Journal of Cellular Biochemistry 58:517–526 (1995)

Differential Regulation of $p34^{cdc2}$ and $p33^{cdk2}$ by Transforming Growth Factor-β_1 in Murine Mammary Epithelial Cells

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Abstract Cyclin-dependent kinases (cdks) are a family of proteins whose function plays a critical role in cell cycle traverse. Transforming growth factor-β_1 (TGF-β_1) is a potent growth inhibitor of epithelial cells. Since cdks have been suggested as possible biochemical markers for TGF-β growth inhibition, we investigated the effect of TGF-β_1 on cdc2 and cdk2 in a normal mouse mammary epithelial cell line (MME) and a TGF-β-resistant MME cell line (BG18.2). TGF-β_1 decreases newly synthesized cdc2 protein levels within 6 h after addition. Coincident with this decrease in newly synthesized cdc2 protein was a marked reduction in its ability to phosphorylate histone H1. This decrease in kinase activity is not due to a change in steady-state levels of cdc2 protein, since mRNA and total protein levels of cdc2 are not reduced until 12 h after TGF-β_1 addition. This suggests that the kinase activity of cdc2 is dependent on newly synthesized cdc2 protein. Moreover, the protein synthesis of another cyclin-dependent kinase, cdk2, is not effected by TGF-β_1 addition, but its kinase activity is substantially reduced. Thus, it appears that TGF-β decreases the kinase activity of both cdc2 and cdk2 by distinct mechanisms.

Key words: cyclin-dependent kinase, cell cycle, serine-threonine kinase

Transforming growth factor-β (TGF-β) is a 25-kDa polypeptide that exists as a disulfide-linked homodimer. There are three mammalian forms of TGF-β with similar, but not identical, activities [Massague, 1990; Roberts and Sporn, 1990]. TGF-β_1 has a variety of growth effects on various cultured cells. Growth of cells derived from mesenchymal tissues can be stimulated by TGF-β_1, while growth inhibition of many epithelial, hematopoietic, and endothelial cells has been observed [Barnard et al., 1990; Nielson-Hamilton, 1990; Massague et al., 1992]. Although the precise mechanism by which growth effects occur is not well understood, progress in this regard has come with the cloning of the TGF-β cell surface receptors. These are known as the type I, II, and III TGF-β receptors. Type I and II receptors are both transmembrane receptors that contain a cytoplasmic serine/threonine kinase domain [Lin et al., 1992; Ebner et al., 1993; Franzen et al., 1993; Attisano et al., 1993]. Studies using TGF-β mutant cell lines have shown that the type I and II TGF-β receptors form a heteromeric complex that is believed to mediate the biological actions of TGF-β [Wrana et al., 1992, 1994]. While the specific interactions within this complex are not completely understood, it appears that the type I receptor requires the type II receptor for ligand binding and the type II receptor requires the type I receptor to signal TGF-β responses through its kinase domain. In addition, the type III receptor, otherwise known as β-glycan, appears to increase TGF-β binding to the type I–type II heteromeric protein kinase receptor complex and regulate access of TGF-β to the signaling receptor(s). Although the majority of evidence indicates that TGFβ signaling requires heteromeric receptor complexes, recent data show that the type II–III receptor complexes that form following ligand binding constitute only a minor fraction of the receptor population [Moustakas et al., 1993]. Thus, it is unclear how the TGF-β signal is transmitted following receptor activation.

The events between surface receptor binding of TGF-β and effects on growth inhibition are

Received December 15, 1994; accepted January 13, 1995.

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even less well understood. However, the actions of TGF-β have been shown to be cell cycle dependent [Laiho et al., 1990; Howe et al., 1991; Pietenpol et al., 1992]. TGF-β has been shown to arrest cells in late G1, prior to commitment of the cells to S-phase. Recent publications have identified a family of proteins called the cyclin-dependent kinases (cdks) as having a critical regulatory role in cell cycle traverse. One of these proteins is the serine-threonine kinase $p34^{cdc2}$ (cdc2). cdc2 has been shown to be an important protein component involved in cell cycle progression through G1/S as well as G2/M in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [Nurse, 1985; Hayles and Nurse, 1986]. Complementation studies using human cdc2 have successfully rescued temperature-sensitive yeast cdc2 mutants, suggesting that cdc2 has evolutionarily conserved its biological activity from yeast to humans [Lee and Nurse, 1987]. In higher eukaryotes, cdc2 is a critical component of maturation promoting factor (MPF), which is required for initiation of mitotic division. This gives cdc2 a vital function at the G2/M transition. However, the role of cdc2 at G1/S in higher eukaryotes is less well understood. Some studies have shown that addition of antisense oligonucleotides made to cdc2 resulted in inhibition of DNA synthesis [Furukawa et al., 1990; Mercer et al., 1992]. This suggests that, along with its importance at G2/M, cdc2 may also play a critical role during the G1/S-phase transition. Along with cdc2, another related serine–threonine protein kinase called $p33^{cdk2}$ (cdk2) has been isolated [Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991; Tsai et al., 1991]. Immunodepletion of cdk2 from *Xenopus* oocyte cell-free extracts or expression of dominant negative cdk2 mutants clearly shows that DNA replication is blocked [Fang and Newport, 1991; van den Heuvel and Harlow, 1993]. Therefore, similar to cdc2, cdk2 appears to also be important in cell cycle progression with a more defined activity at the G1/S border.

