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# Transforming Growth Factor β1 Inhibition of p34$^{cdc2}$ Phosphorylation and Histone H1 Kinase Activity Is Associated with G1/S-Phase Growth Arrest

PHILIP H. HOWE,[1]† GIULIO DRAETTA,[2] AND EDWARD B. LEOF[1]*

*Department of Cell Biology, Vanderbilt University, Nashville, Tennessee 37232,[1] and EMBL, Post Fach 10-2209, Meyerhof Strassel, Heidelberg D6900, Federal Republic of Germany[2]*

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**Transforming growth factor β1 (TGFβ1) is a potent inhibitor of epithelial cell proliferation. We present data which indicate that epithelial cell proliferation is inhibited when TGFβ1 is added throughout the prereplicative G1 phase. Cultures become reversibly blocked in late G1 at the G1/S-phase boundary. The inhibitory effects of TGFβ1 on cell growth occur in the presence of the RNA synthesis inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Associated with this inhibitory effect is a decrease in the phosphorylation and histone H1 kinase activity of the p34$^{cdc2}$ protein kinase. These data suggest that TGFβ1 growth inhibition in epithelial cells involves the regulation of p34$^{cdc2}$ activity at the G1/S transition.**

The entry of quiescent cells into G1 and their commitment to DNA synthesis is regulated by a determined sequence of events under hormonal control (for reviews, see references 2 and 28). Purified growth factors have been shown to regulate distinct portions of G1 and entry to S phase (21). Once cells have progressed beyond the final arrest point in G1, referred to as the R (restriction) or W point, they are irrevocably committed to enter DNA synthesis (4, 30). The R point is temporally 1 to 3 h from the S phase, and continued G1 traverse from the R point to DNA synthesis does not require additional growth factor stimulation (5). While there are numerous means by which cell cycle traverse can be initiated (16), these diverse pathways are believed to ultimately converge at the R point as the final G1-regulated event prior to the S phase. Thus, modulation of events in late G1 might be a general mechanism by which the actions of a variety of growth-promoting polypeptides could be inhibited.

One such modulator of late G1 cell cycle events is a 34-kDa protein (p34$^{cdc2}$) encoded by the *cdc2* and *CDC28* genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively. p34$^{cdc2}$ is a serine-threonine kinase whose activity is required both before DNA synthesis (G1/S boundary) and before mitosis (G2/M boundary) (8, 15, 27). Recent studies have shown that p34$^{cdc2}$ kinase is a component of M-phase-specific histone H1 kinase (1, 19) in addition to being a component of maturation-promoting factor, an activity required for the initiation of mitotic division in multicellular eukaryotes (11, 13). Homologous proteins have been found in every eukaryotic species studied including humans, leading to the proposal that p34$^{cdc2}$ kinase participates in a universal and conserved regulatory pathway (9, 10, 26). While it is postulated that p34$^{cdc2}$ is regulated by both phosphorylation and dephosphorylation in a cell cycle-dependent manner (8, 10, 12, 20, 25, 33), the kinase(s) and phosphatase(s) involved in vivo remain unidentified. Also not clearly understood is the role of phosphorylation in the cell cycle-dependent activity of the p34$^{cdc2}$ kinase. For example, while tyrosine dephosphorylation has been shown to be a prerequisite for kinase activation at mitosis (8, 11, 25), the role that other alterations of p34$^{cdc2}$ play in kinase activation, such as the reported changes in serine and threonine phosphorylation observed in proliferating human cells (10), remain to be elucidated.

Transforming growth factor β1 (TGFβ1) is a potent inhibitor of epithelial cell proliferation (7). The molecular mechanism of inhibition by TGFβ1 remains poorly understood. While guanine nucleotide binding proteins have been implicated in TGFβ1 signaling (17, 18), a number of studies have shown that TGFβ1 does not affect early G1 events induced by growth factors (6, 22). For instance, the antiproliferative effect of TGFβ1 is apparently not a consequence of effects on phosphoinositide breakdown, ribosomal protein S6 phosphorylation, epidermal growth factor (EGF) binding and autophosphorylation, or $Na^+/H^+$ antiport. Thus, cell cycle-dependent late G1/S-phase events, in contrast to early G0/G1-associated events, are likely targets through which growth inhibition might be regulated. In this report, we establish that the inhibition of epithelial cell proliferation by TGFβ1 occurs in late G1. Associated with this growth arrest at the G1/S transition is a decrease in the phosphorylation and histone H1 kinase activity of p34$^{cdc2}$.

## MATERIALS AND METHODS

**Cell culture.** Mink lung epithelial cells (ATCC CCL64) were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). After 5 to 7 days of growth at 37°C, the quiescent density-arrested cultures were used for subsequent studies. BALB/MK-2 keratinocytes were grown in minimal essential medium containing 0.05 mM calcium supplemented with 8% dialyzed FBS and 4 ng of EGF per ml. These cells were made quiescent by incubating 50 to 60% confluent cultures in EGF-deficient medium as described previously (7).

**DNA synthesis measurements.** Inhibition of DNA synthesis by TGFβ1 in CCL64 and BALB/MK-2 (see Fig. 2C) cells was evaluated by [$^3$H]thymidine incorporation into trichloroacetic acid (TCA)-insoluble material. Cells were plated at a density of $2 \times 10^4$ cells per cm$^2$. After 5 to 7 days of growth for CCL64 cells or 2 days of growth in EGF-deficient

* Corresponding author.
† Present address: Department of Vascular Cell Biology, FF 4-17, Cleveland Clinic Foundation, Cleveland, OH 44195.

medium for BALB/MK-2 cells, the density-arrested cultures were stimulated with the indicated growth factors. At the times indicated, [$^3$H]thymidine (1 μCi/ml) was added for a 2-h pulse and the radioactivity incorporated into TCA-precipitable material was determined by scintillation counting. Alternatively, when labeled nuclei were indexed (see Fig. 1B), quiescent cultures were stimulated with the indicated reagents in the presence of 1 μCi of [$^3$H]thymidine per ml for 24 h. The cells were fixed in 100% methanol and processed for autoradiography by coating with NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N.Y.).

**Immunoprecipitations and immunoblotting.** For immunoprecipitation studies, cells were plated at a density of $2.3 \times 10^4$ cells per cm$^2$ in 6-cm plates (22 cm$^2$). Quiescent density-arrested CCL64 cells were stimulated with FBS and EGF in the presence or absence of TGFβ1 (5 ng/ml). At various times after stimulation, the cells were pulsed for the last 2 h of their incubation period at 37°C with [$^{35}$S]methionine (300 μCi/ml) or $^{32}PO_4^{3-}$ (250 μCi/ml) in methionine-free medium or phosphate-free medium, respectively. At the times indicated in each figure (end of the 2-h pulse), cell lysates were prepared and immunoprecipitated by using antiserum to p34$^{cdc2}$ (G6) as previously described (8). Equal amounts of [$^{35}$S]methionine acid-precipitable counts per minute or equal amounts of total protein (nonlabeled lysates) were used. For $^{32}PO_4^{3-}$ immunoprecipitations, lysates from equivalent cell numbers were utilized. TGFβ1 did not have any detectable effect on [$^{35}$S]methionine or $^{32}PO_4^{3-}$ incorporation as determined from the acid-precipitable counts per minute. The immunoprecipitates were analyzed by gel electrophoresis and autoradiography on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. For immunoblotting, cells were plated and stimulated as described above. At the times indicated, protein extracts were prepared by sonication for 15 s in 50 mM Tris-HCl (pH 8.0)–150 mM NaCl. Proteins were electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose, and processed for immunoblotting as described previously (8) with the G6 anti-p34$^{cdc2}$ serum and $^{125}$I-labeled immunoglobulin G.

