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Transforming growth factor β effects on expression of G_1 cyclins and cyclin-dependent protein kinases

(cell cycle progression)

YAN GENG AND ROBERT A. WEINBERG

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

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ABSTRACT **Transforming growth factor β_1 (TGF-β_1) is a potent growth-inhibitory polypeptide. The mechanism of TGF-β_1 inhibition has been related to its ability to prevent the hyperphosphorylation of retinoblastoma protein (pRb). Several lines of evidence have suggested that cell cycle-regulated protein kinases are responsible for the hyperphosphorylation of pRb. We demonstrate here that TGF-β_1 has profound effects on the expression of genes encoding certain G1 cyclins and their associated kinases, which provides one explanation of TGF-β_1 effects on pRb hyperphosphorylation. These results also suggest that the growth-inhibitory effects of TGF-β_1 in many cells are attributable to its effects on the cell cycle apparatus involved in programming G1 transit.**

Transforming growth factor β (TGF-β) is a multifunctional cytokine which is involved in controlling cell cycle progression, cell differentiation, cell adhesion, and extracellular matrix deposition in a variety of cell lineages (1, 2). Of the three mammalian forms of this protein (TGF-β_1, -β_2, and -β_3), TGF-β_1 has been studied most extensively over the past decade. One of the readily measured biological activities of TGF-β_1 is its antiproliferative action on a variety of cell types. For example, TGF-β_1 arrests epithelial cells in the G1 phase of their cell cycle (3, 4). However, the mechanism of growth inhibition of TGF-β_1 remains unclear.

Several cellular genes that are important in the control of cell proliferation have been shown to be affected by TGF-β_1 treatment—e.g., the transcription of the c-*myc* gene is inhibited by TGF-β_1 (5). The negative growth effects of TGF-β_1 have been linked to a tumor suppressor gene product, the retinoblastoma protein, pRb. Specifically, it has been shown that upon TGF-β_1 treatment, the hyperphosphorylation of pRb is prevented, pRb remains hypophosphorylated, and cells become arrested in late G1 (3). Since the hyperphosphorylation of pRb, which normally occurs in late G1, is thought to represent a molecular switch that converts pRb from an active growth-suppressing form into an inactive form (6–9), it has been hypothesized that TGF-β_1 exerts its inhibitory effects on the cell cycle progression by preventing this hyperphosphorylation.

Because hyperphosphorylation of pRb appears to be the key regulator of its function, the identity of the responsible kinases has attracted much interest. Tryptic mapping of the sites of phosphorylation on pRb *in vivo* suggested that pRb is phosphorylated by a subset of cdc2-related kinases, termed cyclin-dependent protein kinases (cdks) (10, 11). Recently a number of known or putative cdks have been described (12, 13). The activity and substrate specificity of cdks are thought to be regulated in turn by association with their regulatory subunits, the cyclins. Five classes of mammalian cyclins have been enumerated to date: A, B (including B1 and B2), C, D (including D1, D2, and D3), and E (14–17).

These various cyclins have been assigned roles in specific phases of the cell cycle. Thus, cyclin B in association with cdc2 has been shown to promote G_2/M transition (18). The expression of cyclin A occurs at the onset of S phase, and this cyclin associates with both cdc2 and cdk2 (12, 19, 20). Cyclins C, D, and E are defined as G1 cyclins, as their expression occurs mainly in G1. The expression of cyclin E occurs with sudden onset in mid/late G1 in parallel with the appearance of cyclin E·cdk2 complexes having strong kinase activity (15, 20). In contrast, cyclins C and D showed rather constant expression through a cell cycle (15). It has been recently found that cyclin D1 can form a complex with cdk4 (21); however, no kinase activity has yet been found to associate with cyclin C.

The timing of initial pRb hyperphosphorylation in late G1 coincides closely with the appearance of cyclin E mRNA. For this reason, it has been speculated that cyclin E is responsible for the initial hyperphosphorylation of pRb, while cyclin A is responsible for subsequent maintenance of pRb hyperphosphorylation through S phase and into G_2.

