorously at intervals for 60 min, then centrifuged at 15,000*g* for 5 min at 4°C and the supernatant stored at −80°C. Before immunoprecipitation, 500 μg of lysate was precleared by incubation with protein-A-Sepharose beads (1 h, 4°C). The precleared lysate was then immunoprecipitated (3 h, 4°C) using protein-A-Sepharose beads conjugated with either preimmune rabbit serum or the rabbit polyclonal cyclin D1 antiserum. The immunoprecipitated proteins on the beads were then washed four times with 500 μl of ice-cold lysis buffer B and three times with ice-cold 50 mM Hepes (pH 7.5), 1 mM DTT. The beads were suspended in 30 μl of kinase buffer (50 mM Hepes, pH 7.5, 1 mM DTT, 2.5 mM EGTA, 10 mM $MgCl_2$, 20 μM ATP, 10 μCi [γ-^{32}P] ATP, 0.1 mM orthovanadate, 1 mM NaF, 10 mM β-glycerophosphate) with 3 μg pRB(379-928) fusion protein substrate. After 30 min at 30°C the reaction was terminated as described above for histone H1 kinase assays. The samples were then incubated at 90°C for 2 min, separated using 10% SDS-PAGE and exposed to X-ray film.

RESULTS

Effect of Cyclin D1 Induction on Cyclin and CDK Abundance

Cyclin D1 induction following zinc treatment of T-47D ΔMTcycD1 human breast cancer cells, which express cyclin D1 under the control of a truncated human metallothionein IIA promoter, leads to accelerated G_1 phase transit with a consequent marked increase in the proportion of cells in S phase after ~12 h [Musgrove et al., 1994]. To identify events occurring during G_1 which were consequences of cyclin D1 induction, cyclin and CDK mRNA levels were examined after zinc induction of cyclin D1 in T-47D ΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium. Under these conditions T-47D cells proliferate exponentially, at a rate which is less than that observed under optimum conditions in serum-supplemented me-

dium, allowing ready detection of both stimulation and inhibition of proliferation [Musgrove et al., 1991, 1993]. Exogenous cyclin D1 mRNA increased within 1 h of zinc treatment (Fig. 1), followed by increased cyclin E mRNA abundance after 3 h. Cyclin E mRNA abundance remained elevated until 24 h, the conclusion of the experiment (Fig. 1). Histone H4 mRNA abundance increased at 9–12 h and remained elevated at 24 h (Fig. 1), consistent with the time-course for entry into S phase [Musgrove et al., 1994].

No change in the Cdk4 mRNA level was detected after zinc induction of cyclin D1 in T-47DΔMTcycD1 cells (Fig. 1), although a small increase in Cdk4 mRNA follows mitogen induction of cyclin D1 induction in these cells (unpublished data). Similarly, despite the increase in Cdk2 mRNA following mitogen stimulation of T-47D cells [Musgrove et al., 1993], no change in Cdk2 mRNA was detected after zinc induction of cyclin D1 (Fig. 1).

Since induction of cyclin E mRNA was an early response to induction of cyclin D1, cyclin E expression was investigated in clonal cell lines. The responses of the 7 stable cyclin D1-transfected cell lines examined were identical and data from representative lines are shown in Fig. 2. Some basal expression from the metallothionein promoter was apparent in cells transfected with cyclin D1 coding sequences (for example T-47D ΔMTcycD1-1 and -3), leading to increased cyclin D1 expression without zinc induction (Fig. 2A, see also Fig. 6). This did not affect the levels of cyclin E mRNA in the absence of zinc treatment (Fig. 2A and data not shown). However, zinc treatment of T-47D ΔMTcycD1-1 and -3 cells resulted in increases in the levels of both the exogenous cyclin D1 mRNA and the endogenous cyclin E mRNA (Fig. 2A). By comparison, there was little or no effect of zinc on cyclin D1 or cyclin E mRNA in T-47D ΔMT-1 cells. Treatment of T-47D ΔMTcycD1-3 cells with 20 or 50 μM zinc for 9 h resulted in a concentration-dependent increase in the levels of both cyclin D1 and cyclin E protein (Fig. 2B). Together, these data confirm that the induction of cyclin E resulted from cyclin D1 induction