**Protein kinase assays.** Immunoprecipitates from stimulated cells were prepared as described above and suspended in a reaction mixture (40 μl) containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM ATP, 5 μCi of [γ-$^{32}$P]ATP, and 0.83 mg of histone H1 (Boehringer-Mannheim) per ml or 1 mg of casein (Sigma) per ml (9). Reactions were incubated for 5 min at 30°C, stopped with 40 μl of 2× sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. As a control for the histone H1 kinase activity, parallel immunoprecipitations were performed with antiserum pretreated with 100 nmol of blocking peptide (G6 peptide used to generate the G6 antiserum). Following peptide pretreatment, histone H1 kinase activity was not detectable (data not shown).

## RESULTS

**TGFβ1 growth inhibition occurs in late G1.** Rapidly growing CCL64 epithelial cells are extremely sensitive to growth inhibition by TGFβ1 (22). However, to analyze the cell cycle parameters and potential targets for TGFβ1 growth inhibition, it was first necessary to define conditions in which synchronized G0-arrested cells would undergo cell cycle traverse. A combination of 10% FBS and 20 ng of EGF per ml stimulated maximal DNA synthesis in density-arrested cultures (Fig. 1A). Addition of various concentrations of TGFβ1 at the time of growth stimulation resulted in a dose-dependent inhibition of [$^3$H]thymidine incorporation into DNA (Fig. 1A). The half-maximal inhibitory dose was approximately 1.0 ng of TGFβ1 per ml, which is 10- to 30-fold greater than that required to inhibit sparse, rapidly growing cells (22; data not shown). The suboptimal level of DNA synthesis stimulated by either FBS or EGF alone was similarly inhibited by TGFβ1 (Fig. 1A). Comparable results were also observed if the cultures were processed for autoradiography and the percentage of labeled nuclei was determined (Fig. 1B). For instance, 12% labeled nuclei were seen in the control untreated cultures, 82% labeled nuclei were seen in FBS-EGF-stimulated cultures, and 18% labeled nuclei were seen in FBS-EGF-TGFβ1-treated plates.

Experiments designed to determine the cell cycle kinetics of stimulation and of TGFβ1 growth inhibition were performed next (Fig. 2). Addition of FBS and EGF to density-arrested CCL64 cells resulted in entry to S phase after a minimum G1 of approximately 12 h, with maximal DNA synthesis occurring 20 to 24 h after growth factor stimulation (Fig. 2). Similar kinetic data have also been observed in a variety of other cell types (2). If the only target(s) for TGFβ1-mediated growth inhibition was in early G0/G1, once cultures had progressed temporally into late G1 they should have been insensitive to TGFβ1. However, when TGFβ1 was added to FBS-EGF-stimulated cultures at any time during the 12-h prereplicative G1 period, DNA synthesis was inhibited to an extent (approximately 80 to 100%) similar to that when TGFβ1 was added at the time of stimulation (Fig. 2). In fact, maximal growth inhibition was routinely observed when TGFβ1 was added between 6 and 10 h after initial growth factor addition (Fig. 2 and data not shown). If, however, cultures progressed beyond commitment to DNA synthesis (i.e., >12 h following addition of FBS and EGF) before TGFβ1 was added, then a loss in growth inhibition was observed.

Since TGFβ1 could inhibit cell cycle traverse when added at any time during the G1 phase (Fig. 2), we next determined whether a specific arrest point was in fact associated with growth inhibition. To perform these experiments, we stimulated quiescent CCL64 cells with FBS and EGF plus TGFβ1 for 20 h at 37°C. The cultures were then washed and placed in FBS-containing medium without TGFβ1. At various times after TGFβ1 removal, the cells were fixed and DNA synthesis was determined (Fig. 3A). When TGFβ1-treated cells were released from growth arrest, there was a lag of only 1 to 2 h prior to initiation of DNA synthesis, which was maximal by 12 h (Fig. 3A). Since quiescent G0-arrested cultures had a minimum G1 of 12 h (Fig. 2), pretreatment with FBS and EGF plus TGFβ1 permitted cell cycle progression to a point temporally 1 to 2 h from the S phase. A similar late G1 arrest and rate of entry was seen when BALB/MK-2 cells (mouse keratinocytes inhibited by TGFβ1) (7) were utilized (Fig. 3B) or if autoradiography was performed and the percentage of labeled nuclei was determined (data not shown). Thus, in the continual presence of TGFβ1, epithelial cells progress temporally into G1 and arrest at the G1/S-phase border.

**TGFβ1 growth inhibition occurs in the presence of the RNA synthesis inhibitor DRB.** The previous data indicated that effects on early G0/G1 events might not be the primary mechanism for TGFβ1 growth inhibition. Since traverse of the last half of G1 and commitment to DNA synthesis can occur in the absence of new RNA synthesis (34, 35) (Table 1), and TGFβ1 can inhibit epithelial cell proliferation when added in late G1 (Fig. 2), we next determined whether