In breast cancer, mRNA levels for cdc2 as well as cyclins A and B have been shown to be overexpressed in 90% of all tumor cell lines tested [Keyomarsi and Pardee, 1993]. Moreover, their expression does not appear to be tightly cell cycle regulated. For instance, breast cancer cell lines express moderately high levels of cdc2, cyclin A, and cyclin B in G1-phase. This is in contrast to normal breast cell lines that show a tightly regulated mRNA expression pattern with peaks in early S-phase for cdc2 and cyclin A, and late S-phase for cyclin B [Keyomarsi and Pardee, 1993]. It is postulated that the inappropriate expression of the cyclins and cyclin-dependent kinases may lead to a loss of normal growth control in breast cell lines. Since previous work had indicated that the cdks might be biochemical markers for TGF-β_1 growth inhibition, we wished to investigate the effect of TGF-β_1 on cdc2 and cdk2 in a normal mouse mammary epithelial cell line (MME) and in a TGF-β-resistant mouse mammary epithelial cell line (BG18.2). Consistent with previous data, the level of newly synthesized cdc2 protein was reduced following TGF-β addition to MME cells, while no effect was seen in BG18.2 cells. Coincident with this decrease in cdc2 protein synthesis was a marked reduction in cdc2 kinase activity. However, while TGF-β similarly decreased cdk2 kinase activity, there was no effect on newly synthesized cdk2 protein. Thus, while TGF-β decreases the kinase activity of both cdc2 and cdk2, this occurs by distinct mechanisms.

MATERIALS AND METHODS

Cell Culture and Growth Studies

Mouse mammary epithelial (MME; CRL1636/NMuMG) or BG18.2 cells were grown in Dulbecco's modified Eagle (DME) media containing 10% fetal bovine serum (FBS) supplemented with epidermal growth factor (EGF) (5 ng/ml) and insulin (10 μg/ml). Stock cultures were split at a 1:4 dilution every 3–4 days. For all experiments cells were plated at the indicated concentrations and used following an approximate 24-h incubation at 37°C. BG18.2 cells were mutagenized with EMS and selected in the presence of 1 ng/ml TGF-β_1 as described previously [Howe et al., 1990].

The concentration of TGF-β_1 required to inhibit cellular growth was determined by plating MME cells at a density of 5.2×10^3 cells/cm^2 and observing growth after 6 days in the presence of various concentrations of TGFβ1. On day 3, the cultures were refed with fresh medium containing the indicated concentration of TGF-β; following 3 additional days' incubation, the cells were fixed with methanol and stained with hematoxylin.

For DNA synthesis, MME cells were plated in 10% DME at a density of 2×10^4 cells/cm^2 in 24-well plates (2.0 cm^2/well) and incubated at 37°C overnight. The following day, TGF-β_1 (Aus-

tral Biologicals) was directly added to the final indicated concentration. After a 22-h incubation, cells were pulsed with 1 μCi of ^{3}H-thymidine for 2 h. Trichloroacetic acid (TCA)-insoluble material was assayed for ^{3}H-thymidine incorporation by scintillation counting.

Immunoprecipitations and Immunoblotting

Cells were plated at a density of 1.36×10^4 cells/cm^2 in 6-cm plates (22 cm^2). After 24-h incubation, cells were washed in methionine-free media and placed at 37°C for 1 h in methionine-free medium containing 300 μCi/ml of ^{35}S-methionine, 10% dialyzed FBS, 5 ng/ml EGF, 10 μg/ml insulin, ± 5 ng/ml TGF-β_1. After harvest, cells were lysed on ice in lysis buffer [50 mM Tris, 0.5% Triton X-100, 0.5% deoxycholate, 0.5% sodium dodecyl sulfate (SDS), 137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 50 mM NaF, 0.1 mM Na vanadate, 75 μg/ml PMSF, 0.1 TIU/ml aprotinin, 1 μg/ml leupeptin, and 1.48 mg/ml iodoacetimide]. Samples were sonicated, the insoluble material removed by centrifugation at 10,000*g* for 5 min, and then equalized for TCA-precipitable counts. Samples were incubated with a polyclonal antibody generated to the human COOH-terminal sequence of p34^{cdc2} (DNQIKKM) or with a polyclonal antibody generated to the human COOH-terminal sequence of p33^{cdk2} (CQDVTKPVPHLRL). Immunoprecipitated pellets were washed three times in RIPA buffer (50 mM Tris pH 8.0, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 137 mM NaCl, 3 mM KCl, 8 mM, $Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 50 mM NaF, 0.1 mM Na vanadate, 75 μg/ml PMSF, 0.1 TIU/ml aprotinin, 1 μg/ml leupeptin, and 1.48 mg/ml iodoacetimide), resuspended in 1× Laemmli sample buffer, boiled, and electrophoresed on a 10% polyacrylamide gel electrophoresis (PAGE)–SDS gel. Gels were dried, exposed to Kodak XAR-5 film, and band intensity analyzed by densitometry. Antisera specificity was determined by incubating the antibody with 100 nmole peptide, to which it was generated (data not shown).