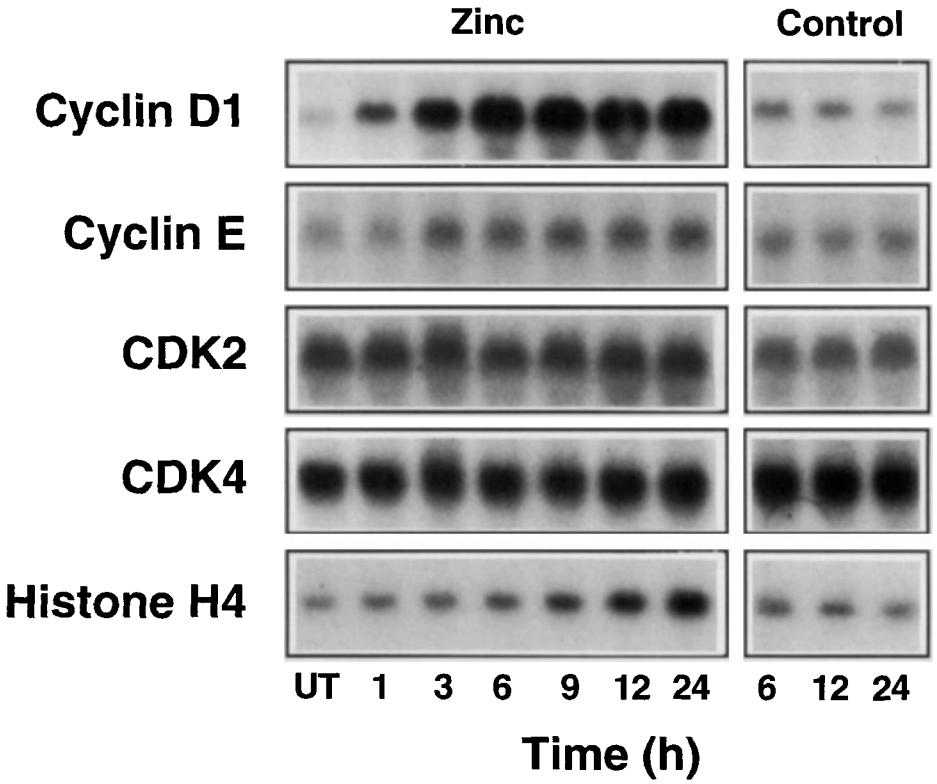

Fig. 1. Effect of zinc induction of cyclin D1 on cyclin and CDK mRNA abundance. T-47DΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium were treated with 50 μM $ZnSO_4$ or vehicle (Control) and harvested at intervals for Northern blot analysis of total cellular RNA. UT: untreated. Data shown were obtained using three replicate filters. In this exposure, only the exogenous cyclin D1 transcript is apparent.

and that the level of cyclin E induction depended on the relative cyclin D1 induction.

Further experiments examined the temporal relationship between the increased abundance of cyclin D1 protein and cyclin E protein. In the experiment shown in Figure 3, cyclin D1 protein abundance was increased approximately 2.5-fold at 6 h and reached a maximum 3.7-fold induction by 9 h. Cyclin E protein increased in abundance at 9–12 h (2-fold, Fig. 3), approximately 3 h after the increase in cyclin D1 protein level, and a similar time before the increase in S phase fraction [Musgrove et al., 1994]. Although the increase in cyclin E abundance was modest, it was consistently observed in independent experiments using either T-47D ΔMTcycD1 or T-47D ΔMTcycD1-3 cells. No change in the abundance of Cdk2 or Cdk4 protein was detected (Fig. 3), consistent with the constant abundance of the corresponding mRNAs (Fig. 1).

Effect of Cyclin D1 Induction on Cyclin/CDK Complex Formation and CDK Activity

In T-47D breast cancer cells, Cdk4 is the predominant CDK associated with cyclin D1 [Tam et al., 1994]. Although cyclin D1/Cdk2

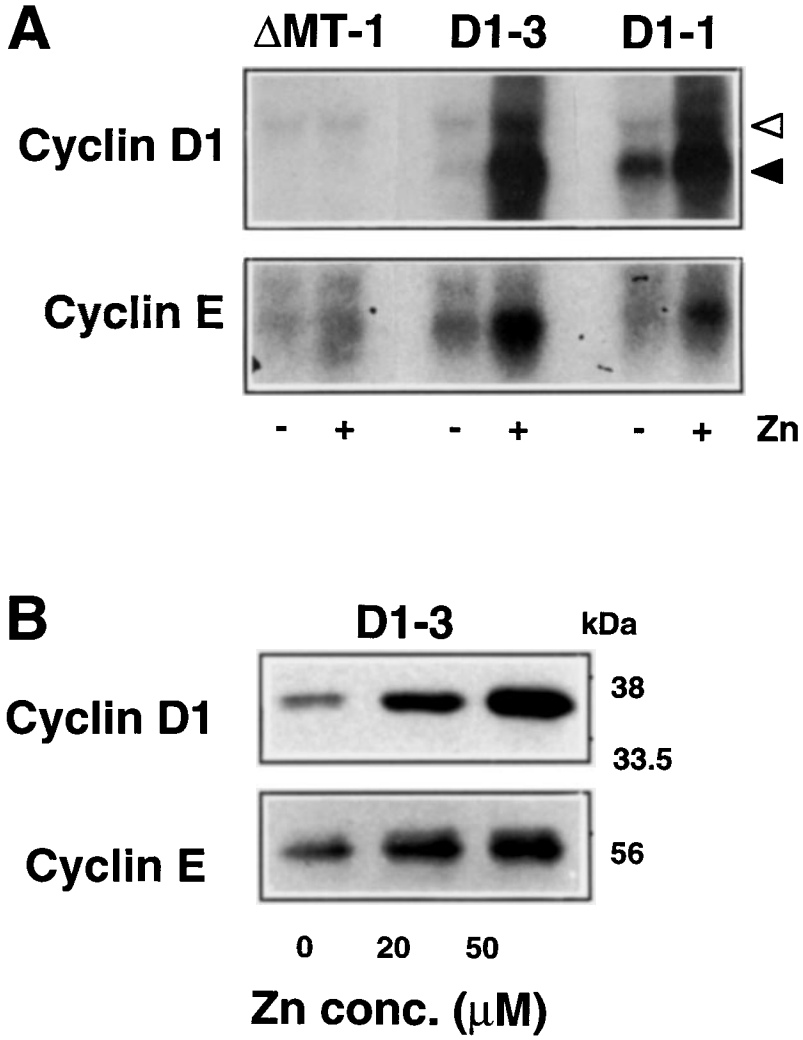

Fig. 2. Effect of zinc induction of cyclin D1 on cyclin E mRNA and protein abundance in clonal cell lines. **A:** Clonal cell lines, containing either vector sequences only (T-47D ΔMT-1) or exogenous cyclin D1 (T-47D ΔMTcycD1-1 and -3), proliferating in insulin-supplemented serum-free medium were harvested after 6 h treatment with 50 μM $ZnSO_4$ or vehicle and total cellular RNA extracted for Northern blot analysis. *Open arrowhead* indicates the major endogenous cyclin D1 transcript; *closed arrowhead* indicates the exogenous cyclin D1 transcript. The same filter was successively hybridised with cyclin D1 and cyclin E cDNAs. **B:** T-47D ΔMTcycD1-3 cells proliferating in insulin-supplemented serum-free medium were harvested after 9 h treatment with the indicated concentrations of $ZnSO_4$ or vehicle and lysates prepared for immunoblotting. The same filter was sequentially blotted for cyclins D1 and E.