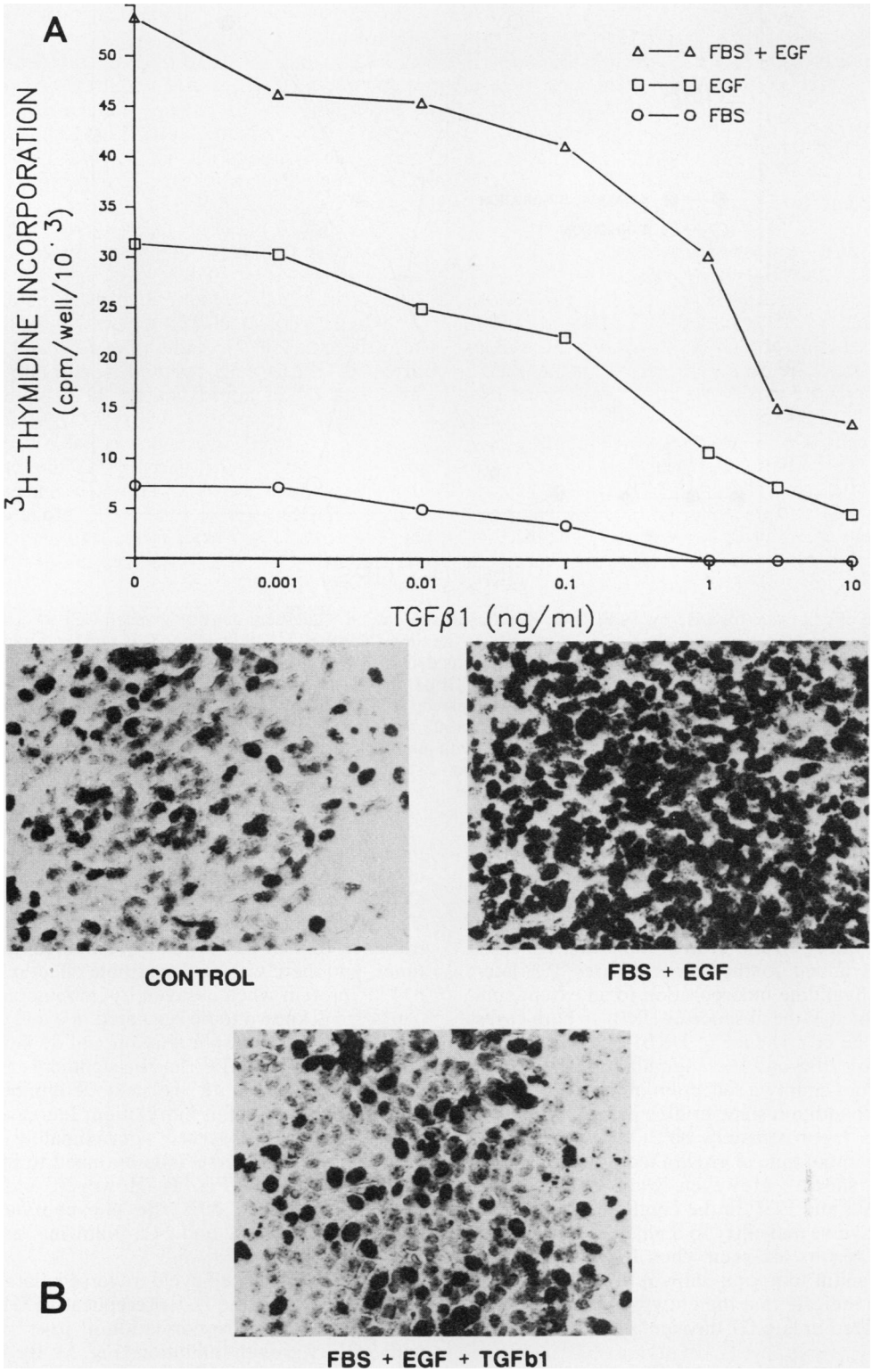


FIG. 1. Inhibition of DNA synthesis by TGFβ1. (A) Quiescent density-arrested CCL64 cell cultures were stimulated with FBS (10%), EGF (20 ng/ml), or FBS and EGF in the presence of the indicated concentrations of TGFβ1 for 22 h at 37°C. At that time, 1 μCi of [$^3$H]thymidine per ml was added for a 2-h pulse and the radioactivity incorporated into TCA-insoluble material was measured. The basal level of thymidine incorporation was 5,789 cpm. (B) Quiescent cultures were stimulated with the indicated reagents in the presence of 1 μCi of [$^3$H]thymidine per ml for 24 h. The cells were fixed in 100% methanol and processed for autoradiography.

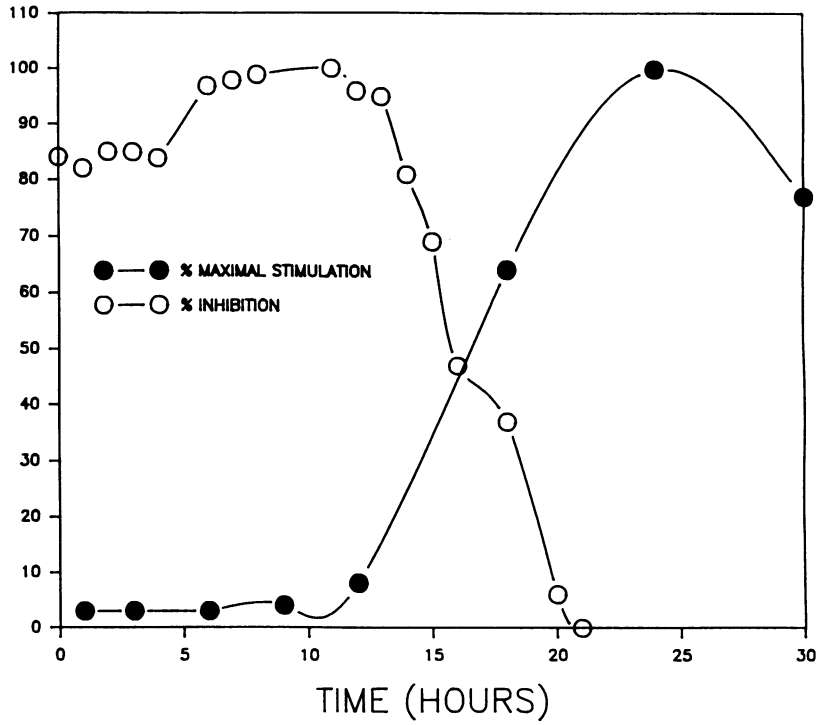


FIG. 2. Inhibition of cell cycle traverse by TGFβ1. ●, Kinetics of stimulation. Quiescent density-arrested CCL64 cells were stimulated in fresh medium containing 10% FBS and 20 ng of EGF per ml. At the times indicated [$^3$H]thymidine (1 μCi/ml) was added for a 2-h pulse and the radioactivity incorporated into TCA-precipitable material was determined. The data are expressed as percent maximal stimulation, which at the 24-h point represented 36,236 cpm. ○, Kinetics of TGFβ1 growth inhibition. Quiescent density-arrested CCL64 cells were stimulated with FBS and EGF as described above. At the times indicated, TGFβ1 was directly added to a final concentration of 1 ng/ml. The cultures were pulsed with 1 μCi of [$^3$H]thymidine per ml for 2 h at 22 h following initial FBS and EGF stimulation. Value for maximal stimulation in the absence of TGFβ1 was 25,417 cpm. Maximal growth inhibition was observed when TGFβ1 was added 10 h after FBS and EGF and was 4,630 cpm. The basal level of thymidine incorporation in serum-free medium was 4,867 cpm.

TGFβ1 growth inhibition could occur in the absence of RNA synthesis. As shown in Table 1, addition of the RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) at 8 h following FBS and EGF stimulation had no detectable effect on the growth inhibition observed when TGFβ1 was added to the same cultures 1 h later; TGFβ1 inhibited thymidine incorporation to an extent similar to that when added in the absence of DRB. In both cases, in either the absence or presence of DRB, TGFβ1 was able to completely inhibit FBS and EGF stimulation when added in late G1. This is not simply a reflection of the differing cell cycle times of DRB addition since uridine incorporation was similarly inhibited (approximately 80%) when DRB was added either at the initial time of growth factor addition or in late G1 (data not shown). However, when cultures were stimulated with FBS and EGF in the continued presence of DRB, cell cycle traverse and entry to S phase was prevented (Table 1). Similar results are seen when RNA synthesis is inhibited by α-amanitin (data not shown). The data (Fig. 2 and 3 and Table 1) indicate that the growth-inhibitory action of TGFβ1 is mediated in late G1 through a DRB-insensitive pathway(s).