To understand the mechanism of action of TGF-β_1 in regulating cell cycle progression, we have further explored its effects on the actions of cyclins in the G1 phase of the cell cycle. As described here, TGF-β_1 has profound effects on the expression of genes encoding G1 cyclins and their associated cdks. These effects provide important insights into its mechanism of cell growth regulation.

MATERIALS AND METHODS

Cell Culture. Human keratinocytes [HaCaT cells (22), a gift from P. Boukamp] were cultured in α minimal essential medium (αMEM, GIBCO) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) in a 5% CO_2 incubator at 37°C.

Cell Synchronization and TGF-β_1 Treatment. Cells were plated at the density of 1–2 × 10^6 cells per 10-cm^2 dish and grown in αMEM containing 10% FBS for 36–48 hr until the cell monolayer became confluent. The cells were then cultured in αMEM in the absence of serum for 3 days to achieve quiescence. The cells were restimulated with fresh medium containing 10% FBS. Bovine TGF-β_1 (kindly provided by P. Segarini, Celtrix) was added to a final concentration of 2 ng/ml at the beginning of the serum addition or at 2-hr intervals after serum stimulation.

DNA Synthesis Measurement. DNA synthesis of the synchronized cells was determined by detection of 5-bromodeoxyuridine (BrdUrd) incorporation into DNA. The cells were grown on glass coverslips and BrdUrd was added with serum.

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Abbreviations: TGF-β_1, transforming growth factor β_1; cdks, cyclin-dependent protein kinases; pRb, retinoblastoma protein; FBS, fetal bovine serum; BrdUrd, 5-bromodeoxyuridine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

The coverslips were taken out at 2-hr intervals after serum restimulation and fixed in 90% ethanol/5% acetic acid/5% water (vol/vol). Labeled cell nuclei were detected with a Cell Proliferation Kit (Amersham). All nuclei were counterstained with Hoechst 33258 (Sigma) at 50 μg/ml.

RNA Extraction and Northern Blot Analysis. Total RNA was extracted by phenol/chloroform treatment and precipitated by an equal volume of 4 M LiCl. Total RNA (25 μg) was resolved by electrophoresis in a formaldehyde/1.2% agarose gel and blotted onto Hybond-N membrane (Amersham) in 10× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The following ^{32}P-radiolabeled human probes were used for hybridization of the filters: the cDNAs of cyclin E, cyclin A, cyclin D1, and cyclin D3 (23); cyclin D2 (24); cdk2 (12); and cdk4 (13). The amount of RNA in each sample was normalized by hybridizing the filter with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA probe or a tubulin cDNA probe. The signals were quantified by densitometric scanning.

Western Blot Analysis. Cells were lysed in ELB buffer containing protease inhibitors (23). The lysates corresponding to 10^6 cells were fractionated on an SDS/10% polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell). For the immunoblotting a monoclonal anti-cyclin E antibody (HE12, cell culture supernatant, a gift from E. Lees, Massachusetts General Hospital, Charlestown) was used as the primary antibody at 1:4 dilution, and a peroxidase-conjugated donkey anti-mouse immunoglobulin (Jackson ImmunoResearch) was used at 1:4000 dilution as the secondary antibody, followed by the detection using the ECL chemiluminescence system (Amersham).

RESULTS

Inhibition of DNA Synthesis by TGF-β_1 in HaCaT Cells. We undertook this work with the HaCaT line of TGF-β_1-responsive human keratinocytes. These cells were synchronized by complete serum deprivation. After 3 days, we found that about 98% of the HaCaT cells were in the quiescent state. After addition of fresh serum to a concentration of 10%, these cells reentered the cell cycle, proceeded through G1 phase, and entered S phase beginning 14 hr later. DNA synthesis peaked between 18 and 22 hr after serum addition, at which time about 50% of the cells were incorporating BrdUrd into DNA (Fig. 1*A*). However, when TGF-β_1 was added together with the serum to a concentration of 2 ng/ml, only 5–8% of the cells entered S phase. Thus, as widely reported, TGF-β_1 is a potent inhibitor of G1 progression in keratinocytes (1, 2).