Histone H1 Kinase Assays

MME or BG18.2 cells were plated at a density of 1.36×10^4 cells/cm^2 on 6-cm plates. After overnight incubation, cells were treated in the presence or absence of TGF-β_1 (5 ng/ml). At the indicated times, cells were washed in PBS and harvested. Cells were lysed in kinase lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 0.1% Triton X100, 5 mM EDTA, 5 mM NaF, 0.1 mM Na vanadate, 50 μg/ml PMSF, 0.1 TIU/ml aprotinin, and 1 μg/ml leupeptin) and immunoprecipitated with either cdc2 or cdk2 polyclonal antibodies (described above). Samples were washed twice with kinase lysis buffer and twice with kinase buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM DTT). H1 kinase assays were performed in a 50-μl reaction consisting of kinase buffer, 100 μg/ml histone H1 (Boerhinger Mannheim), 5 μM cold ATP, and 5 μCi [^{32}P]-γ-ATP. After 5-min incubation at 30°C, the reaction was stopped with 2× Laemmli sample buffer, boiled, and electrophoresed on a 10% PAGE–SDS gel. Gels were dried down and exposed to Kodak XAR-5 film. Intensity of radiographic bands was analyzed by densitometry.

RNA Isolation and Northern Blot Analysis

MME cells were plated at a concentration of 1.6×10^4 cells/cm^2 in 10-cm petri dishes (64 cm^2) 1 day prior to experimental treatment. Total RNA was isolated using RNAzol (Cinna/Biotex). Equivalent quantities of total RNA were electrophoresed on a 1.2% agarose/2.2M formaldehyde gel and stained with ethidium bromide for RNA integrity. The RNA was transferred to nitroplus membranes (MSI) and crosslinked by UV irradiation (Stratagene). A human cdc2 clone (provided by Paul Nurse) was radiolabeled using a random primer labeling system (Boehringer-Mannheim), and the constitutively expressed 1B15 gene (rat cyclophilin A) was used as an internal marker to normalize RNA loading [Danielson et al., 1988]. Blots were exposed to Kodak XAR-5 film and the intensity of radiographic bands was quantified by densitometry.

RESULTS

Effect of TGF-β_1 on Cycling Mouse Mammary Epithelial Cells

MME cells are an aneuploid mouse mammary epithelial cell line derived from normal glandular tissue that exhibit many features of secretory epithelium. These cells also obey many generally accepted in vitro growth criteria such as high serum dependence, density-dependent growth arrest, and lack of colony formation in soft agar. TGF-β is a strong growth inhibitor of most epithelium-derived cells. To determine the effect of TGF-β_1 on sparsely growing MME cells, increasing concentrations of TGF-β_1 were added

to proliferating MME cultures. Figure 1A shows that as little as 0.1 ng/ml TGF-β_1 results in inhibition of cell growth and maximal growth inhibition is seen with 3–5 ng/ml TGF-β_1. A similar growth inhibitory response to TGF-β_1 is observed if autoradiography and labeled nuclei are counted (data not shown). Although the mechanism of this inhibition is unknown, the action of TGF-β appears to be cell cycle dependent, with growth arrest occurring approximately 1–3 h prior to S-phase [Howe et al., 1991; Thoresen et al., 1992; Eblen et al., 1994]. Similar growth inhibitory affects of TGF-β_1 are observed if DNA synthesis is monitored by ^{3}H-thymidine incorporation (Fig. 1C). For instance, the addition of 1–3 ng/ml TGF-β_1 to cycling MME cells results in 70–80% inhibition of DNA synthesis. As a means to further investigate the action of TGF-β_1 on mammary cell growth, we have selected for a TGF-β_1-resistant MME cell line called BG18.2. As can be seen in Figure 1B, TGF-β_1 has little effect on sparsely growing BG18.2 cells after 8 days in culture. In addition, BG18.2 cells grown in the presence of TGF-β_1 show little effect on DNA synthesis over a 24-h period as compared to MME cells (Fig. 1C).

Effect of TGF-β_1 on Protein Synthesis of Cyclin-Dependent Kinases

Previous reports have suggested that the cyclin-dependent kinases might be potential targets modulated during TGF-β_1-induced growth arrest [Howe et al., 1991; Abraham et al., 1992; Eblen et al., 1994]. Abraham et al. [1992] reported that the addition of TGF-β_1 to cycling mink lung Mv1Lu cells (CCL64) decreased the steady-state levels of cdc2, while Eblen et al. [1994] demonstrated that TGF-β_1-treated CCL64 cells showed a decrease in newly synthesized cdc2 protein prior to a reduction in steady-state protein levels. Since cdk expression and/or activity is believed to be directly coupled to the proliferative potential of cultured cells, we decided to examine the effect of TGF-β_1 on the expression of cdc2 and cdk2 in mammary epithelial cells. Immunoprecipitations from cellular