**Cell cycle effects of TGFβ1 on p34$^{cdc2}$ protein.** Since the previous results (Fig. 2 and 3 and Table 1) suggested that the primary mechanism(s) by which TGFβ1 inhibits cellular proliferation is not by modulating events in early G0/G1, targets acting in late G1 were considered. One such target for TGFβ1 might be the p34$^{cdc2}$ protein kinase shown previously to regulate unique portions, G1/S and/or G2/M transition, of the eukaryotic cell cycle (10, 15, 27). The de novo synthesis of p34$^{cdc2}$ was relatively constant in quiescent restimulated CCL64 cells and essentially unaffected by TGFβ1 (Fig. 4). The slight decrease in *cdc2* protein seen at 6 h in the presence of TGFβ1 was not maintained at later cell cycle times, and there was no discernible effect of TGFβ1 on total p34$^{cdc2}$ protein when assayed by immunoblotting (Fig. 4B).

p34$^{cdc2}$ is known to be regulated in a cell cycle-dependent fashion by both phosphorylation and dephosphorylation (8, 10, 20, 33). Since TGFβ1 had no significant effects on p34$^{cdc2}$ protein levels (Fig. 4), we next determined the effect of TGFβ1 on p34$^{cdc2}$ phosphorylation. Increased p34$^{cdc2}$ phosphorylation was observed approximately 12 h following initiation of G1 traverse and continued to increase throughout the next 12 h (Fig. 5). However, addition of TGFβ1 inhibited by 70 to 80% the phosphorylation of p34$^{cdc2}$ observed between 12 and 24 h following restimulation (Fig. 5).

TGFβ1 inhibited cell cycle traverse in late G1 when added at any time during the 12-h prereplicative G1 period (Fig. 2). Since decreased phosphorylation of p34$^{cdc2}$ was associated with TGFβ1 growth inhibition (Fig. 5), we next determined whether decreased p34$^{cdc2}$ phosphorylation was simply a result of growth inhibition and whether the effect of TGFβ1 on p34$^{cdc2}$ phosphorylation was regulated in a cell cycle-dependent, in contrast to a growth factor-dependent, manner. When quiescent density-arrested CCL64 cells were stimulated with FBS and EGF in the presence of methotrexate (a folate analog), resulting in an early S-phase block, no

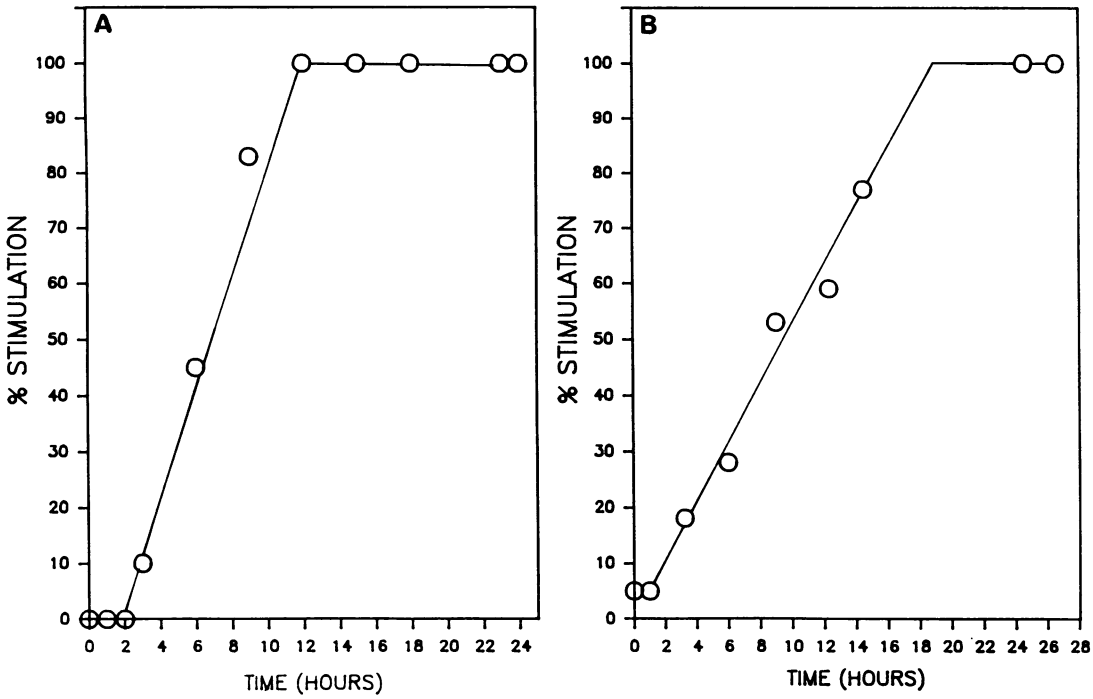


FIG. 3. CCL64 (A) and BALB/MK-2 (B) cell entry to S phase after release from TGFβ1 growth inhibition. Quiescent density-arrested cells were stimulated with FBS and EGF in the continuous presence of TGFβ1 (1 ng/ml) and [$^3$H]thymidine (1 μCi/ml) for 20 h at 37°C. The cultures were washed, and FBS plus [$^3$H]thymidine-containing medium was added without TGFβ1. At various times after TGFβ1 removal (0 to 24 h), the cells were fixed with 300 μl of 1 M ascorbic acid and the TCA-insoluble material was determined. BALB/MK-2 cells were grown and made EGF dependent as described previously (7). Results of panel A represent the mean of two experiments done in duplicate, and maximal stimulation was 25,846 cpm. Results of panel B represent the mean of three experiments done in duplicate, and 100% stimulation was 85,302 cpm.

decrease in p34$^{cdc2}$ phosphorylation was observed (Fig. 6A, lane 4). However, addition of TGFβ1 with the methotrexate reduced p34$^{cdc2}$ phosphorylation to basal levels (Fig. 6A, lane 5). These results indicate that decreased p34$^{cdc2}$ phosphorylation is not a general consequence of growth inhibition but is possibly a specific component of TGFβ1 action. Additionally, the action of TGFβ1 on p34$^{cdc2}$ phosphorylation was cell cycle specific (Fig. 6B). If quiescent cells were stimulated with FBS and EGF in the presence of methotrexate and then released from methotrexate inhibition in either the absence (Fig. 6B, lane 4) or presence (lane 5) of TGFβ1, no inhibitory effects of TGFβ1 on p34$^{cdc2}$ phosphorylation were observed. Once the cells bypassed the TGFβ1 inhibition point in late G1, they became refractile to its inhibitory action on both cell growth (Fig. 2) and on p34$^{cdc2}$ phosphorylation (Fig. 6B). Further specificity for the effects of TGFβ1 on p34$^{cdc2}$ phosphorylation is shown in Fig. 6C, where it is shown that in MLE-M cells, a TGFβ1-resistant CCL64 cell line (18), TGFβ1 addition had no effect on the FBS- and EGF-stimulated phosphorylation of p34$^{cdc2}$.