Time Course of Action of TGF-β_1 in G1. To define more precisely the stage of G1 at which TGF-β_1 exerts its growth-inhibitory effect, TGF-β_1 was added to synchronized cells at different times after serum addition. All cells were collected at 19 hr after serum stimulation, when the number of cells in the S phase normally reached its highest level.

When TGF-β_1 was added at 0–6 hr after serum, subsequent DNA synthesis was reduced to a level similar to that observed when TGF-β_1 was added with fresh serum (Fig. 1*B*). However, with increasing delay between serum stimulation and TGF-β_1 addition, TGF-β_1 gradually lost its ability to inhibit DNA synthesis. Thus, when TGF-β_1 was added 11 hr after serum stimulation, it reduced subsequent DNA synthesis by only 50%. The complete loss of this inhibition was seen when TGF-β_1 was added at 13–15 hr (Fig. 1*B*). Therefore, TGF-β_1 can arrest these cells only if it is added before they reach a point in late G1, which we estimate to be 3 hr before the onset of S phase. This result is compatible with the earlier studies which showed that TGF-β_1 loses its growth inhibition in late G1 (3, 4).

TGF-β_1 Effects on the Expression of G1 Cyclins. Progression of cells through various phases of their cell cycle appears to be controlled by the action of cdks and associated cyclins.

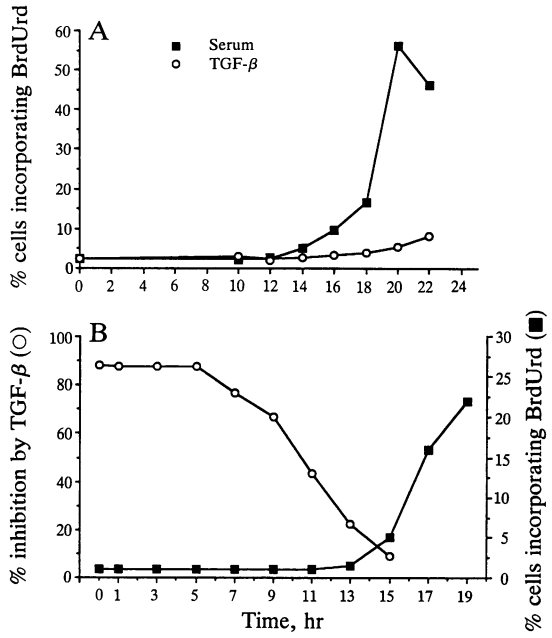

FIG. 1. (*A*) Inhibition of cell cycle progression by TGF-β_1 in HaCaT cells. Serum starvation-arrested cells were stimulated by 10% FBS without or with TGF-β_1 at 2 ng/ml. DNA synthesis was monitored at the indicated time after serum addition by measurement of BrdUrd incorporation. (*B*) Time course of TGF-β_1 growth inhibition in the cell cycle of HaCaT cells. Serum starvation-arrested cells were stimulated as above. TGF-β_1 was then added at each indicated time after serum addition and the cells were harvested at 19 hr. The percentage of inhibition by TGF-β_1 was calculated as follows: % inhibition = $(a - b)/a$, where a is the percentage of BrdUrd-labeled cells stimulated with serum for 19 hr and b is the percentage of labeled cells with TGF-β_1 added at the indicated time (shown on the left axis). The normal cell cycle progression was monitored as above (shown on the right axis).

Given the previously demonstrated inhibition of G1 cell cycle traverse by TGF-β_1 (Fig. 1*A*), we speculated that TGF-β_1 may affect G1 cyclin expression and in this way achieve its inhibitory effects. To examine this possibility, we first determined the expression patterns of G1 cyclins upon mitogen stimulation. HaCaT cells were synchronized as above. Total RNA was prepared from the cells harvested at 2-hr intervals after serum addition and analyzed by Northern blotting.