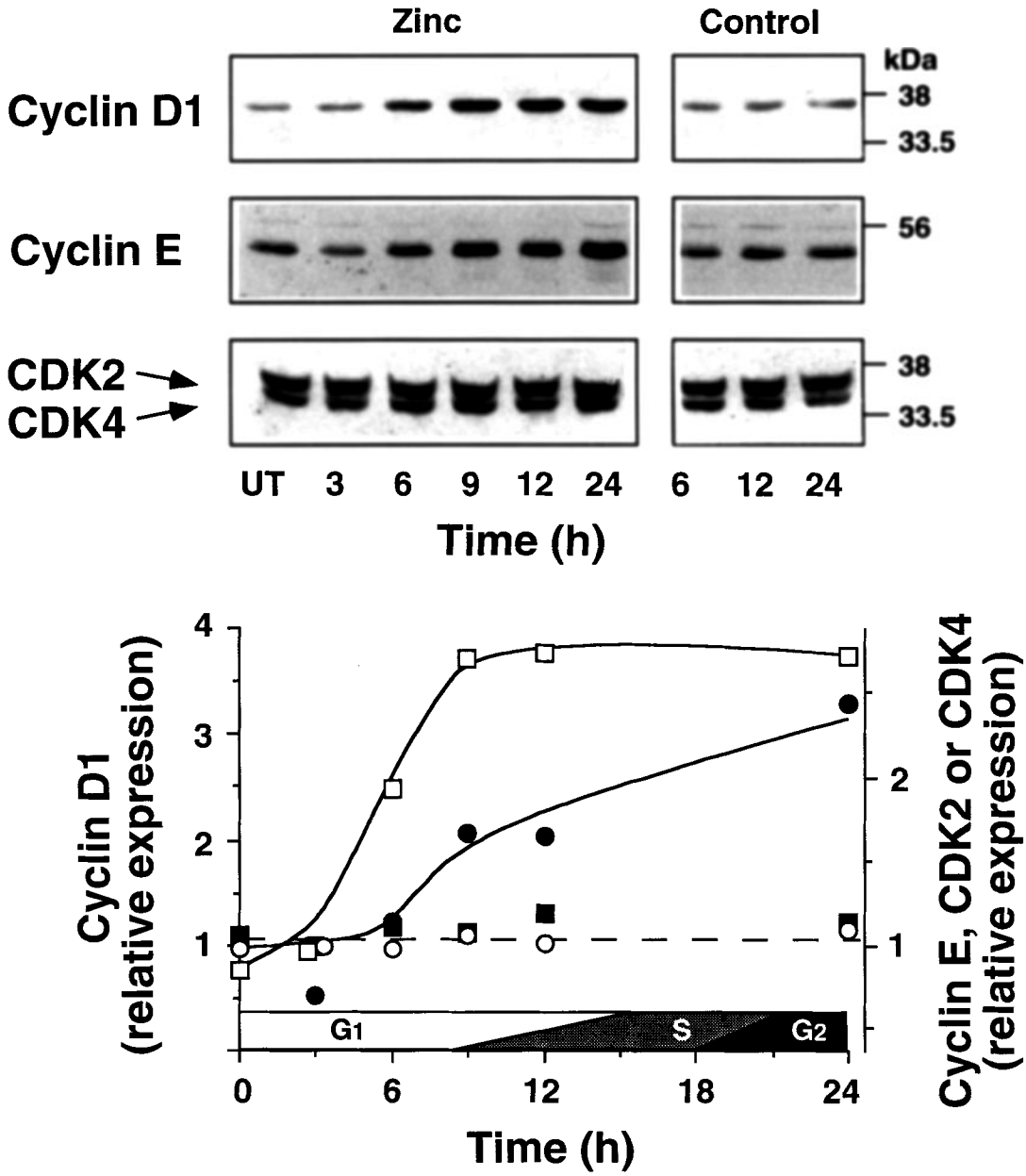

Fig. 3. Effect of zinc induction of cyclin D1 on cyclin and CDK protein abundance. T-47DΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium were treated with 50 μM $ZnSO_4$ or vehicle (Control) and lysates prepared for immunoblotting. UT: untreated. Data for cyclins D1 and E were obtained using the same filter. Densitometric analysis of these immunoblots is presented in the lower panel. Cyclin D1, □; cyclin E, ●; Cdk2, ○; Cdk4, ■.

complexes have been reported in some cell lines including MCF-7 breast cancer cells [Xiong et al., 1992; Tam et al., 1994], they do not appear to be present in all cells [Bates et al., 1994] and are not detectable in T-47D cells (see below). To determine whether overexpression of cyclin D1 in neoplastic cells might alter its association with CDKs, the composition of cyclin D1 immunoprecipitates was examined after zinc induction of cyclin D1 in T-47D breast cancer cells. The rabbit polyclonal cyclin D1 antiserum used for these experiments specifically recognised cyclin D1 on the basis of the following evidence: the antiserum immunoprecipitated a protein which was not present in parallel immunoprecipitations performed using preimmune serum; this protein comigrated with cyclin D1 in SDS-PAGE of T-47D cell lysates and was recognised

by cyclin D1 antibodies but not by antibodies to other cyclins (Fig. 4A and data not shown).

To allow determination of the effects of alterations of cyclin D1 expression over a wide range, T-47D ΔMTcycD1-3 cells proliferating in insulin-supplemented serum-free medium were treated with 20 or 50 μM zinc for 6 h, resulting in approximately 4.5-fold or 7.5-fold inductions of cyclin D1 protein relative to vehicle-treated cells (see Fig. 2B). The lysates were immunoprecipitated using anti-cyclin D1 serum or anti-Cdk2 antibodies. Cdk4 was co-immunoprecipitated with cyclin D1 while cyclin E was co-immunoprecipitated with Cdk2 (Fig. 4B). However, there was no evidence for co-immunoprecipitation of cyclin D1 and Cdk2, even in the lysate containing the highest concentration of cyclin D1 protein (Fig. 4B). Thus, even 7.5-fold overexpression of cyclin D1 did not lead to the formation of cyclin D1/Cdk2 complexes. Furthermore, cyclin D1/Cdk2 complexes were not detected in similar experiments using other cyclin D1-transfected cell lines (data not shown). Increased expression of cyclin D1 was accompanied by increased association with Cdk4, since approximately 2-fold more Cdk4 was detected in cyclin D1 immunoprecipitates following zinc induction of cyclin D1 (Fig. 4B). To determine whether the increased formation of cyclin D1/Cdk4 complexes was associated with activation of Cdk4, the kinase activity of cyclin D1 immunoprecipitates was measured using bacterially-expressed pRB(379-928) fusion protein as a substrate. No kinase activity was detected in lysates immunoprecipitated using pre-immune serum, but phosphorylation of pRB(379-928) fusion protein was detectable in cyclin D1 immunoprecipitates (Fig. 4C). Kinase activity was present at low levels in vehicle-treated control cells and increased with increasing cyclin D1 abundance after 7 h zinc treatment (Fig. 4C).