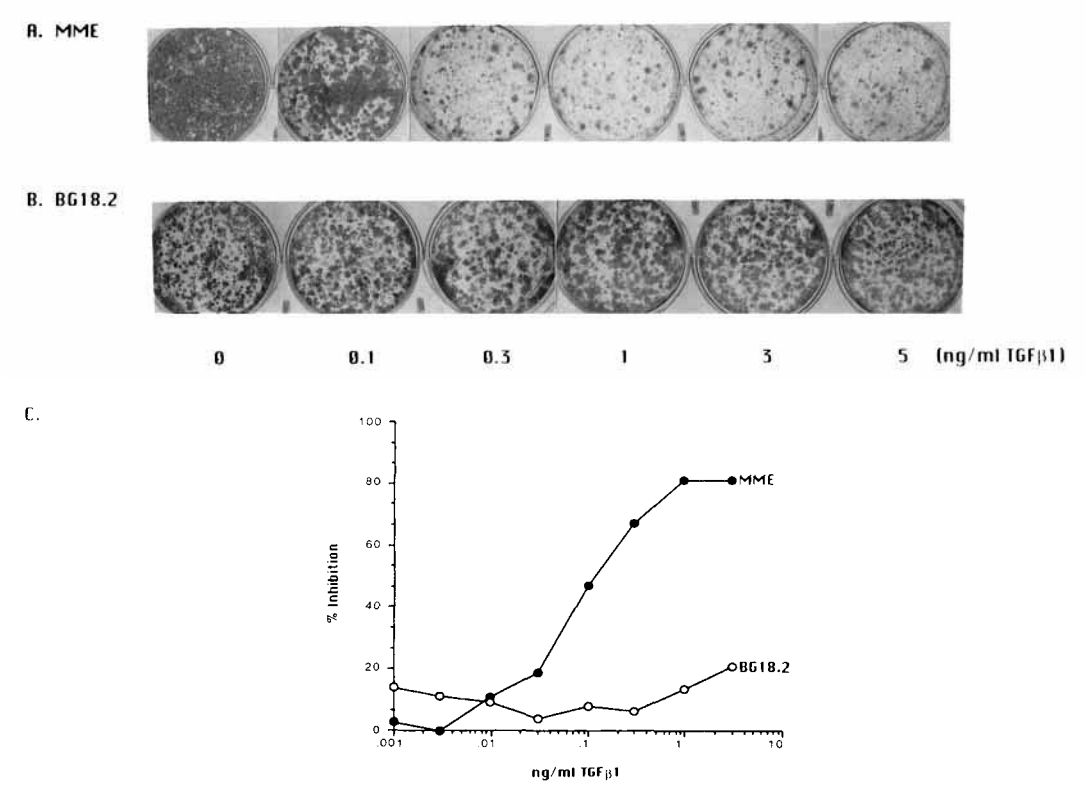

Fig. 1. Effect of TGF-β_1 on cycling MME or BG18.2 cells **A:** Cycling MME cells were grown in DME containing 10% fetal bovine serum (FBS), 5 ng/ml EGF, and 10 μg/ml insulin. After a 6-day incubation with various concentrations of TGF-β_1, cells were fixed and stained with hematoxylin. **B:** Cycling BG18.2 cells were grown and stained as described above. **C:** Graph depicts DNA synthesis results from both cycling MME cells (●) and BG18.2 cells (○). Control cycling MME cells incorporated approximately 19,000 cpm ^{3}H-thymidine, while control BG18.2 cells incorporated approximately 16,500 cpm.

extracts made from either MME cells or BG18.2 cells grown in the presence or absence of TGF-β_1 were performed. As shown in Figure 2A, while cdc2 protein synthesis is reduced in MME cells treated with TGF-β_1, BG18.2 cells show no reduction in cdc2 protein synthesis as compared to control levels observed in the absence of TGF-β_1 (Fig. 2B). In order to determine whether the effect on cdc2 synthesis seen at 24 h appropriately reflected the cellular response to TGF-β_1, the kinetics of this inhibition over a 24-h period was investigated. As shown in Figure 2C, a reduction in newly synthesized cdc2 protein is observed approximately 6 h following the addition of TGF-β_1. Moreover, by 16 h, these levels had decreased approximately 70% compared to control. While this reduction in cdc2 correlates directly with the decrease in DNA synthesis observed following addition of TGF-β_1 (Fig. 1C), it does not demonstrate a causal relationship. Answers to those questions await a more complete understanding of the signaling pathways induced and/or inhibited by TGF-β_1. However, the observed decrease in cdc2 synthesis does not appear to be simply a reflection of TGF-β_1-mediated growth arrest prior to the cell cycle location in which cdc2 synthesis occurs. For instance, while cdc2 synthesis begins to decline about 6 h following TGF-β_1 addition (Fig. 2C), inhibition of DNA synthesis is not observed for an additional 4–6 h (data not shown). These results are similar to what we have observed in the mink lung epithelial cells and normal human mammary epithelial cells [Eblen et al., 1994; data not shown].

As discussed previously, cdc2 belongs to a family of protein kinases believed to regulate distinct portions of the eukaryotic cell cycle. For this reason, we decided to look at the effects of TGF-β_1 on another cyclin-dependent kinase, cdk2. Interestingly, newly synthesized protein levels of cdk2 are not affected by TGF-β_1 (Fig. 2C). Over a 24-h period of TGF-β_1 treatment, the expression of cdk2 protein levels is approximately 90% of its controls. This suggests that TGF-β_1 is specific for its effects on newly synthesized cdc2 protein levels but has no effect on its cell cycle counterpart cdk2. It also shows that TGF-β_1 effects are not a result of an overall reduction in newly synthesized proteins.