In G_0, cyclin E and cyclin A mRNAs were undetectable (Fig. 2*A*). Upon serum stimulation, the cyclin E mRNA first appeared at 7–9 hr and reached its peak at 19 hr. The onset of cyclin A expression occurred about 4 hr later than that of cyclin E. Since Fig. 1*A* shows the length of G1 phase to be about 14 hr, we concluded that the appearance of cyclin E mRNA started in mid/late G1 and that the appearance of cyclin A mRNA occurred close to or at the G1/S transition. This is consistent with previously reported results (15).

Cyclin D1 mRNA showed a different pattern of expression (Fig. 2*A*). In contrast to the mRNA levels of cyclins E and A, it was quite abundant in G_0. After serum stimulation, the cyclin D1 mRNA showed a very moderate increase in the following hours and then decreased to its previous, basal, level.

In cells to which TGF-β_1 was added at the time of serum addition, the expression patterns of these various cyclins were affected in very different ways (Fig. 2*A*). Cyclin E mRNA was greatly reduced to an almost nondetectable level; similarly, the induction of cyclin A expression was almost completely blocked. In contrast, TGF-β_1 had a very small effect on cyclin D1 expression.

As there are in fact three types of D cyclins (D1, D2, and D3), we further examined their patterns of expression and

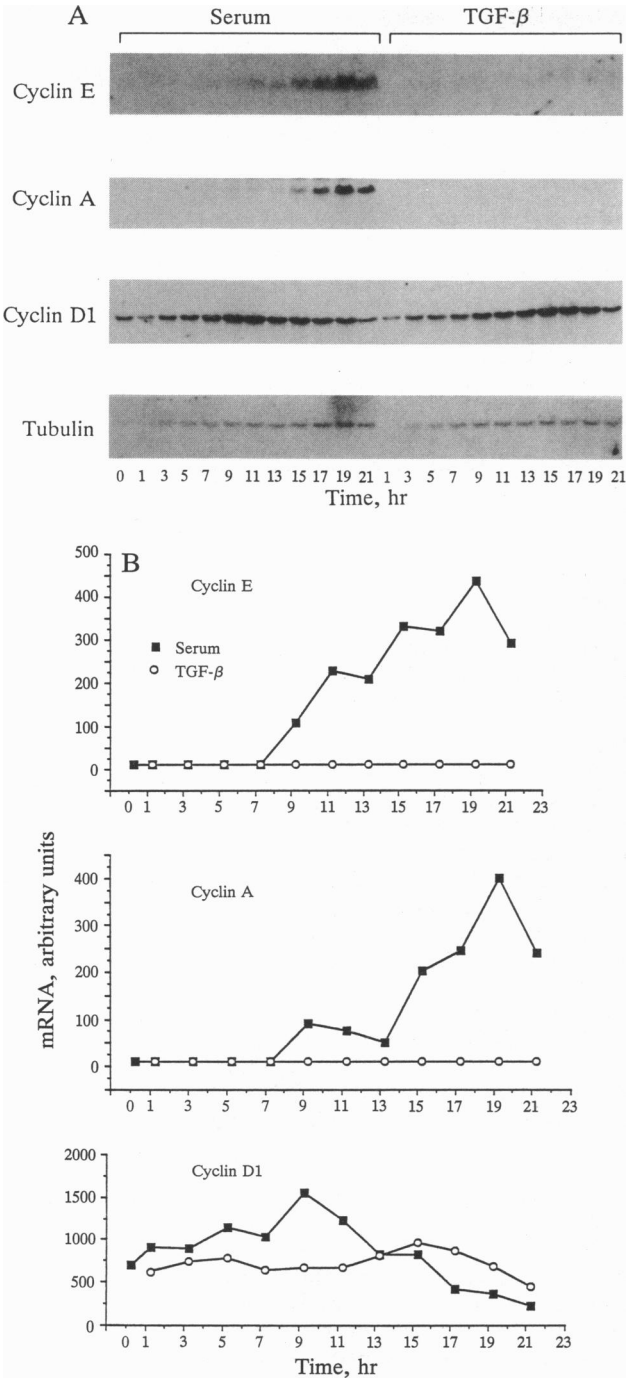

FIG. 2. TGF-β_1 effects on the expression of G1 cyclins. (*A*) The cells were synchronized as described for Fig. 1*A* and harvested at the indicated time after serum stimulation. Total RNA was extracted and Northern blot analyses were performed. Human probes of cyclin E, cyclin A, and cyclin D1 were used sequentially. The samples on the left half of the gels were stimulated with 10% FBS and the samples on the right half of the gels were stimulated with FBS plus TGF-β_1 at 2 ng/ml. (*B*) Expression of cyclins E, A, and D1 normalized to tubulin mRNA by densitometric scanning of the gels in *A*.