Since the cyclin E abundance in Cdk2 immunoprecipitates increased by >2-fold following zinc induction of cyclin D1 (Fig. 4B), the kinase activity of Cdk2 immunoprecipitates was measured using histone H1 as a substrate. In untreated or vehicle-treated T-47D ΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium, histone H1 kinase activity was readily detected in Cdk2 immunoprecipitates (Fig. 5A). After zinc induction of cyclin D1, a 1.5–2-fold increase in Cdk2 activity was observed, apparent at 6 h and maintained until the conclusion of the experiment at 24 h (Fig. 5A). In this experiment cyclin D1 protein was increased 2-fold by 3 h, a more rapid timecourse than the experiment presented in Figure 3 (data not shown). Thus, the activation of Cdk2 occurred approximately 3 h after cyclin D1 induction.

Although Cdk4 phosphorylates pRB but not histone H1 in vitro, Cdk2 phosphorylates both substrates [Tsai et al., 1991; Akiyama et al., 1992; Matsushime et al., 1992; Kato et al., 1993]. Since it was possible that cyclin D1/Cdk2 complexes below the level of detection of the immunoblotting experiments presented in Figure 4 might display detectable kinase activity, cyclin D1 immunoprecipitates from the same lysates were also assayed for histone H1 kinase activity. Vehicle-treated T-47D ΔMTcycD1 cells had no detectable cyclin D1-associated histone H1 kinase activity compared with Cdk2 immunoprecipitates of the same amount of total cellular protein (Fig. 5B). Furthermore, no cyclin D1-associated histone H1 kinase activity was detected after either 6 or 24 h zinc treatment, even after extended exposure times (Fig. 5B and data not shown). Since Cdk2-associated histone H1 kinase activity was increased at these timepoints (Fig. 5A), these data demonstrate that neither the increased pRB(379-928) fusion protein kinase activity (Fig. 4C) nor the increased histone H1 kinase activity (Fig. 5) resulted from cyclin D1 directly binding and activating Cdk2, consistent with the failure to detect cyclin D1/Cdk2 complexes even in the presence of significantly increased cyclin D1 abundance (Fig. 4). Nevertheless, no increase in Cdk2 activity was observed after 6 h zinc treatment of vector-transfected cells (Fig. 5C), showing that increased cyclin D1 abundance was required for the increased Cdk2 kinase activity.

Effect of Cyclin D1 Induction on pRB Phosphorylation

Hyperphosphorylation of pRB in late G_1 phase in mitogen-stimulated cells occurs in parallel with increased Cdk2 activity [Akiyama et al., 1992] and results in reduced mobility of the protein on SDS-PAGE [Buchkovich et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989]. Therefore, to examine the effect of cyclin D1 induction and subsequent Cdk2 and Cdk4 activation on pRB phosphorylation, the mobility of the protein was investigated after zinc treatment of T-47D ΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium. Untreated