To investigate further whether the decrease in newly synthesized cdc2 protein was due to an overall decrease in cdc2 protein levels, Western analysis on cycling MME cells grown in the

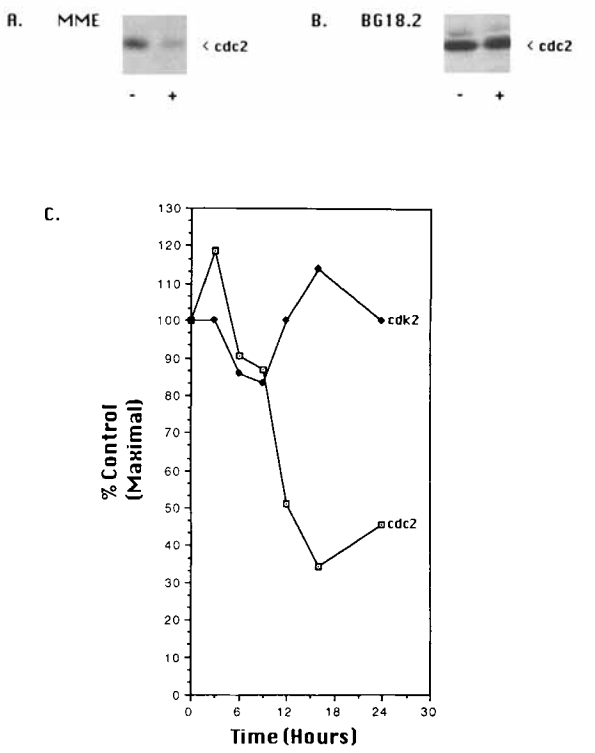

Fig. 2. Effect of TGF-β_1 on newly synthesized cdc2 and cdk2 proteins. **A:** Cycling MME cells were grown as described in Figure 1. After an initial 24-h incubation, cells were grown in the presence or absence of TGF-β_1 (5 ng/ml). One h prior to harvest, cells were labeled with 300 μCi/ml of ^{35}S-methionine. Protein extractions and cdc2 Immunoprecipitations were performed as described in Materials and Methods. **B:** Immunoprecipitation of BG18.2 cells with cdc2 antibody. **C:** Time course depicting the effect of TGF-β_1 (5 ng/ml) on newly synthesized cdc2 (□) or cdk2 (■) protein. Graph represents densometric readings of three independent cdc2 experiments and four independent cdk2 experiments.

presence or absence of TGF-β_1 was performed. As can be seen in Figure 3, overall protein levels of cdc2 remain unchanged over a 48-h time period. In addition, cycling MME cells treated with TGF-β_1 show no change in cdc2 protein levels 12 h after ligand addition. This is contrasted by the results of Figure 2C, where newly synthesized cdc2 protein is decreased by 50% following 12 h TGF-β_1 treatment. However, steady-state levels of cdc2 are decreased 12–24 h after addition of TGF-β_1. Thus, the reduction in total cdc2 protein levels occurs 6–12 h following the observed decrease in newly synthesized cdc2 protein.

TGF-β_1 Effect on cdc2 mRNA

In normal breast cell lines, cdc2 mRNA synthesis is tightly regulated with peak expression

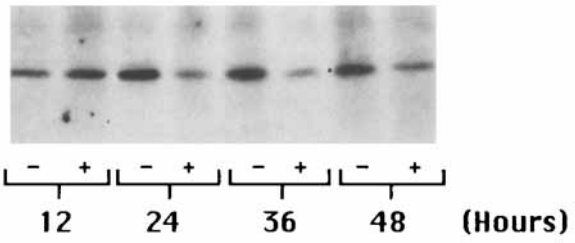

Fig. 3. Western blot analysis of total cdc2 protein. Cycling MME cells were harvested at the indicated times in the presence (+) or absence (−) of TGFβ1 (5 ng/ml). Samples were normalized for protein and immunoprecipitated with an antibody to cdc2. After immunoprecipitation, lysates were run on a 10% SDS–PAGE gel and processed for Western analysis as described (Materials and Methods).

seen early in S-phase [Keyomarsi and Pardee, 1993]. By contrast, breast cancer cell lines have shown that cdc2 mRNA is overexpressed and not tightly cell cycle regulated [Keyomarsi and Pardee, 1993]. In order to explore the possibility that transcription of cdc2 in cycling MME cells was affected by TGF-β_1, Northern blot analysis of total RNA isolated from MME cells treated for various times with TGF-β_1 was performed. As shown in Figure 4, cdc2 mRNA levels decrease with similar kinetics to that seen for newly synthesized cdc2 protein (Figs. 2C, 4). For instance, an approximate 70% decrease in both newly synthesized protein and mRNA accumulation is observed following 16-h TGF-β_1 treatment. In order to determine whether the decrease in cdc2 mRNA was due to a TGF-β_1-regulated increase in mRNA degradation, the experiment shown in Figure 5 was performed. Cycling MME cells were treated with actinomycin D in the presence or absence of TGF-β_1 and the decay in cdc2 mRNA determined at the indicated time points. As can be seen in Figure 5, these experiments demonstrate that the decrease in cdc2 mRNA levels observed following TGF-β_1 addition is not due to any significant change in the stability of the cdc2 message. While TGF-β_1 decreased the half-life of cdc2 mRNA from approximately 12 h to 9 h, it is unlikely that this reduction can fully account for the decrease in cdc2 message and protein observed in Figures 2 and 4.