responsiveness to TGF-β_1. As shown in Fig. 3, cyclins D1 and D2 mRNAs demonstrated high levels in G_0. Upon serum stimulation both cyclins D1 and D2 mRNAs increased about 2-fold between 6 and 9 hr and TGF-β_1 had very little effect on their expression. The level of cyclin D3 mRNA was very low in G_0 and increased about 2- to 3-fold after 6 hr of serum stimulation. TGF-β_1 also had no effect on cyclin D3 expression.

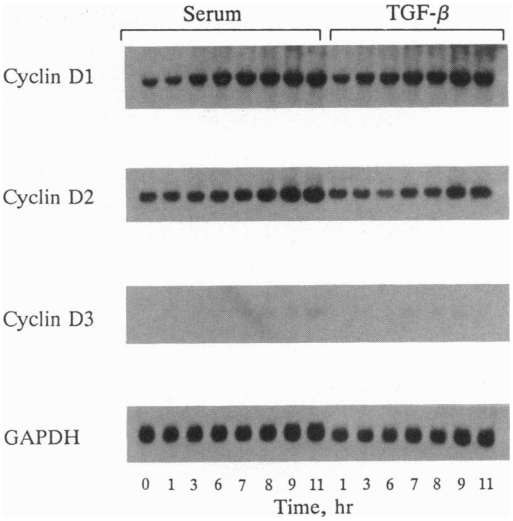

FIG. 3. TGF-β_1 effects on the expression of D cyclins in G1. Northern blot analyses were performed as described for Fig. 2. Human probes of cyclins D1, D2, and D3 were used sequentially.

Comparative Effects of TGF-β_1 on Cyclin E Expression and S Phase Entrance. As shown in Fig. 1*B*, TGF-β_1 can exert its growth-inhibitory effects on HaCaT cells only if added 0–9 hr after serum stimulation; thereafter it rapidly loses its potency. We noted that the time at which TGF-β_1 loses its inhibitory effects coincided approximately with the time of appearance of cyclin E mRNA. Accordingly, we speculated that the expression of cyclin E might define the end point of the period when TGF-β_1 could inhibit G1 progression. For this reason, we proceeded to compare directly the timing of these two G1 events.

To do so, TGF-β_1 was added at different time points after serum stimulation of HaCaT cells, which were then harvested at 19 hr after serum stimulation, a time when cyclin E expression normally reaches its peak. As shown in Fig. 4, the inhibition of cyclin E expression by TGF-β_1 occurred only when TGF-β_1 was added at 0 to 7–9 hr after serum stimulation. Adding TGF-β_1 at later stages of G1 had no effect on the expression of cyclin E (Fig. 4*A*, right part of gels). This result shows that the schedule of loss of inhibition of cyclin E expression by TGF-β_1 is virtually superimposable on that describing the loss of inhibition of DNA synthesis by TGF-β_1 (Fig. 4*B*). For this reason, we concluded that the expression of cyclin E occurs very close in time with the loss of responsiveness of cell cycle progression to TGF-β_1 inhibition.

TGF-β_1 Effects on Cyclin E Protein Level. Because TGF-β_1 prevents the accumulation of cyclin E mRNA, we further analyzed the effects of TGF-β on cyclin E protein levels by Western blot analysis. As shown in Fig. 5, cyclin E protein was induced about 10-fold by serum, starting at 12 hr after serum addition. The time lag between the initial appearance of cyclin E mRNA (Fig. 2) and the substantial increase in cyclin E protein was 3–5 hr. The induction of cyclin E protein was almost completely blocked by adding TGF-β_1 together with serum. Thus, this mid/late G1 increase in cyclin E protein is likely attributable in large part to the increased cyclin E mRNA observed several hours earlier, since both are strongly inhibited by TGF-β.