TABLE 1. RNA synthesis is not required for TGFβ1-mediated growth inhibition[a]

| Initial addition | Late G1 addition | [$^3$H]thymidine incorporation (cpm) | % Stimulation |
|---|---|---|---|
| FBS + EGF | | 17,125 | 100 |
| FBS + EGF + TGFβ1 | | 5,468 | 13 |
| FBS + EGF + DRB | | 3,437 | 0 |
| FBS + EGF | TGFβ1 | 3,818 | 1 |
| FBS + EGF | DRB | 13,913 | 76 |
| FBS + EGF | DRB + TGFβ1 | 2,547 | 0 |

[a] Quiescent density-arrested CCL64 cells were stimulated in fresh medium containing FBS (10%) and EGF (20 ng/ml) in the presence or absence of TGFβ1 (2 ng/ml) or DRB (20 μM). At 22 h after stimulation, 1 μCi of [$^3$H]thymidine per ml was added for a 2-h pulse, and radioactivity incorporated into TCA-precipitable material was determined. Initial addition indicates that these reagents were added at the time of stimulation, and late G1 addition indicates that they were added at 8 h poststimulation for DRB and 9 h poststimulation for TGFβ1. Values for percent stimulation were determined after subtraction of the control value (medium alone), which was 3,730 cpm. The data represent the mean of two experiments performed in duplicate.

**TGFβ1 inhibits p34$^{cdc2}$ histone H1 kinase activity.** Since TGFβ1 affected the cell cycle-dependent phosphorylation of p34$^{cdc2}$, we next examined whether its kinase activity was similarly affected. It has previously been shown that p34$^{cdc2}$ phosphorylates histone H1 in a cell cycle-dependent manner, while its use of casein as a substrate is independent of cell cycle events (3). Histone H1 kinase activity was detectable 10 to 12 h following FBS and EGF stimulation of quiescent cultures and was maximal (18-fold above control, nonstimulated cells) at 16 to 24 h (Fig. 7). Addition of TGFβ1, however, decreased the H1 kinase activity of p34$^{cdc2}$ by approximately 70 to 80% at the 16- and 24-h points. In contrast, while basal p34$^{cdc2}$ casein kinase activity was higher, it showed essentially no cell cycle regulation (less than twofold), and furthermore, it could not be inhibited by TGFβ1 (Fig. 7).

Histone H1 kinase activity is believed to reflect a mitotic action of p34$^{cdc2}$ (8, 23, 24, 26). Since the peak in H1 kinase activity occurred at a cell cycle time earlier than that expected for an M-phase-specific function, we determined

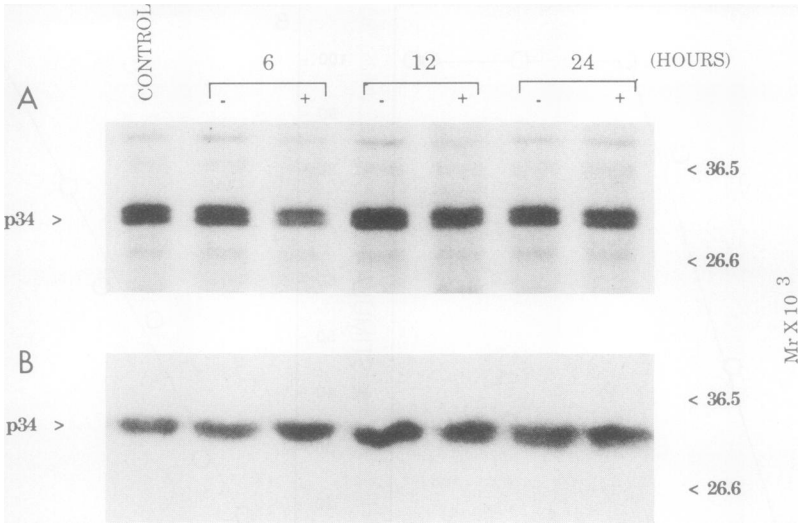


FIG. 4. TGFβ1 does not affect p34$^{cdc2}$ protein levels. (A) Immunoprecipitations. Quiescent density-arrested CCL64 cells were stimulated to undergo G1 traverse by the addition of FBS and EGF in the presence (+) or absence (−) of TGFβ1 (5 ng/ml). The cells were pulsed for the last 2 h of their respective incubation period with [$^{35}$S]methionine (300 μCi/ml) in methionine-free medium in the presence or absence of TGFβ1. At the indicated times, cells were harvested and immunoprecipitated using G6 sera as described in Materials and Methods. (B) Immunoblot. CCL64 cells were stimulated with FBS and EGF with or without TGFβ1 for the indicated times. Cell lysates were prepared at the indicated times and then immunoblotted with the anti-p34$^{cdc2}$ G6 serum.

whether this increase was simply a consequence of contaminating G2/M cells. Fluorescence-activated cell sorter (FACS) analysis indicated an approximate 9, 6, and 7% G2/M pool at 0 h (control), 6 h, and 16 h of stimulation, respectively. Thus, there is no apparent difference in the G2/M content of the culture at cell cycle times when H1 kinase activity is low (i.e., 0 and 6 h of stimulation) or maximal (i.e., 16 h of stimulation) (Fig. 7 and 8).

## DISCUSSION

Cell cycle traverse and commitment to DNA synthesis can be initiated by numerous growth factors and in higher eukaryotes are believed to be controlled primarily in G1 (2, 28). Thus, growth-inhibitory molecules would be most effective if they acted at a point(s) common to a variety of mitogenic stimuli. Our results demonstrated that quiescent restimulated epithelial cells are inhibited when TGFβ1 is added throughout the G1 phase of the cell cycle (Fig. 2). Thus, TGFβ1 is capable of inhibiting cellular proliferation when added following the expression of those early G0/G1 events proposed as the primary regulators of cell cycle traverse. These results suggest that the transient expression of early response modifiers does not begin a series of events which, once initiated, confers resistance to TGFβ1 growth inhibition. The results shown in Fig. 3A and B demonstrate that in the continual presence of TGFβ1, stimulated cells can progress to the G1/S-phase boundary but not enter the S phase. The cultures become reversibly growth arrested at a point 1 to 2 h from the S phase. The temporal location of this inhibition correlates well with an R point-like arrest (5). Furthermore, the data of Table 1 indicate that TGFβ1 might not require de novo RNA polymerase II transcription for mediating its inhibitory effects, suggesting that an activity, operative during the last 1 to 2 h of G1, is the primary target for TGFβ1. This, of course, does not eliminate the possibility of a DRB-insensitive transcript(s) being required for TGFβ1 action. However, these results suggest that events in late G1, as opposed to early G0/G1 events, are common targets for a variety of growth arrest molecules and/or states (14, 31).

