Journal of Cellular Biochemistry 48:305–315 (1992)

TGF-β_1 Inhibits DNA Synthesis and Phosphorylation of the Retinoblastoma Gene Product in a Rat Liver Epithelial Cell Line

Robert H. Whitson, Jr., and Keiichi Itakura

Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, California 91010

Abstract In the rat liver epithelial cell line, WB, the ability of TGF-β_1 to inhibit DNA synthesis was shown to correlate with its ability to inhibit phosphorylation of the protein product of the retinoblastoma susceptibility gene, pRb. When WB cells were serum-starved, then refed with serum-containing medium, a peak of DNA synthesis occurred at about 18 h. Autoradiographs showed that 43.6% of cell nuclei could be labeled with ^{3}H-thymidine at this time. When TGF-β_1 was added simultaneously with serum, it blocked DNA synthesis and reduced the number of labeled nucleii to 6.3%. Cells treated with serum alone for 18 h also showed a pronounced increase in the highly phosphorylated form of pRb, as shown by mobility shifts in immunoblots, and in active phosphorylation of pRb, as shown by ^{32}P incorporation. Simultaneous addition of TGF-β_1 with serum abolished both ^{32}P incorporation into pRb and its mobility shift on immunoblots. The effect of TGF-β_1 on DNA synthesis measured at 18 h was sharply reduced if the cells were incubated with serum for 8 h (and thus allowed to enter S) before the addition of TGF-β_1. If TGF-β_1 was added after 8 h of serum treatment, its ability to inhibit pRb phosphorylation at 18 h was unchanged. If TGF-β_1 was added after 13 h of serum treatment, its effects on pRb phosphorylation were reduced. Thus, as the cell population moved into S, the ability of TGF-β_1 to inhibit both pRb phosphorylation and DNA synthesis was lost. In higher passages of WB cells the dose-response for inhibition of DNA synthesis by TGF-β_1 was shifted to the right. Inhibition of pRb phosphorylation by TGF-β_1 was also lost in higher passage WB cells. Thus, the passage-dependent loss of sensitivity to inhibition of DNA synthesis accompanied the loss of sensitivity to inhibition of pRb phosphorylation. Since the phosphorylation of pRb is believed to be required for the progression of cells from G_1 to S, inhibition of pRb phosphorylation may be either a cause or a consequence of the G_1 arrest of WB cells by TGF-β_1.

Key words: TGF-β_1, retinoblastoma susceptibility protein, cell cycle, growth inhibitors

In recent years, there has been increasing interest in negative regulation of cell growth. Inhibitory growth factors and tumor suppressor genes have both proved to be important elements of negative growth regulation. A possible functional interaction between these two elements, represented by transforming growth factor β_1 (TGF-β_1) and the protein product of the retinoblastoma susceptibility gene (pRb), has attracted increasing interest. TGF-β_1 is known to exert a variety of effects in vitro, depending on the cell types studied and the specific growth conditions used. TGF-β_1 either stimulates or inhibits differentiation of myoblasts, adipoblasts, bronchial epithelial cells, hematopoietic stem cells, and a variety of lymphoid cells. TGF-β_1 also stimulates production and transport of extracellular matrix proteins and promotes cellular adhesion to these proteins [reviewed in 1,2]. In addition, TGF-β_1 (and its closely related analogues TGF-β_2 and TGF-β_3) are among the most potent known inhibitors of cell division in epithelial cells. Despite this wealth of information about the nature of TGF-β_1's activities, very little is known of its mechanisms of action. In lung epithelial cells and keratinocytes, TGF-β_1 appears to arrest growth in G_1 [3,4] and probably acts through the type I TGF-β receptor [5], but the intracellular factors which mediate this effect have yet to be identified.

The retinoblastoma susceptibility gene (Rb), as its name implies, was first discovered as a gene in which mutational inactivation led to the

Received August 23, 1991; accepted November 15, 1991.

Address reprint requests to Dr. Robert H. Whitson, Jr., Department of Molecular Genetics, Beckman Research Institute of the City of Hope, Duarte, CA 91010.

development of ocular tumors in children [6,7]. The finding that the Rb gene was inactivated in other tumor types [8] and is expressed in normal cells [9] led to the suggestion that the product of the retinoblastoma gene is a tumor suppressor. This was shown directly when transfection of the gene into retinoblastoma cells led to a suppression of the transformed phenotype [10]. The protein product of the Rb gene, pRb, is a 105 kDa nuclear protein which undergoes cell-cycle-dependent phosphorylation and de-phosphorylation [11–14]. pRb is least phosphorylated in G_1, becomes multiply phosphorylated near the G_1/S boundary, and remains highly phosphorylated through G_2 and into M. pRb is then dephosphorylated as the cell moves into late M or early G_1 [11–14]. Phosphorylation of pRb alters its mobility on polyacrylamide gels, allowing the overall phosphorylation state of the pRb pool to be assessed in Western blots [11–14].