TGF-β_1 Effects on the mRNA Levels of cdks. Since TGF-β_1 showed very strong inhibition of cyclin E and cyclin A mRNA accumulation but no effect on cyclin D1 mRNA levels, we were interested to determine whether the mRNAs encoding cdks were similarly affected. To this end, the Northern blot used in Fig. 2*A* was probed with cdk2 and cdk4 cDNAs (Fig. 6). Both cdk2 and cdk4 mRNAs showed relatively high basal levels in G_0. Upon serum stimulation, the

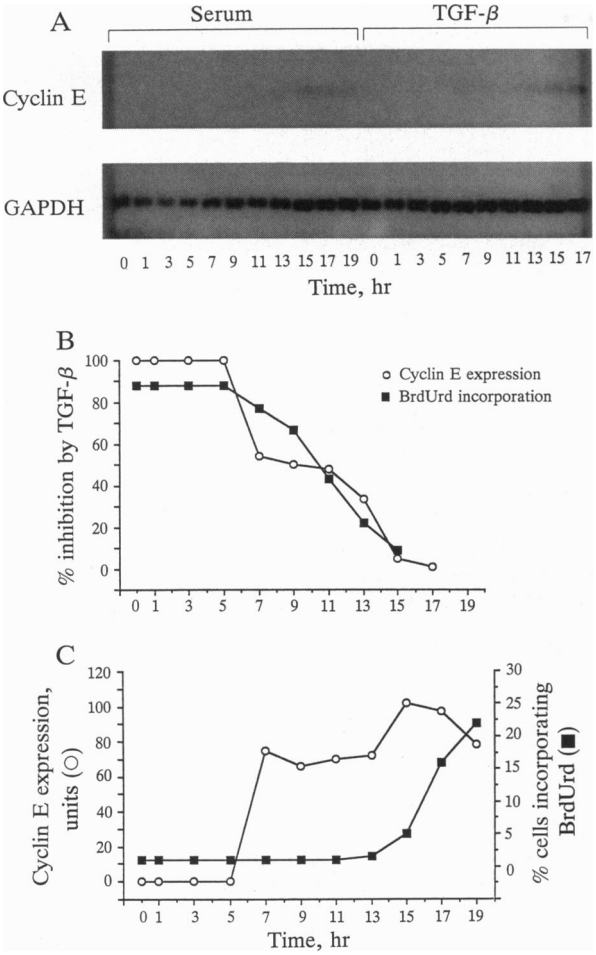

FIG. 4. Loss of inhibition of the expression of cyclin E by TGF-β_1. (*A*) Northern blot analysis was performed with the cells harvested in the experiment shown in Fig. 1*B*. TGF-β_1 (2 ng/ml) was added at the indicated times after serum addition and cells were collected at 19 hr (the right half of the gels). On the left half of the gels the normal cyclin E induction by serum in the absence of TGF-β_1 was measured. (*B*) Time course of TGF-β_1 inhibition of the expression of cyclin E. TGF-β_1 (2 ng/ml) was added at the indicated times after serum stimulation. The relative amounts of cyclin E mRNAs were quantified by densitometric scanning. The percentage of inhibition on cyclin E expression by TGF-β_1 was calculated as follows: % inhibition = 100% − (c/d), where c is the arbitrary units of cyclin E mRNA in TGF-β_1-treated cells and d is the arbitrary units of cyclin E mRNA in serum-stimulated cells analyzed at 19 hr after serum addition. For comparison, the time course of TGF-β_1 inhibition on cell cycle progression, as measured by BrdUrd incorporation, is also shown. (*C*) Normal cyclin E expression pattern in a cell cycle. Serum induction of cyclin E expression in this experiment is normalized to GAPDH mRNA by densitometric scanning and is shown on the left axis. The normal cell cycle progression monitored by BrdUrd incorporation is shown on the right axis.

mRNA levels of cdk2 and cdk4 showed 4-fold increase beginning at 7 hr. In TGF-β_1-treated cells, high induction of both cdk2 and cdk4 mRNAs by serum was blocked.