p34$^{cdc2}$ gene function is required both in late G1, prior to the initiation of DNA synthesis, and in G2 before entry to mitosis (10, 15, 27). The mechanisms that regulate activation of p34$^{cdc2}$ kinase are still to be determined. It has previously been shown that the abundance of p34$^{cdc2}$ remains constant throughout the cell cycle but that its activity oscillates dramatically (8, 25). Histone H1 activity is minimal in early G1 but increases steadily and is maximal during mitotic interphase. Consistent with these findings are the data presented in Fig. 4, where it is shown that the synthesis (A) or steady-state (B) levels of p34$^{cdc2}$ protein are relatively constant in quiescent and restimulated CCL64 cells. It is also shown that the p34$^{cdc2}$ protein levels are unaffected by the addition of TGFβ1. Thus, regulation of p34$^{cdc2}$ protein levels does not appear to be the primary control mechanism for either p34$^{cdc2}$ kinase activation or TGFβ1 growth inhibition.

p34$^{cdc2}$ activity, however, has been shown to be associated both with cell cycle-dependent phosphorylation-dephosphorylation and with interaction with associated regulatory components of the kinase complex (8, 10, 12, 20, 25). For example, in HeLa cells p34$^{cdc2}$ is phosphorylated on serine, threonine, and tyrosine residues (8, 10). The phosphotyrosine content increases during the S phase and is maximal in G2 (25). Tyrosine dephosphorylation occurs at the onset of mitosis, and no phosphotyrosine is detectable during G1. While phosphorylation of p34$^{cdc2}$ is required for complex formation with other regulatory components (29) (i.e., cyclins) during late G1/S, tyrosine-phosphorylated p34$^{cdc2}$ is inactive as a histone H1 kinase. In its most active mitotic form, p34$^{cdc2}$ is dephosphorylated on tyrosine (25). Thus, a complex and yet to be fully determined picture emerges between the phosphorylation state of p34$^{cdc2}$ and its kinase activity during the cell cycle. Our data (Fig. 5) show that when density-arrested epithelial cells are stimulated in the presence of serum and EGF, p34$^{cdc2}$ becomes increasingly phosphorylated as the cells progress through the cell

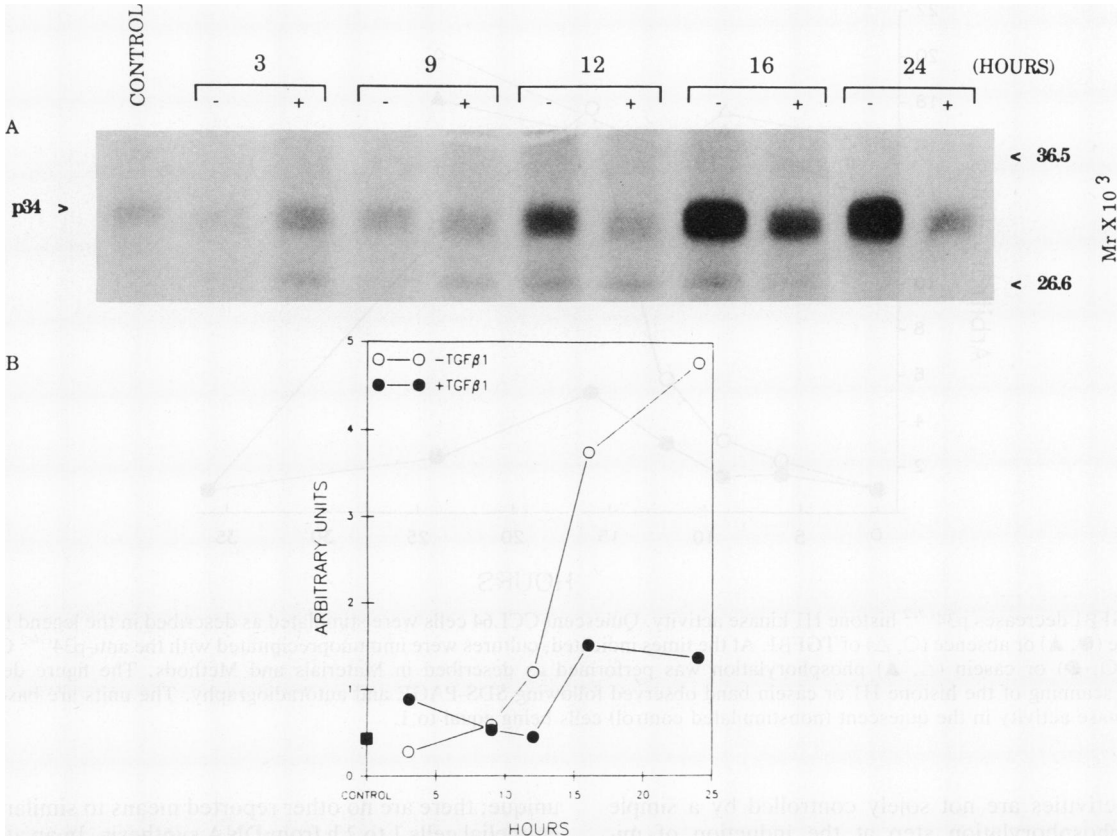


FIG. 5. TGFβ1 inhibits p34$^{cdc2}$ phosphorylation. (A) CCL64 cells were treated in the presence (+) or absence (−) of TGFβ1 as described in the legend to Fig. 2A, except that the cultures were pulsed in phosphate-free medium containing $^{32}PO_4^{3-}$ (250 μCi/ml). At the indicated times, cellular lysates were immunoprecipitated with anti-p34$^{cdc2}$ G6 serum. (B) Diagrammatical presentation of the data from the autoradiograph, in which the relative intensities (normalized to the control level) of the phosphorylated p34$^{cdc2}$ bands were determined by using an LKB UltroScan XL laser densitometer.

cycle. Increased p34$^{cdc2}$ phosphorylation is initially observed 12 h following initiation of G1 traverse, and in the mid to late S phase, p34$^{cdc2}$ phosphorylation is increased 8- to 10-fold over control (quiescent) levels. These results are identical to previous studies (20) in which it was shown that p34$^{cdc2}$ initially becomes phosphorylated at the G1/S-phase boundary when quiescent fibroblasts are stimulated to enter the cell cycle. Although kinase activity was not reported in these studies, it was suggested that this phosphorylation of p34$^{cdc2}$ at the beginning of the S phase is required for activation and continual cell cycle traverse. Thus, it becomes apparent that the various cell cycle-dependent p34$^{cdc2}$