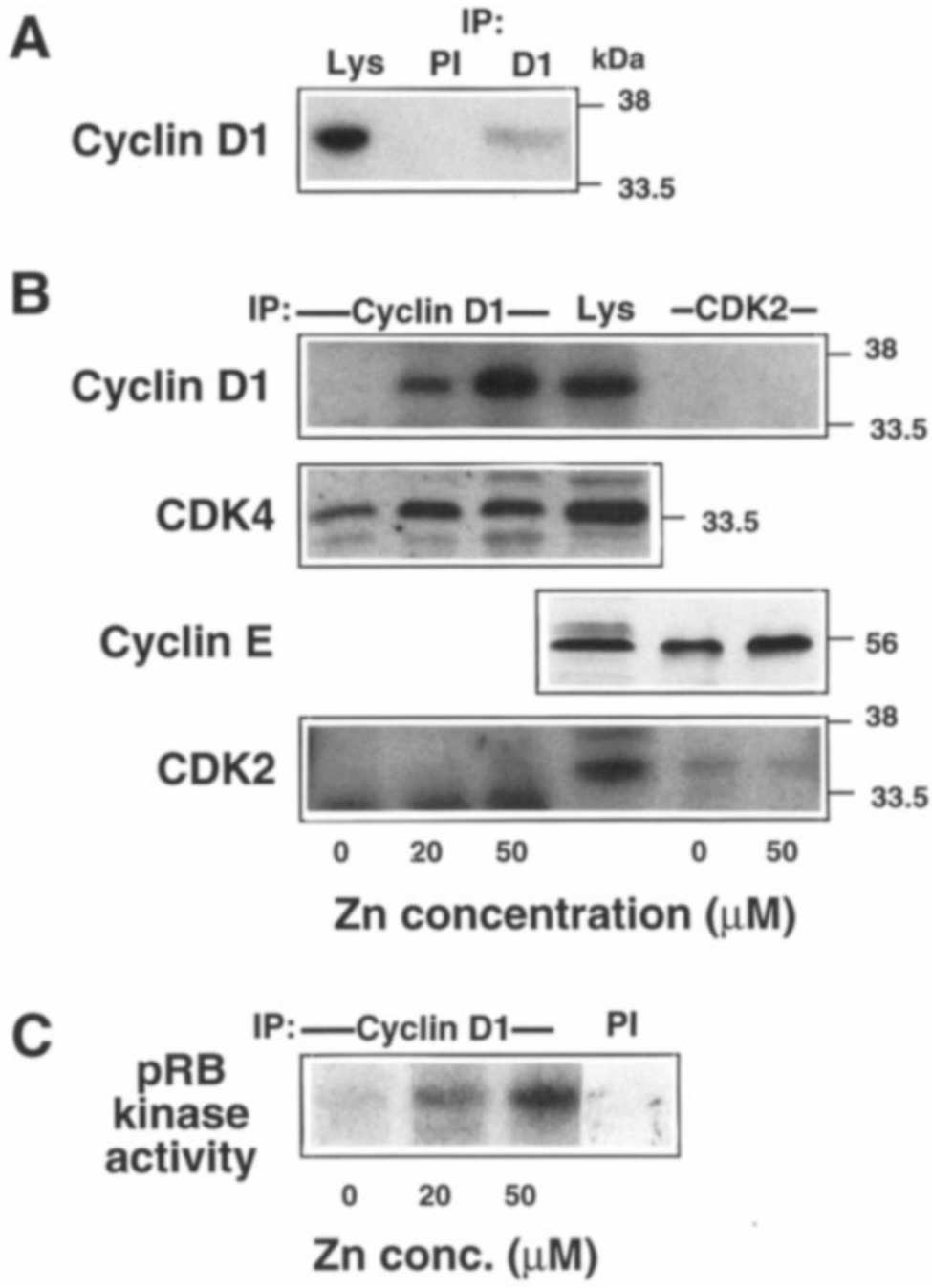

Fig. 4. Effect of zinc induction of cyclin D1 on cyclin/CDK association and kinase activity. **A:** T-47D cell lysate was immunoprecipitated using either anti-cyclin D1 antiserum (D1) or pre-immune serum (PI) and then blotted with cyclin D1 polyclonal antibody (PRAD1, Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were visualised using [^{125}I]protein A. T-47D cell lysate (Lys) was run in parallel. **B:** T-47DΔMTcycD1-3 cells proliferating in insulin-supplemented serum-free medium were treated with $ZnSO_4$ (20 or 50 μM) or vehicle. Lysates were immunoprecipitated using either anti-cyclin D1 serum or anti-Cdk2 antibodies. The immunoprecipitated proteins and, as a control, lysate from T-47D cells (Lys), were separated on replicate gels, immunoblotted using cyclin D1, Cdk2 or Cdk4 polyclonal antibodies, or cyclin E monoclonal antibody, and visualised using either ECL (cyclin E, Cdk4) or [^{125}I]protein A (cyclin D1, Cdk2). Cyclin D1 protein was detectable in cyclin D1 immunoprecipitates from vehicle-treated cells upon longer exposure of the blot. **C:** T-47DΔMTcycD1-3 cells proliferating in insulin-supplemented serum-free medium were treated with $ZnSO_4$ (20 or 50 μM) or vehicle. Lysates were immunoprecipitated using anti-cyclin D1 serum or pre-immune serum (PI) and the kinase activity measured using pRB(379-928) fusion protein as a substrate.