TGF-β_1 Effect on cdc2 and cdk2 Histone H1 Kinase Activity

TGF-β_1 has been previously reported to inhibit cdk histone H1 activity in mink lung epithelial cells [Howe et al., 1991; Abraham et al., 1992; Koff et al., 1993; Eblen et al., 1994]. Since

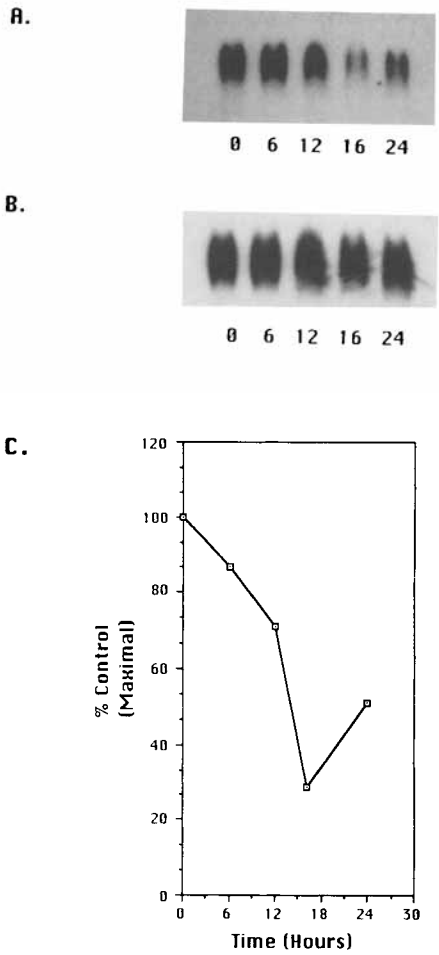

Fig. 4. Analysis of TGFβ1 effects on RNA levels of cdc2. Total RNA was isolated from cycling MME cells after various times of incubation with TGF-β_1 (5 ng/ml); 15 μg of total RNA from each timepoint was run on a 1.2% agarose/2.2 M formaldehyde gel. Total RNA was transferred to nitrocellulose and (**A**) probed with cdc2 or (**B**) probed with 1B15 (rat cyclophilin A). **C**: Effect of TGF-β_1 on cdc2 mRNA levels as a percentage of control (time 0) following normalization to the 1B15 signal is shown. Results depict the mean of two independent experiments.

MME cells showed distinct effects of TGFβ1 on cdc2 and cdk2 protein synthesis (Fig. 2), it was of interest to determine whether the activity of these kinases was differentially regulated by TGF-β_1. To address that question, the experiments shown in Figure 6 were performed. TGF-β_1 was added to cycling cultures of MME cells and cdk histone H1 activity was determined in the immunoprecipitates at the indicated times. As can be seen in Figure 6, cdc2 and cdk2 show a decrease in kinase activity almost immediately following addition of TGF-β_1. The decreased ability of both kinases to phosphorylate histone H1 is essentially identical when both graphs are combined (Fig. 6C). The de-

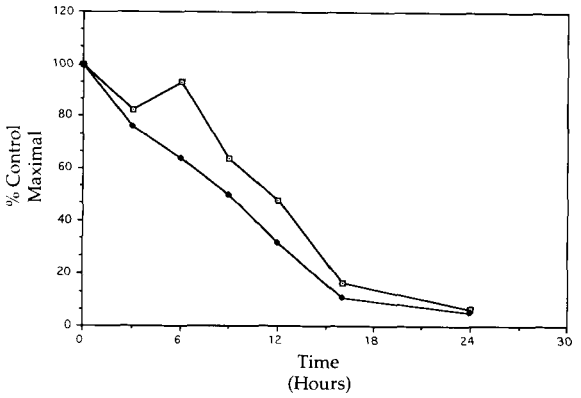

Fig. 5. Decrease in cdc2 mRNA caused by treatment with TGF-β_1 is not due to a change in its degradation pattern. Cycling MME cells were treated with actinomycin D (5 μg/ml) in the presence (■) or absence (□) of TGF-β_1 (5 ng/ml) for the indicated period of time. Cells were collected at each time point and total RNA prepared; 15 μg of total RNA from each time point was loaded on a 1.2% agarose/2.2M formaldehyde gel and probed with cdc2 or 1B15. Graph depicts cdc2 mRNA degradation after normalization for RNA content by 1B15. ■, presence of TGF-β_1; □, absence of TGF-β_1.