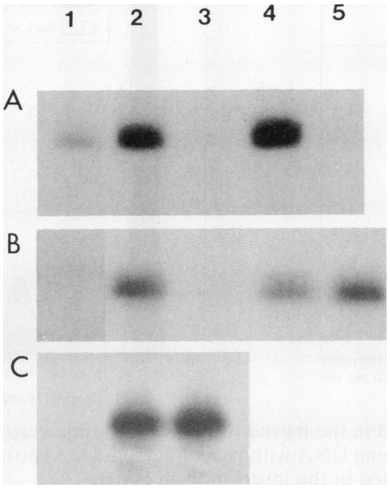


FIG. 6. Specificity of TGFβ1 inhibition of p34$^{cdc2}$ phosphorylation. (A) Quiescent CCL64 cells were stimulated with the indicated reagents for 14 h at 37°C. At that time, the medium was replaced with phosphate-free medium containing the same reagents and 250 μCi of $^{32}PO_4^{3-}$ per ml for an additional 2 h. Cellular lysates were immunoprecipitated with the G6 serum as described in Materials and Methods. Lanes: 1, control (no addition); 2, FBS (10%) and EGF (20 ng/ml); 3, FBS and EGF and TGFβ1 (5 ng/ml); 4, FBS and EGF and methotrexate (200 nM); 5, FBS and EGF and methotrexate and TGFβ1. (B) Cells were treated with the indicated reagents for 22 h at 37°C. The medium was removed, and the cells were pulsed for 2 h in phosphate-free medium containing the same supplements and 200 μCi of $^{32}PO_4^{3-}$ per ml. Lanes: 1, methotrexate (200 nM); 2, FBS and EGF and methotrexate; 3, FBS and EGF and methotrexate and TGFβ1; 4 and 5, FBS and EGF and methotrexate for 22 h and then cells were released in labeling medium for an additional 2 h in the presence of FBS and EGF (lane 4) or FBS and EGF and TGFβ1 (lane 5). (C) Immunoprecipitations from $^{32}PO_4^{3-}$-labeled TGFβ1-resistant cells. Quiescent MLE-M cells were stimulated with FBS and EGF in the absence (lane 2) or presence (lane 3) of TGFβ1 for 14 h. At that time, phosphate-free medium containing the same reagents and $^{32}PO_4^{3-}$ (250 μCi/ml) was added for an additional 2 h. Cellular lysates were then prepared and immunoprecipitated as described above. Lane 1 indicates control (no addition) cultures.

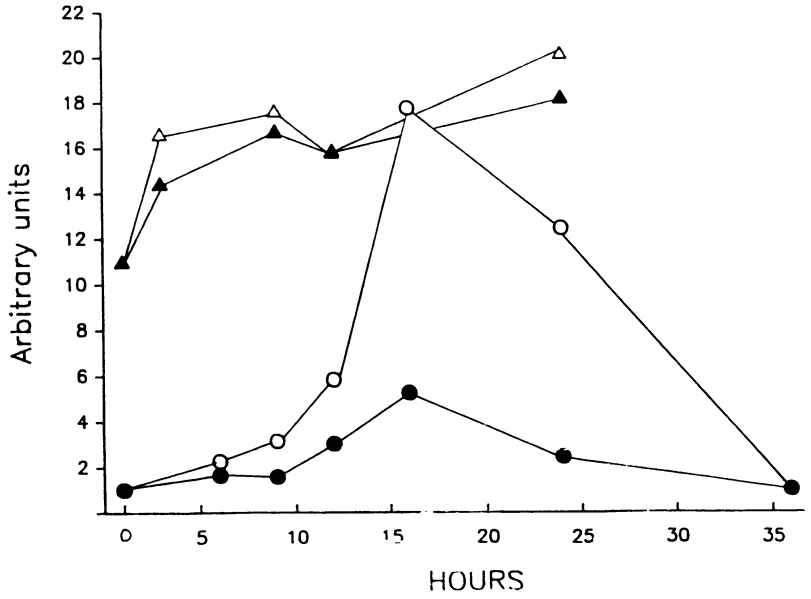


FIG. 7. TGFβ1 decreases $p34^{cdc2}$ histone H1 kinase activity. Quiescent CCL64 cells were stimulated as described in the legend to Fig. 2A in the presence (●, ▲) or absence (○, △) of TGFβ1. At the times indicated, cultures were immunoprecipitated with the anti-$p34^{cdc2}$ G6 serum, and histone (○, ●) or casein (△, ▲) phosphorylation was performed as described in Materials and Methods. The figure depicts the densitometric scanning of the histone H1 or casein band observed following SDS-PAGE and autoradiography. The units are based on the histone H1 kinase activity in the quiescent (nonstimulated control) cells being equal to 1.

forms and activities are not solely controlled by a simple tyrosine dephosphorylation step at the induction of mitosis.

Addition of TGFβ1 inhibited the phosphorylation of $p34^{cdc2}$ seen between 12 and 24 h following stimulation (Fig. 5), correlating well with its inhibitory effects in late G1 on cellular proliferation (Fig. 2). At present, it cannot be conclusively determined whether the decrease in $p34^{cdc2}$ phosphorylation is a specific event associated with TGFβ1 growth inhibition. The particular arrest with TGFβ1 is unique; there are no other reported means to similarly arrest epithelial cells 1 to 2 h from DNA synthesis. In an attempt to address this question of specificity, we performed the experiments shown in Fig. 6. If decreased $p34^{cdc2}$ phosphorylation by TGFβ1 was merely a nonspecific consequence of growth arrest, then an agent that inhibits cells in the early S phase should also decrease $p34^{cdc2}$ phosphorylation. However, as can be seen in Fig. 6A, the inhibition of $p34^{cdc2}$ phosphorylation by TGFβ1 is not a consequence of growth arrest per se since stimulation of quiescent cells in the presence of meth-