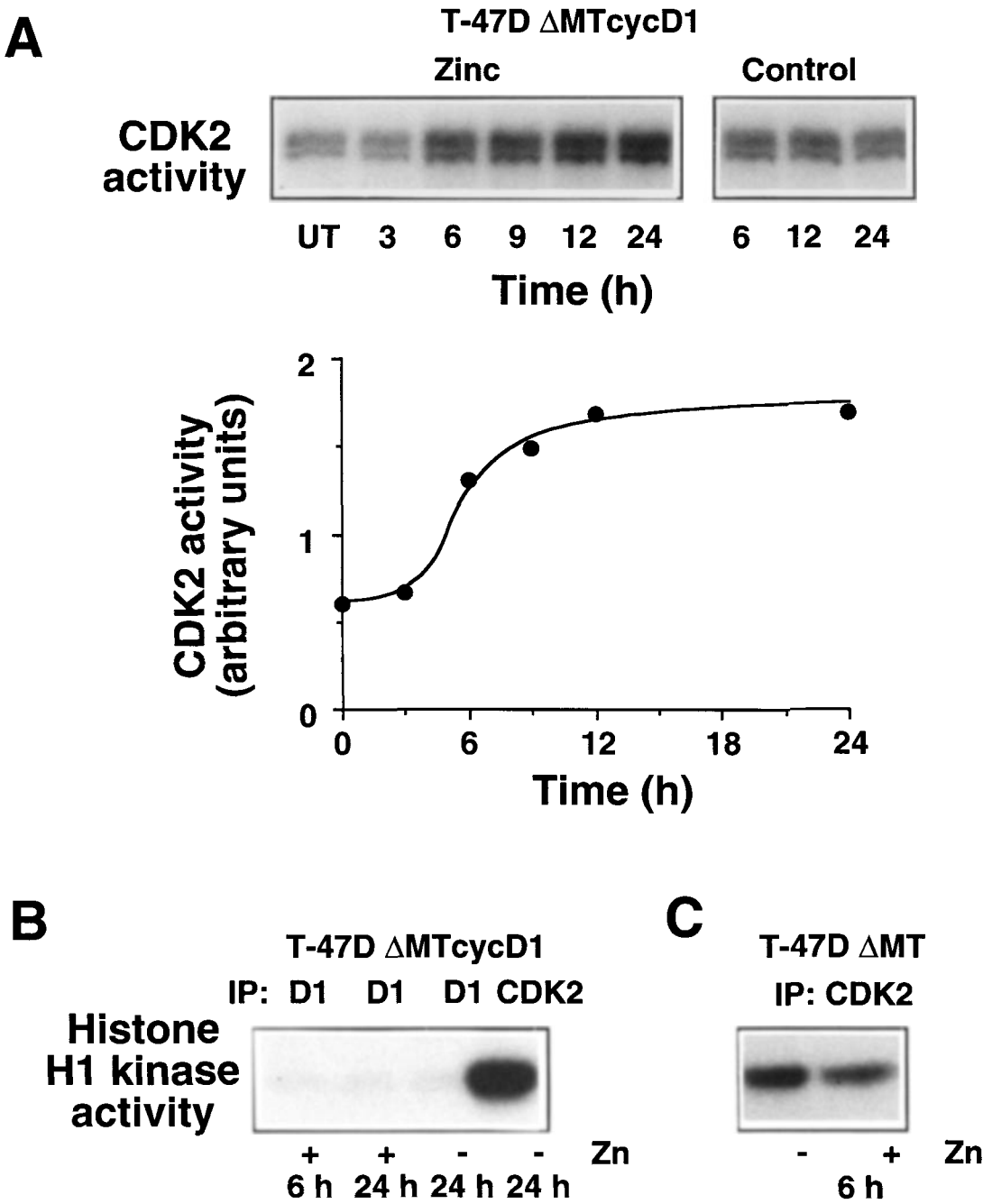

Fig. 5. Effect of zinc induction of cyclin D1 on immunoprecipitated histone H1 kinase activity. Cells proliferating in insulin-supplemented serum-free medium were treated with 50 μM $ZnSO_4$ or vehicle (Control) and lysates prepared. **A:** Equal amounts of total protein from T-47DΔMTcycD1 cells were immunoprecipitated using anti-Cdk2 antibodies and the kinase activity measured using histone H1 as a substrate. UT: untreated. Densitometric analysis of this autoradiogram is also presented. **B:** Equal amounts of total protein from T-47DΔMTcycD1 cells treated for 6 or 24 h were immunoprecipitated using either anti-cyclin D1 serum or anti-Cdk2 antibodies (as indicated, IP) and the histone H1 kinase activity measured. **C:** Equal amounts of total protein from T-47DΔMT cells treated for 6 h were immunoprecipitated using anti-Cdk2 antibodies and the histone H1 kinase activity measured.

and vehicle-treated cells displayed both the hypophosphorylated and hyperphosphorylated forms of pRB, as expected for proliferating cells (Fig. 6). After 9 h zinc treatment, although hypophosphorylated pRB was still present, an increased proportion of pRB was in the lower mobility, hyperphosphorylated form (ppRB) (Fig. 6). In each of two experiments, there was a delay of approximately 3 h between cyclin D1 induction and pRB hyperphosphorylation, which was thus apparently coincident with cyclin E induction and Cdk2 activation (Figs. 3 and 5).

To confirm that phosphorylation of pRB was dependent on cyclin D1 induction, several clonal cell lines were examined. Zinc treatment (9 h) did not alter cyclin D1 protein abundance in untransfected or vector-transfected cells (T-47D (7-2) and T-47D ΔMT-1, respectively) (Fig. 6B). Cyclin D1 protein abundance was significantly increased in T-47D ΔMTcycD1-1 and -3 cells following zinc treatment, although the high basal expression from the metallothionein promoter in the serum-containing medium used for these experiments reduces the relative induction of cyclin D1 protein (Fig. 6B). A shift of pRB to the hyperphosphorylated form after zinc treatment was observed in T-47D ΔMTcycD1-1 and -3 cells but not T-47D (7-2) or T-47D ΔMT-1 cells (Fig. 6B), i.e., only after cyclin D1 induction.

DISCUSSION

Progress through G_1 phase requires the successful completion of steps including induction of cyclins D1 and E, consequent activation of cyclin D1/Cdk4, and cyclin E/Cdk2 complexes and hyperphosphorylation of pRB (see Introduction). These steps occur in a well-defined sequence common to many cell types but the degree to which the early events are a prerequisite for those following has not been firmly established. The data presented in this manuscript show that induction of cyclin D1 in T-47D breast cancer cells is sufficient for induction of cyclin E, activation of Cdk2, and hyperphosphorylation of pRB, providing further evidence for a critical role of cyclin D1 in cell cycle progression and mitogenesis.

Induction of cyclin D1 mRNA is an early response to mitogen stimulation of T-47D breast cancer cells, and is apparent within 2 h [Musgrove et al., 1993]. The progression of stimulated cells through the cell cycle is accompanied by sequential induction of mRNA for other cyclins and their associated CDKs [Musgrove et al., 1993]. Increased expression of cyclin E, Cdk2, and Cdc2 accompanies entry into S phase [Musgrove et al., 1993], and is preceded by a small (25–50%) but consistent increase in Cdk4 mRNA levels in mid G_1 phase (unpublished data). In many respects, the events following inducible expression of cyclin D1 in T-47D cells paralleled those following mitogenic stimulation and induction of endogenous cyclin D1. The increase in cyclin E mRNA after zinc treatment preceded entry into S phase by 6–9 h, while in mitogen-stimulated cells increased expression of cyclin E mRNA is apparently coincident with entry into S phase [Musgrove et al., 1993]. However, following zinc induction of cyclin D1, induction of cyclin E protein and Cdk2 activation occurred in late G_1, timing consistent with that observed following mitogen induction of cyclin D1 and also in synchronised cells [Dulic et al., 1992; Koff et al., 1992; Rosenblatt et al., 1992]. No increase in Cdk2 levels was observed, in contrast with results from mitogen-stimulated cells, where Cdk2 abundance increases during G_1 progression [Rosenblatt et al., 1992; Musgrove et al., 1993], but consistent with data suggesting that CDK abundance displays little cell cycle dependence in proliferating cells [Elledge et al., 1992; Tsai et al., 1993]. The constant level of Cdk2 is thus likely to reflect the fact that the cells were already proliferating, albeit suboptimally, at the time of zinc induction of cyclin D1.