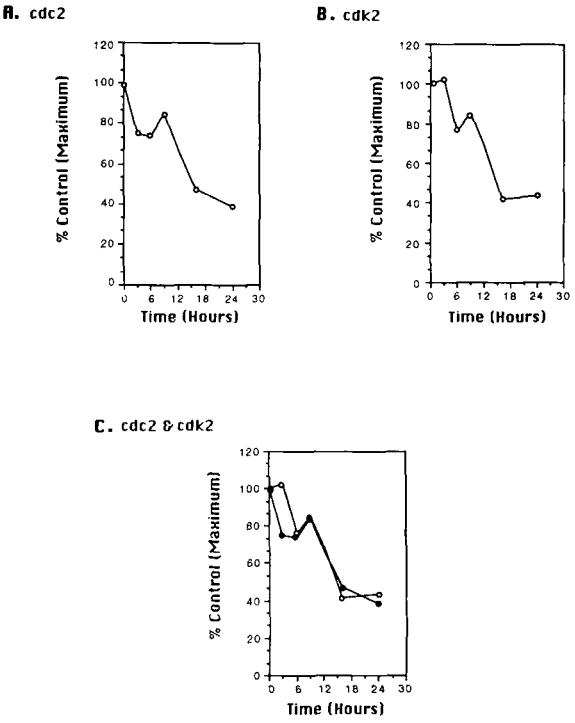

Fig. 6. Time course showing TGFβ1 effect on cdc2 and cdk2 ability to phosphorylate histone H1. Cells were harvested at the indicated times, and cellular extracts were normalized for protein content. Ability of cdc2 immunoprecipitate (**A**) or ability of cdk2 immunoprecipitate (**B**) to phosphorylate histone H1 as compared to control (time 0) are depicted. **C**: cdc2 and cdk2 graphs superimposed to compare directly the effect of TGF-β_1 on kinase activity. Graphs indicate the means of the densometric readings from three independent experiments.

crease in the ability of cdc2 to phosphorylate histone H1 precedes its overall decrease in total protein but correlates well with a decrease in newly synthesized cdc2 protein. This indicates that the ability of cdc2 to phosphorylate histone H1 may be dependent on a requirement for newly synthesized cdc2 protein.

Since TGF-β_1 similarly decreases cdk2 kinase activity but has no detectable effect on cdk2 protein synthesis, we decided to look at cdk2 and determine whether the addition of TGF-β_1 had any effect on its phosphorylation state. Immunoprecipitations from cellular extracts made from ^{32}P-orthophosphate-labeled cycling MME cells grown in the presence or absence of TGF-β_1 were performed. As can be seen in Figure 7, the phosphorylation state of cdk2 is decreased by at least 50% in cells grown with TGF-β_1. We are presently attempting to map the affected sites. These results (Figs. 2, 6, 7) suggest that there are multiple mechanisms by which TGF-β_1 modulates cdk activity.

DISCUSSION

TGF-β_1 is unique in its ability to stimulate or inhibit various cell types. Depending on the individual responses of specific cells, the effects of TGF-β_1 can lead to either increased cellular proliferation or to growth inhibition as seen in epithelial cells [Barnard et al., 1990; Nielson-Hamilton, 1990; Massague et al., 1992]. Although the signal transduction pathway(s) for TGF-β_1 is relatively unknown, the identification of some of its effectors will be important for understanding the mechanism(s) regulating these distinct activities. To this end, the cdks have recently been proposed as possible mediators of TGF-β_1 activity [Howe et al., 1991; Abraham et al., 1992; Koff et al., 1993; Ewen et al., 1993; Eblen et al., 1994]. For instance, addition of TGF-β_1 to mink lung epithelial cells results in the inhibition of cdc2, cdk2, and cdk4 kinase activity, as well as protein synthesis. Because mammary cancer is a major cause of morbidity and mortality in woman, we wished to determine whether TGF-β_1 and the cdks showed a similar relationship in mammary cell growth.

Using a mouse mammary epithelial cell line (MME), we have shown that TGF-β_1 regulates cdc2 at multiple levels. First, newly synthesized protein levels are reduced 6 hours after addition of TGF-β_1 (Fig. 2A,C). This appears to be di-

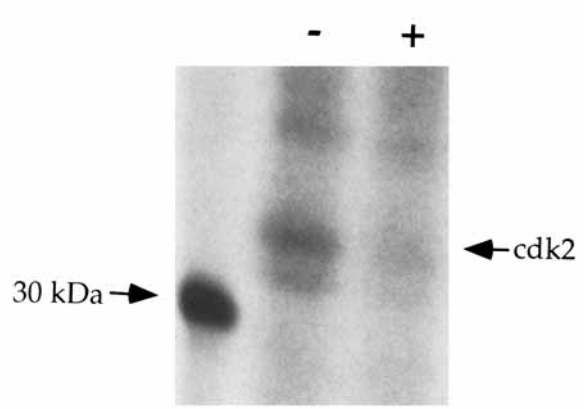

Fig. 7. Decrease in phosphorylation of cdk2 following the addition of TGF-β_1. Cycling MME cells were grown for 24 h in the absence (−) or presence (+) of 5 ng/ml TGF-β_1. Two h prior to harvest, ^{32}P-orthophosphate was added to a final concentration of 250 μCi/ml. Cellular extracts were normalized for protein content and immunoprecipitated with an antibody to cdk2. Lysates were run on a 10% SDS–PAGE gel and processed for autoradiography.

rectly related to the growth inhibitory activity of TGF-β_1, since similar experiments performed on a TGF-β_1-resistant MME cell line (BG18.2) did not result in a reduction of newly synthesized cdc2 protein (Fig. 2B). The inhibition of cdc2 protein synthesis by TGF-β_1 can be kinetically dissociated from effects of TGF-β_1 on cell growth in that cdc2 synthesis is decreased by 6 h following the addition of TGF-β_1, while decreased DNA synthesis is not evident for an additional 4–6 h (data not shown). Moreover, this decrease in cdc2 synthesis is not the result of TGF-β_1 causing a general reduction in new protein synthesis, nor are all cdk proteins similarly affected. For instance, as shown in Fig. 2C, cdk2 synthesis is relatively unaffected over a 24-h treatment with TGF-β_1. In addition, since TGF-β_1 has been shown to decrease the synthesis of cdk4 in early G1 [Ewen et al., 1993], this provides additional support that the lack of an effect of TGF-β_1 on cdk2 synthesis is not simply a reflection of the late G1 arrest mediated by TGF-β_1.