DISCUSSION

While much research has focused on growth factors, their receptors, and immediate downstream signaling pathways, relatively little is known about the mechanisms through which these peripheral signals impinge upon and control the centrally acting cell cycle clock. One insight has come from an earlier report in which it was demonstrated that, in macrophages, the mitogen colony-stimulating factor 1 is a

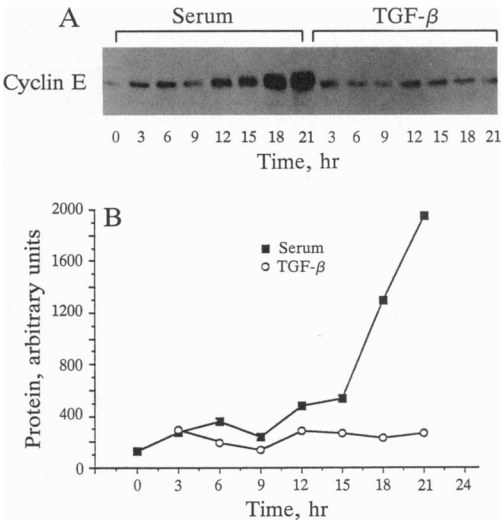

FIG. 5. TGF-β_1 effects on the levels of cyclin E protein. (*A*) Western blot analysis of the levels of cyclin E protein upon TGF-β_1 treatment. Cells were harvested at the indicated time after serum stimulation without TGF-β_1 (left half of the gel) or with TGF-β_1 at 2 ng/ml (right half of the gel). Immunoblotting used a monoclonal anti-cyclin E antibody (HE12). (*B*) The levels of cyclin E protein were quantified by densitometric scanning.

potent inducer of cyclin D1 (26). The current work presents a complementary and distinct set of findings.

As described above, TGF-β can potently inhibit the expression of multiple essential components of the cell cycle apparatus required for G1 transit and S phase entrance, including cdk2, cdk4, and cyclin A. In particular, TGF-β_1 inhibits the appearance of cyclin E at both mRNA and protein levels (Fig. 2 and Fig. 5). Furthermore, the cyclin E expression can be inhibited by TGF-β_1 added at the time of serum addition or for a number of hours thereafter. Once the transcription of cyclin E has begun, TGF-β_1 appears to lose its ability to affect further expression of this gene (Fig. 4*A*).

Simultaneous with this loss of ability to inhibit cyclin E expression is a loss of the ability of TGF-β_1 to inhibit subsequent entrance into S phase (Fig. 4*B*). A simple interpretation of these data would be that the onset of cyclin E expression signals a point in G1 after which all steps in cell cycle progression have lost responsiveness to TGF-β_1 and its inhibitory effects. It may therefore be that the cyclin E protein, together with associated cdk2 kinase (20), establishes a physiologic state in which further cell cycle progression becomes independent of TGF-β_1. We cannot say with certainty that the two events—cyclin E expression and escape from TGF-β inhibition—occur at precisely the same

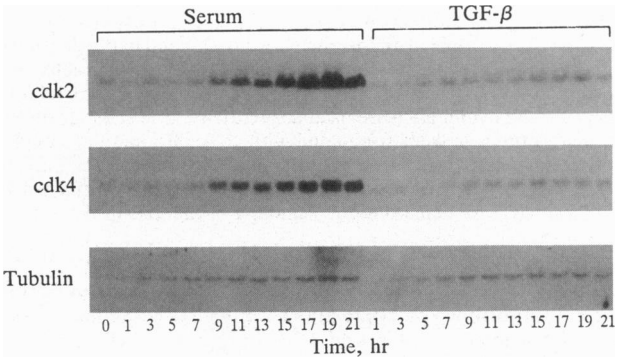

FIG. 6. TGF-β_1 effects on the expression of cdk2 and cdk4. The Northern blot used in Fig. 2*A* was reprobed with human cDNAs of cdk2 and cdk4.

moment in G1. For this reason, it remains possible that the initial increase of cyclin E is followed in short order by other events that result in the final, irreversible, escape from TGF-β's inhibitory effects.