At least three protein products of viral oncogenes are known to bind to pRb. They are: the large T antigen of SV-40 [15,16], the E7 protein of human papilloma virus 6 [17], and the EIA protein of adenovirus [18–19]. The ability of these oncoproteins to transform efficiently depends upon their ability to bind pRb [14–17]. Moreover, large T antigen binds only to the underphosphorylated form of pRb [15,16]. These facts, taken together, suggest that pRb (and specifically the underphosphorylated form) may be a regulator of the cell cycle [16]. Thus, alteration of pRb, either by phosphorylation or binding to viral (or cellular) proteins, may be necessary to allow progression of the cell from G_1 into S.

In this paper, we report studies that show a correlation between inhibition of pRb phosphorylation and inhibition of DNA synthesis by TGF-β_1 in the WB rat liver epithelial cell line. While this work was completed, similar results were reported for keratinocytes and mink lung epithelial cells [4].

MATERIALS AND METHODS

Cells

WB-F334 [20] cells were obtained at passage 9 from Brian I. Carr at the City of Hope. The cells were maintained in Richters Improved Minimal Essential Medium (Irvine Scientific) plus 10% fetal bovine serum (Gibco). To arrest cell growth in G_1, cells were grown to 50%–70% of confluency, then placed in the above medium without fetal bovine serum for 6 days.

^{3}H-Thymidine Uptake Measurements

Cells were grown in six-well plates (9.6-cm^2 wells) and serum-arrested as described above. The cells were refed with 2 ml of medium containing 10% serum for the times indicated in the figures. ^{3}H-thymidine was added at a concentration of 1 μCi/ml for 2 or 3 h, then the medium was removed and the cells were washed two times with 2 ml of Hanks buffer. 0.5 ml of a solubilizing buffer (20 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100) was added and the plates were shaken vigorously at room temperature for 20 min. The plates were then scraped with a teflon scraper and the extracts were collected into glass test tubes. 1.5 ml of 10% TCA was added, the tubes were placed on ice for 20 min, and the mixture was then filtered through Whatman GF/C glass fiber filters. The filters were washed twice with ice-cold 10% TCA, dried overnight, and counted by liquid scintillation in Ecolume (ICN).

Nuclear Labeling Studies

Cells were grown in 3.5-cm dishes and starved and refed as described above, then incubated with 1.0 μCi ^{3}H-thymidine for 3 h. The cells were rinsed once with 2 ml PBS, then 2 ml of ice-cold 10% TCA was added and the dishes were placed on ice for 20 min. The dishes were washed five times with ice-cold 10% TCA, then twice with 2 ml ice-cold 70% ethanol, and finally with 2 ml 95% ethanol. The plates were allowed to dry overnight, then coated with Kodak NTE2 nuclear track emulsion and placed in the dark for 3 days. The emulsion was developed with Kodak D19 developer. The cells were counterstained with methyl green and the positive nucleii were counted.

Analysis of Retinoblastoma Protein Phosphorylation

These analyses were performed essentially according to the methods obtained from the suppliers of monoclonal antibodies to pRb (Pharmigen, San Diego). WB-F344 cells were grown in T-75 tissue culture flasks, and serum-starved or starved and refed in the presence or absence of TGF-β_1 as indicated in the figure legends. The cell monolayers were washed twice with 25 ml of sterile tris-buffered saline (TBS) at pH 7.4, then incubated for 30 min at 37°C in 10 ml of phosphate-free Richter's medium with or without 10% dialysed FBS. Seven milliliters of this me-

dium were removed, 0.5 mCi of ^{32}P-orthophosphate was added to each flask, and the incubation at 37°C was continued for a further 3 h. The medium was then withdrawn and the cell monolayers were washed twice with TBS. One milliliter of lysis buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP.40, 0.2% SDS, 1 mM phenylmethyl sulfonyl fluoride (PMSF) 50 μg/ml aprotinin and 50 μM leupeptin) was added to each flask and the flasks were shaken vigorously on ice for 5 min. The viscous extract was scraped from the flasks with teflon scrapers and pipetted into 1.5 ml microfuge tubes. The tubes were placed on ice for 30 min with vigorous vortexing every 10 min. The lysates were centrifuged and the supernatant was removed to a clean microfuge tube. Five micrograms of purified monoclonal antibody to pRb (PMG3-245.11, Pharmigen, San Diego, California) were added to each tube and the tubes were incubated on ice for 30 min. For each sample to be precipitated, 10 μg of protein A-Sepharose (Sigma) was swollen in 1 ml lysis buffer in a microfuge tube. The beads were pelleted, then resuspended in 80 μl of lysis buffer, and added to 10 mg of rabbit anti-mouse IgG_1 (Pharmigen). The tubes were mixed end-over-end at 4°C for 1 h. The beads were pelleted and washed three times in lysis buffer and 800 ml of anti-pRb-treated cell extract was then added to the beads. After mixing end-over-end for 1 h at 4°C, the beads were pelleted, then washed five times in wash buffer (25 mM Tris, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 0.2% NP40, 1 mM PMSF). The beads were then resuspended and boiled for 5 min in 100 μl of SDS sample loading buffer (1% SDS, 4 M urea, 0.1 M dithiothreitol in 0.01 M sodium phosphate pH 7.0). The beads were then centrifuged in a microfuge and the supernatant was either frozen or applied directly to polyacrylamide gels.