Induction of Cdk2 histone H1 kinase activity and increased cyclin E protein abundance both occurred approximately 3 h after induction of cyclin D1 protein and were of similar magnitude. Furthermore, cyclin E/Cdk2 complexes increased in abundance following zinc induction of cyclin D1. These data suggest that the increased Cdk2 activity resulted largely from the increased cyclin E abundance. Recent data suggest another mechanism linking the activity of cyclin D1/Cdk4 and cyclin E/Cdk2 which is also likely to contribute to the activation of Cdk2 in the present experiments. Since $p27^{Kip1}$ binds to, and inhibits the activity of, both kinase complexes [Polyak et al., 1994a,b; Toyoshima and Hunter, 1994], it has been proposed that increased abundance of cyclin D/Cdk4 complexes may lead to activation of cyclin E/Cdk2 by sequestering $p27^{Kip1}$ [Polyak et al., 1994a], a hypothesis supported by evidence that $p27^{Kip1}$ has a higher affinity for cyclin D/Cdk4 than for

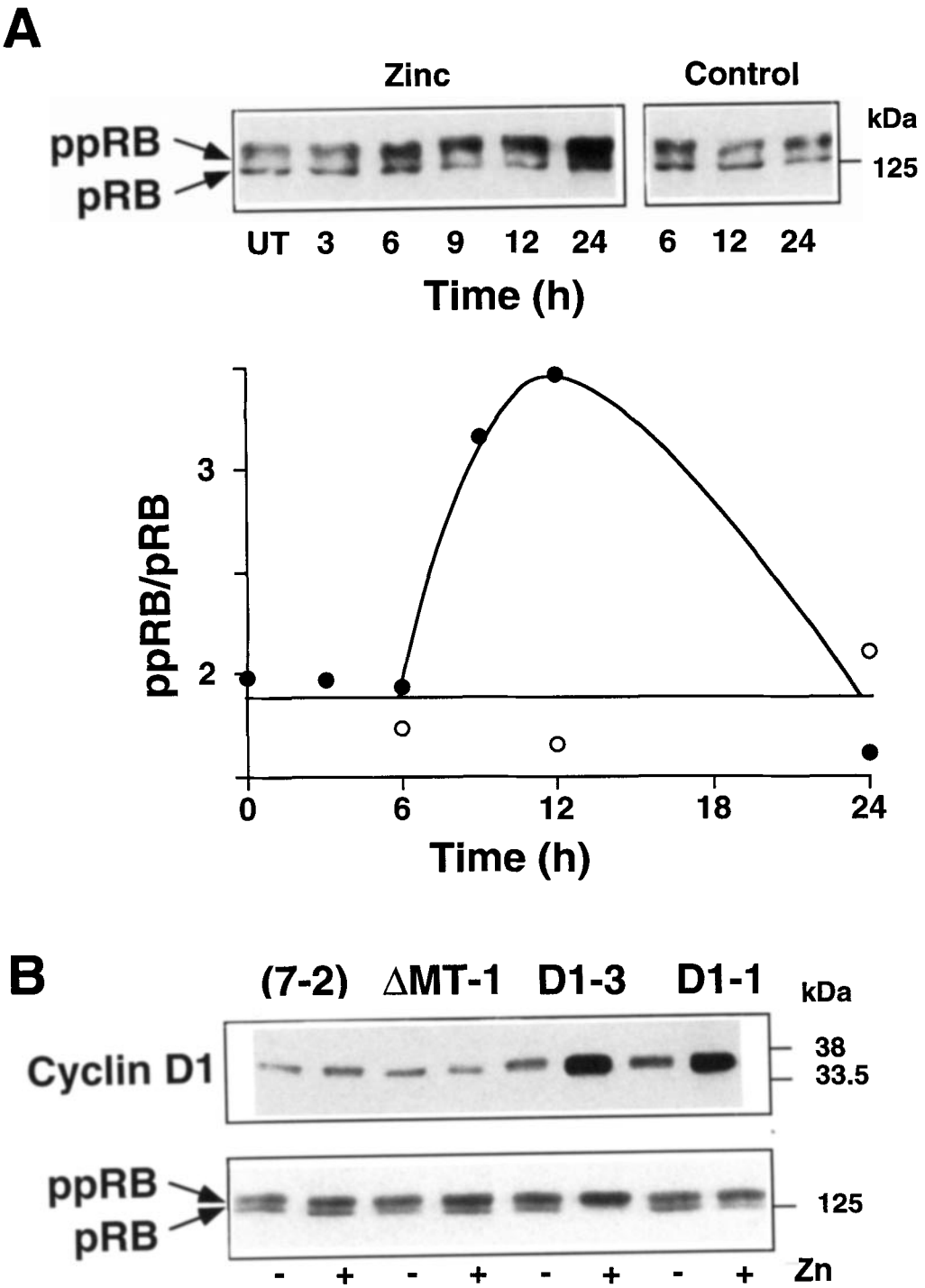

Fig. 6. Effect of zinc induction of cyclin D1 on pRB phosphorylation. **A:** T-47DΔMTcycD1 cells proliferating in insulin-supplemented serum-free medium were treated with 50 μM $ZnSO_4$ or vehicle (Control) and lysates prepared at intervals for immunoblotting. Other data from the same experiment are presented in Figure 3. UT: untreated. Hyperphosphorylated pRB (ppRB) exhibits reduced electrophoretic mobility. Densitometric analysis of this autoradiogram is also presented, as the ratio between the optical densities of the ppRB and pRB bands. Control, ○; Zn, ●. **B:** Lysates were prepared from clonal cell lines, proliferating in medium containing 5% FCS, after 9 h treatment with 50 μM $ZnSO_4$ or vehicle. The cell lines are untransfected (T-47D (7-2)), transfected with vector sequences only (T-47D ΔMT-1) or transfected with exogenous cyclin D1 (T-47D ΔMTcycD1-1 and -3).

cyclin E/Cdk2 [Toyoshima et al., 1994]. The increase in Cdk2 activity following zinc induction of cyclin D1 does not appear to solely reflect the cell cycle position of the stimulated cells. When cell cycle progression of serum-starved T-47D cells was reinitiated by addition of fetal calf serum, the subsequent activation of Cdk2 was approximately 3.6-fold after 12 h, at the G_1/S phase boundary (unpublished data). This is a larger effect than the approximately 2-fold increase following zinc induction of cyclin D1, although the magnitude of the increase in the proportion of cells in S phase following serum stimulation is less than that following induction of cyclin D1 [Musgrove and Sutherland, 1993; Musgrove et al., 1994]. These observations also imply that Cdk2 activation in mitogen-stimulated cells is only partially dependent on cyclin D1/Cdk4 activation.