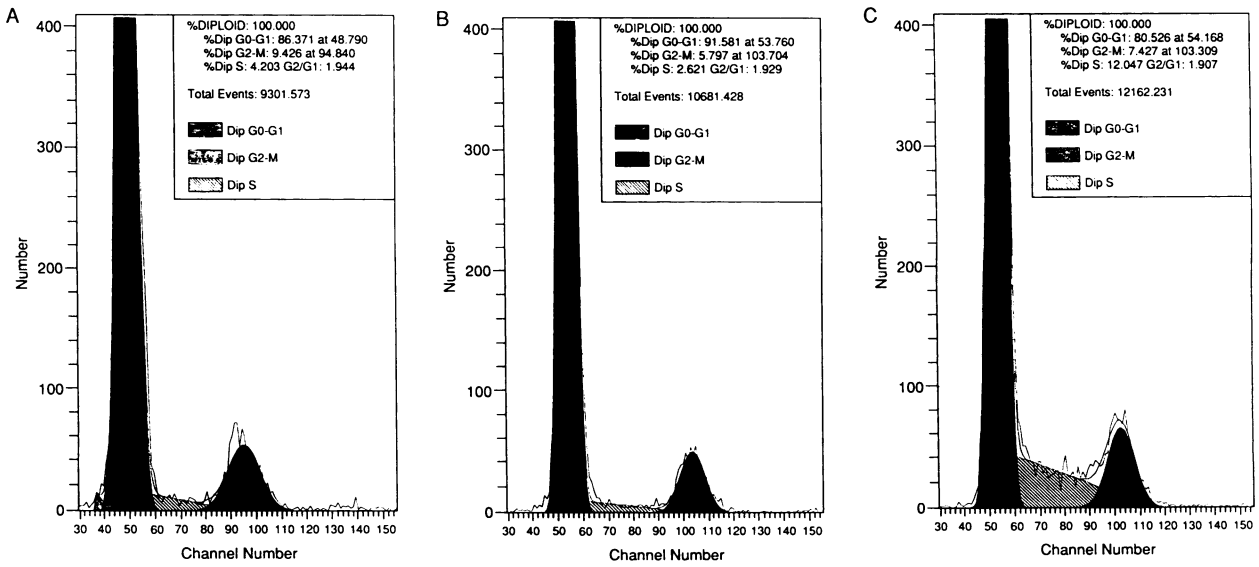


FIG. 8. Cell cycle analysis. Quiescent CCL64 cells were stimulated as described in the legend to Fig. 2A. At time zero (A), 6 h (B), and 16 h (C), stimulation cell cycle analysis was performed by flow cytometry after staining DNA with propidium iodide. Approximately $10^4$ cells were analyzed, and the percentage of cells in G0/G1, G2/M, and S phase is indicated in the insert of each panel.

othrexate had no effect on serum- and EGF-stimulated p34$^{cdc2}$ phosphorylation (Fig. 6A).

Further specificity for the cell cycle-dependent effects of TGFβ1 on p34$^{cdc2}$ phosphorylation is shown in Fig. 6B. When cultures were released from the methotrexate growth inhibition in the early S phase, TGFβ1 had no effect on FBS- and EGF-induced phosphorylation of p34$^{cdc2}$. These results are to be expected if, as we postulate, the effects of TGFβ1 are manifested on p34$^{cdc2}$ prior to the S phase. Once the cells have bypassed or escaped from this inhibitory point, as with an S-phase block, then TGFβ1 should not be expected to inhibit p34$^{cdc2}$ phosphorylation. It might also be suggested that the observed decreases in p34$^{cdc2}$ phosphorylation by TGFβ1 are due to its inhibition of $^{32}$P uptake. While there are no reports of such actions by TGFβ1 in the literature, the results of Fig. 6B also show that this is not the case. When cells were released from the methothrexate S-phase block into $^{32}$P medium containing serum and EGF in the absence (lane 4) or presence (lane 5) of TGFβ1, there was no significant difference in the labeling of the p34$^{cdc2}$ band. Thus, both the phosphorylation of p34$^{cdc2}$ and the TGFβ1 inhibition of p34$^{cdc2}$ phosphorylation are regulated in a cell cycle-dependent fashion. In addition, the inability of TGFβ1 to inhibit p34$^{cdc2}$ phosphorylation in the TGFβ1-resistant MLE-M cells, a cell line lacking the type I TGFβ1 receptor (17), provides further evidence that TGFβ1 growth inhibition might be coupled through its inhibitory effects on p34$^{cdc2}$.

p34$^{cdc2}$ has been shown to phosphorylate both histone H1 (3) and c-*src* (24, 32) in a cell cycle-dependent manner and to phosphorylate casein independent of cell cycle events (3). Activation of histone H1 activity is thought to involve a series of cell cycle-dependent steps which include phosphorylation of p34$^{cdc2}$ kinase and association with cyclin B and the p60 subunit (8, 23, 29). Our results (Fig. 7) are similar to earlier studies (8) showing that when assayed in vitro with casein as substrate, the kinase activity of p34$^{cdc2}$ does not fluctuate in a cell cycle-dependent fashion. When assayed in vitro using histone H1, however, p34$^{cdc2}$ activity correlated well with the cell cycle-dependent increases in p34$^{cdc2}$ phosphorylation shown in Fig. 5. As cells are stimulated to leave quiescence and traverse G1, histone H1 activity increases at the G1/S-phase boundary, is maximal at 16 h poststimulation, and decreases to basal levels by 36 h. While p34$^{cdc2}$ H1 kinase activity is believed to reflect a G2/M function (8, 23, 24, 26), FACS analysis indicated that the peak in H1 kinase activity seen at 16 h was not a consequence of contaminating G2/M cells (Fig. 8). The data demonstrate that the G2/M population at 16 h following growth factor stimulation (i.e., the time when maximal H1 kinase activity is observed) is similar to that seen in control and 6-h-stimulated cultures (i.e., times when no or low H1 kinase is seen). In that regard, when Fig. 2 and 7 are carefully examined, it is seen that approximately 45% of maximal DNA synthesis is observed 16 h following FBS and EGF stimulation. The majority of the population, approximately 55%, has not even entered the S phase at the time (16 h) when maximal H1 kinase activity is seen. Moreover, since the S phase is between 6 and 10 h long and begins approximately 12 h after growth factor addition, the 4-h interval (i.e., 12 to 16 h) when maximal H1 kinase activity is observed is not sufficient time for the fastest-cycling cells to exit S let alone constitute a large G2/M population. Thus, since the initial increase in H1 kinase activity occurs 10 to 12 h following growth factor stimulation (i.e., minimum G1 time, fastest-cycling cells at G1/S border) with peak activity occurring at 16 h (i.e., cell cycle time when majority of the population is at the G1/S border), this is consistent with a G1/S-phase action for p34$^{cdc2}$.

While TGFβ1 has no effect on p34$^{cdc2}$-induced casein phosphorylation the late G1/S histone H1 activity of p34$^{cdc2}$ is inhibited. Whether the decrease in p34$^{cdc2}$ phosphorylation induced by TGFβ1 is causally related to the inhibition in histone H1 kinase activity is difficult to determine and not specifically addressed in these studies. However, the ability of TGFβ1 to preferentially inhibit histone H1 activity compared with casein activity further supports the specific cell cycle-dependent effects of TGFβ1 on p34$^{cdc2}$. If the inhibition of histone H1 activity by TGFβ1 were a consequence of a general growth arrest state, then it might be expected that casein phosphorylation would also be decreased.

Although most studies have focused on the role of p34$^{cdc2}$ protein kinase at the G2/M transition as a mitotic inducer, in mammalian cells cell cycle traverse and commitment to DNA synthesis are primarily controlled in G1 (2, 28). Several earlier reports have implicated a possible regulatory role for p34$^{cdc2}$ kinase at the G1/S-phase border (8, 20, 25). Our results further substantiate the G1/S activity of p34$^{cdc2}$ in mammalian epithelial cells. Furthermore, we show that TGFβ1 arrests epithelial cells in late G1, 1 to 2 h from the onset of DNA synthesis through a DRB-insensitive mechanism. Associated with this growth-inhibitory effect of TGFβ1 is the cell cycle-dependent decrease in p34$^{cdc2}$ phosphorylation and histone H1 kinase activity.

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