For potato acid phosphatase treatment, the beads were washed twice in 1 ml of 100 mM MES buffer (pH 6.0). The beads were incubated with 2 units of potato acid phosphatase (Boeringer Mannheim) at 37°C for 15 min. The beads were then washed twice and treated with SDS sample buffer as above.

The immunoprecipitated extracts were applied to 5%–10% linear gradient polyacrylamide gels containing 0.1% SDS [21]. The resolved proteins were transferred electrophoretically to nitrocellulose [22] at 80 V for 2 h at 4°C. The nitro cellulose was washed once with TBST (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% tween 20) then shaken overnight at 4°C in TBST plus 4% dry nonfat milk and 0.1% NaN_3 (blocking buffer). The blot was then incubated for 2 h at 4°C in the same buffer minus NaN_3, containing 10 μg/ml purified anti-pRb monoclonal antibody. The blot was washed three times for 15 min at 4°C in 50 ml blocking buffer without NaN_3. The blot was then incubated in the same buffer with a 1:100 dilution of peroxidase-conjugated goat anti-mouse IgG (Boehringer-Mannheim) at room temperature for 60 min. The blot was washed at room temperature twice for 15 min with 50 ml of blocking buffer without NaN_3 and three times with 50 ml of TBST. The peroxidase conjugated antibody complex was then visualized by incubating for 5 min at room temperature with 50 ml of phosphate-buffered saline containing 25 mg 3,3′diaminobenzidine, 1 ml 1% $CoCl_2$ and 0.1 ml 30% H_2O_2. The reaction was stopped by washing with water.

In some cases, bands on the Western blots were analyzed by video densitometry using a Biovision video densitometer. The nitrocellulose image was acquired by the video camera after calibration with a gray reflectance scale. The intensities of the diffuse 114–116 kDa phosphorylated pRb bands in each lane were normalized to the intensities of the underphosphorylated 105 kDa pRb bands.

TGF-β_1 was purified from outdated human platelets (donated by the Red Cross) by a modification [23] of the method of Assoian et al. [24].

RESULTS

TGF-β_1 Blocks ^{3}H-Thymidine Incorporation in Serum-Starved and Refed WB Cells

When WB cells at passage 15 were serum-starved for 6 days, then refed with serum, incorporation of ^{3}H-thymidine was detectable by 12 h and reached maximum at 18–20 h (Fig. 1). A moderate dose of TGF-β_1 (200 pM) substantially reduced the incorporation of ^{3}H-thymidine, apparently blocking the entry of cells into S phase. TGF-β_1 did not completely block the incorporation of ^{3}H-thymidine, however. When the data in Figure 1 are replotted to show ^{3}H-thymidine incorporation as a percent of the maximal incorporation, the plots for cells treated with serum and serum plus TGF-β_1 superimpose (not shown). Similar results were obtained using autoradiographic labeling of cell nuclei. In serum-starved cells only 0.34 ± 0.76% of nucleii were labeled with ^{3}H-thymidine. Eighteen hours of

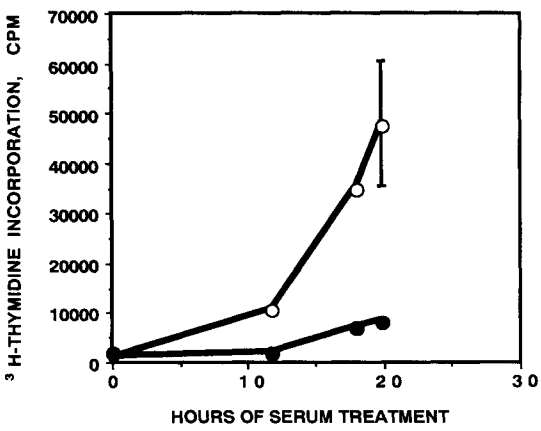

Fig. 1. TGF-β_1 inhibits entry of WB cells into S. WB cells at passage 15 were serum-starved for 6 days then refed for 18 h with 10% fetal bovine serum in the presence (closed circles) or absence (open circles) of 200 pM TGF-β_1. In the last 3 h of the incubation period, ^{3}H-thymidine was added and incorporation of acid-insoluble ^{3}H was measured. Values are means ± S.D. of triplicate determinations.

serum retreatment stimulated labeling to 43.6 ± 9.3% in the absence TGF-β_1, but only 6.3 ± 2.8% in the presence of TGF-β_1. (Values are means ± S.D. of five counts of 100 cells each in randomly selected fields. Cells were at passage 19.)