Cyclin E expression occurs at a time in G1 close to that where pRb undergoes hyperphosphorylation and functional inactivation. It may also occur at a time close to that when the cell acquires independence of stimulation by exogenous mitogens. Thus, these various transitions may all be manifestations of passage by the cell through a distinct point in the cell cycle termed the R or restriction point by Pardee (27, 28).

As TGF-β_1 has been shown to prevent pRb hyperphosphorylation in other cell types (3, 29), we confirmed that addition of TGF-β_1 to HaCaT cells also prevents hyperphosphorylation of pRb (data not shown). pRb hyperphosphorylation in these cells occurs around 13 hr after serum stimulation. The appearance of hyperphosphorylated pRb coincides with the initial increase of cyclin E protein (Fig. 5), which together with several other lines of evidence suggests that cyclin E in association with cdk2 kinase is responsible for this hyperphosphorylation of pRb in mid/late G1 phase (V. Dulic, S. Dowdy, and S. I. Reed, personal communication) (23). In the presence of TGF-β_1, the hyperphosphorylation of pRb does not occur; the induction of cyclin E mRNA and cyclin E protein is also blocked (Fig. 2 and Fig. 5). Therefore, we consider it highly likely that the inhibition of pRb hyperphosphorylation in HaCaT cells by TGF-β_1 is attributable largely and perhaps totally to its ability to prevent cyclin E appearance.

The results of Fig. 2 show that TGF-β_1 blocks the induction of cyclin A expression completely as it was reported by others (30). We have demonstrated here that in HaCaT cells cyclin A expression begins at the G1/S border, while TGF-β_1 loses its inhibitory effects on cell cycle progression in mid/late G1, 2–3 hr before the onset of cyclin A expression. It is likely, therefore, that the inhibition of cyclin A expression by TGF-β_1 is a consequence of the blockage of earlier mid/late G1 steps such as prevention of cyclin E expression.

The D-type cyclin mRNAs show patterns of expression that are very different from those of cyclins E and A (Fig. 3). Thus, the levels of D cyclin mRNAs are only weakly modulated by serum in these cells, in contrast to the effects of mitogens observed in other cell types (21, 25, 26, 31, 32). Such differences are likely attributable to specific responses that vary from one cell type to another. Moreover, the mRNA levels of three D-type cyclins were not substantially affected by the presence of TGF-β_1.

We have also examined the levels of the mRNAs encoding cdk2 and cdk4 subunits of the G1 cyclins. As shown in Fig. 6, both mRNAs were induced by serum, and the inductions were greatly inhibited upon TGF-β_1 addition. However, the activities of the cdk subunits are dependent on their cyclin partners (14, 33, 34), suggesting that the rate-limiting factor in kinase activity is often determined by levels of a cyclin rather than of a kinase. Thus, we propose that the effects of TGF-β_1 on cell cycle progression of these cells are primarily due to its inhibition at the level of cyclin gene expression.

Others have recently reported a second, quite distinct, mechanism by which TGF-β blocks cell cycle progression. Koff *et al.* (35) have found that TGF-β blocks the formation of productive complexes between cyclin E and cdk2 in cells that already express cyclin E. This difference is probably due to the different experimental systems used. The mink lung epithelial cells used in their studies were synchronized by contact inhibition, while the human keratinocytes used in our studies were synchronized by serum starvation. The two synchronization methods could result in different cell-arresting stages, which may lead to different expression levels of cyclin E. Despite these differences, a synthesis of their work and the present data would suggest that TGF-β can block the cell cycle progression by two distinct mechanisms. If it is applied in early/mid G1, TGF-β acts to prevent cyclin E expression. Once cyclin E is expressed, TGF-β may then act to prevent the formation of active cyclin·cdk complexes.

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