Although cdk2 protein synthesis was not affected by the addition of TGF-β_1, both newly synthesized as well as total cdc2 protein was decreased (Figs. 2, 3). In order to investigate these results further, Northern blot analysis of cdc2 mRNA was performed. As shown in Figure 4, cdc2 mRNA levels are reduced by 70% 12–16 h after the addition of TGF-β_1. This does not appear to result solely from an increase in cdc2 mRNA instability. For instance, while TGF-β_1 decreased the half-life of the cdc2 message from approximately 12 h to 9 h, it is unlikely that this could account for the > 70% decrease in mRNA seen by 12–16 h TGF-β_1 treatment. However, these results are consistent with the idea that both transcriptional as well as post-transcriptional mechanisms are used by TGF-β_1 to modulate cdc2 gene expression.

In addition to TGF-β_1 effects on cdc2 protein synthesis and mRNA synthesis, we also investigated TGF-β_1 effects on cdc2 and cdk2 kinase activity in MME cells. As shown in Figure 6, both cdc2 and cdk2 kinase activity were decreased significantly, starting almost immediately after the addition of TGF-β_1. These results suggest that the reduction in cdc2 kinase activity is not simply a reflection of decreased total cdc2 protein synthesis, as this was not evident until 12–24 h following the addition of TGF-β_1 (Fig. 3). Moreover, for the initial 6 h of TGF-β_1 treatment, the reduction in kinase activity slightly precedes the decrease in newly synthesized cdc2 protein, while at subsequent times these two parameters decrease in parallel. This again suggests the possibility that TGF-β_1 is regulating the cdks at multiple levels. For instance, the initial decrease in kinase activity may reflect some modification of a positive acting signal (i.e., threonine 160/161 phosphorylation) and/or activation of a negative cdc2 regulator (see below). Following these initial effects, the decrease in newly synthesized cdc2 protein may also become effective in decreasing the kinase activity. In that regard, the decrease in cdk2 phosphorylation by TGF-β_1 is of interest (Fig. 7). However, it remains to be determined whether the decrease in cdk2 phosphorylation is solely, or in part, responsible for the loss in kinase activity.

Although no affect on cdk2 protein synthesis is seen after the addition of TGF-β_1 to cycling MME cells (Fig. 2), a dramatic reduction in its kinase activity is seen over the same time period (Fig. 6). Since the primary G1-phase H1 kinase activity in MME cells is dependent on cdk2 associated with cyclin A (unpublished), we determined whether the decrease in cdk2 activity was a reflection of decreased cyclin A protein synthesis. The addition of TGF-β_1 for 24 h to cycling MME cells had no effect on newly synthesized cyclin A protein synthesis, while DNA synthesis was inhibited at 40–50% (data not shown). While changes on cdk2 phosphorylation may modulate cdk activity, recent publications have shown that

a variety of negative growth regulators effect activity of the cyclin-dependent kinases [Gyuris et al., 1993; Harper et al., 1993; El-Deiry et al., 1993; Gu et al., 1993; Serrano et al., 1993; Noda et al., 1994]. One in particular is $p27^{Kip1}$ (for cdk inhibitory protein 1) [Polyak et al., 1994a,b]. $p27^{Kip1}$ was identified in Mv1Lu mink lung epithelial cells that were growth arrested by TGF-β_1 or by cell–cell contact. This 27-kDa protein appears to bind to cyclin E–cdk2 complexes and markedly reduce its ability to phosphorylate histone H1. Another inhibitory protein, $p28^{Ick}$ (for inhibitor of cdk) has also been identified with similar properties to $p27^{Kip1}$ [Slingerland et al., 1994; Hengst et al., 1994]. This too appears to inhibit cyclin E–cdk2 kinase activity. Recently, we have identified a 28-kDa protein that in, addition to inhibiting cyclin E–cdk2 complexes, also inhibits cyclin A–cdk2 complexes (data not shown). It is unknown whether p28 is identical or similar to these other inhibitors.

In breast cancer, the mRNA levels for cdc2, cyclin A, and cyclin B are often overexpressed when compared to normal mammary cells [Keyomarsi and Pardee, 1993]. Since the effects on the cdks appear to be biochemical markers for TGF-β_1 action, it will be of interest to determine whether these markers are modulated in epithelial cancers that have lost their growth inhibitory response to TGF-β_1. The kinetics or manner by which marker activity is affected may be of value in staging carcinoma progression.

ACKNOWLEDGMENTS

We thank M. Edens for excellent technical assistance. This work was supported by Public Health Service grants CA42836 and CA59883 from the National Cancer Institute and by grant 2624 from Council for Tobacco Research. M.P.F. was supported in part by grant PF-3892 from the American Cancer Society.

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