Although the formation of cyclin/CDK complexes is generally regulated by cyclin abundance rather than by CDK abundance, in some experimental models at least, Cdk4 abundance is limiting for cyclin D1/Cdk4 complex formation. For example, in growth factor-deprived macrophages stimulated with colony-stimulating factor-1, complex formation is limited by the delay between cyclin D1 induction and the later appearance of Cdk4, which is not present at detectable levels in unstimulated cells [Matsushime et al., 1994]. However, in the experimental conditions used here, Cdk4 protein was present at readily detectable levels which did not increase after induction of cyclin D1 and was more abundant in cyclin D1 immunoprecipitates after zinc induction of cyclin D1 (Figs. 3 and 4). These data argue that Cdk4 abundance was not limiting for complex formation in these experiments. It has been suggested that the assembly or stability of cyclin D/Cdk4 complexes depends on the presence of an assembly factor, present in extracts from proliferating but not growth factor deprived, quiescent cells [Kato et al., 1994]. However, such a factor would be expected to be present in the proliferating cells used for zinc induction of cyclin D1. In other experiments using the same experimental design for zinc induction of cyclin D1, the proportion of cells entering S phase was linearly related to the level of cyclin D1 over a range which extended to a more than 7-fold induction of the protein [Musgrove et al., 1994], i.e., greater than the 4-fold induction of cyclin D1 typically achieved in the present experiments. Furthermore, greater relative induction of cyclin D1 was associated with a larger increase in cyclin D1-associated pRB(379-928) fusion protein kinase activity in vitro. Together these observations imply that cyclin D1 function was regulated by cyclin D1 abundance and not limited by the availability or activation of other proteins.

Increased cyclin D1 expression is a feature of many neoplastic cells, including a significant proportion of breast cancer cells [Buckley et al., 1993; Bartkova et al., 1994; Gillett et al., 1994]. There is accumulating evidence that overexpression of cyclin D1 can lead to oncogenic transformation in a variety of systems, including mammary epithelium [Jiang et al., 1993; Hinds et al., 1994; Lovec et al., 1994; Wang et al., 1994]. One postulated mechanism for oncogenic activity of cyclins is the aberrant expression of other cell cycle-regulated genes as a consequence of cyclin overexpression, leading to loss of checkpoints which normally ensure orderly cell cycle progression [Jiang et al., 1993; Keyomarsi and Pardee, 1993]. In support of this mechanism, c-*myc*, c-*jun*, and cyclin A were overexpressed in fibroblasts transfected with cyclin D1 [Jiang et al., 1993]. However, there was no evidence for altered expression of cyclin E (Fig. 2) or other cyclins including cyclin A (unpublished data) in a series of 7 clonal T-47D breast cancer cell lines under conditions in which constitutive cyclin D1 expression from the metallothionein promoter leads to a 2–3-fold increase in cyclin D1 protein levels (Fig. 6). This relative increase is modest compared with the 10-fold increase in cyclin D1 expression in the cell lines examined by Jiang et al. [1993], but is consistent with that found in cell lines overexpressing endogenous cyclin D1 [Buckley et al., 1993; Bartkova et al., 1994; Tam et al., 1994]. Furthermore, breast cancer cell lines with increased cyclin D1 expression do not typically display increased expression of other cyclin genes [Buckley et al., 1993]. These data suggest that aberrant expression of other cyclin genes may not be a common response to constitutive overexpression of cyclin D1.

In the present experiments cyclin D1 immunoprecipitates contained Cdk4 and displayed pRB(379-928) fusion protein kinase activity but neither Cdk2 protein nor histone H1 kinase activity was detected. Furthermore, the kinase activity of cyclin D1 immunoprecipitates was inhibited by the addition of purified bacterially

expressed $p16^{INK4}$ (unpublished data), an inhibitor specific for Cdk4 and Cdk6 [Serrano et al., 1993; Hannon and Beach, 1994; Parry et al., 1995] as well as by $p21^{WAF1/CIP1}$, which inhibits the activity of Cdk2 as well as that of Cdk4 [Harper et al., 1993; Xiong et al., 1993a]. Although the induction of Cdk2-associated histone H1 kinase activity required the induction of cyclin D1, these data demonstrate the formation of active cyclin D1/Cdk4 complexes but not active cyclin D1/Cdk2 complexes. Similarly, no evidence for active cyclin D1/Cdk2 complexes was found in untransformed rodent fibroblasts overexpressing cyclin D1 [Matsushime et al., 1994]. Thus, activation of CDKs other than the normal partners for cyclin D1, Cdk4, and Cdk6 [Matsushime et al., 1992; Bates et al., 1994; Meyerson and Harlow, 1994], does not appear to contribute to the oncogenic properties of cyclin D1.

Finally, the concordance between cyclin D1-induced and mitogen-induced G_1 progression demonstrates that increased cyclin D1 expression alone is not sufficient to derange the normal ordering of cell cycle control. Together these data argue that overexpression of cyclin D1 in neoplastic cells is more likely to result in independence from exogenous stimuli or amplification of normal pathways of cell cycle control than the postulated generalized derangement of cell cycle control mechanisms [Jiang et al., 1993; Keyomarsi and Pardee, 1993]. Since cyclin D1 complexes contain several proteins in addition to the kinases Cdk4 and Cdk6, including pRB, several CDK inhibitors, and the DNA polymerase δ accessory factor PCNA [Xiong et al., 1992, 1993b; Ewen et al., 1993a; Kato et al., 1993; Polyak et al., 1994a], an important goal of future studies will be to determine whether binding to individual components of these complexes is required for specific cyclin D1 functions in cell cycle progression and oncogenesis.

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