TGF-β_1 Inhibits the Effects of Serum on pRb Phosphorylation

Phosphorylation of the retinoblastoma protein has been shown to retard its mobility on SDS polyacrylamide gels [11,12]. We took advantage of this fact to assess the effects of serum and TGF-β_1 on the phosphorylation state of the overall pool of pRb in WB cells. We also monitored ^{32}P incorporation into pRb to assess the active phosphorylation of pRb over shorter time periods. Serum treatment was carried out for 21 h, and ^{32}P incorporation was carried out during the last 3 h of serum treatment. In serum-starved cells pRb is detected on the Western blot (Fig. 2, left), as a single 105 kDa band. On the

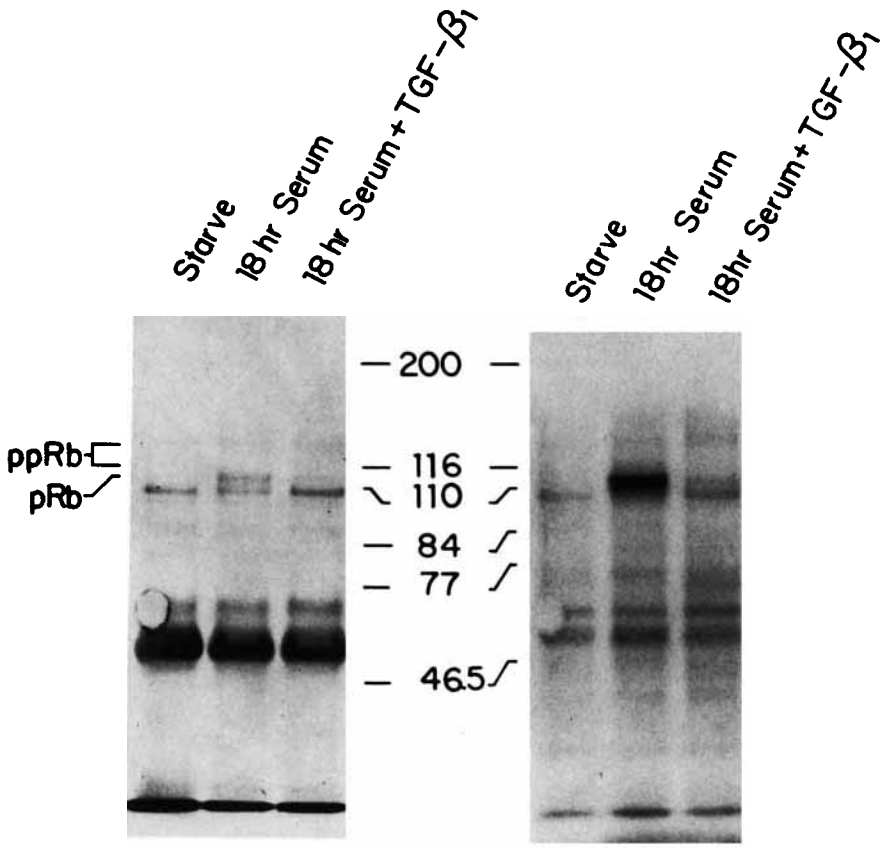

Fig. 2. TGF-β_1 inhibits the phosphorylation of pRb in serum-stimulated WB cells. WB cells at passage 15 were serum-starved, then refed with serum for the indicated times in the presence or absence of 200 pM TGF-β_1. The cells were then incubated for an additional 3 h with ^{32}P. Cells were lysed and immunoprecipitates were prepared, resolved on gels, and immunoblotted as described in the text. Immunoblots are shown in the left panel and autoradiographs of the same blots are shown in the right panel.

TABLE I. Relative Intensities of Phosphorylated and Underphosphorylated pRb in WB Cells After Serum and TGF-β_1 Treatments*

Treatment	Band intensity (mm^2 OD) 114–116 kDa	105 kDa	Ratio 114–116 kDa/105 kDa
Experiment I[1]			
Starved	289	3,620	0.08
21 h serum	2,724	2,282	1.19
21 h serum + TGF-β_1	1,138	4,178	0.27
Experiment II[2]			
Starved	6,155	27,946	0.22
21 h serum	18,188	24,752	0.73
21 h serum + TGF-β_1 0–21 h	4,147	28,293	0.15
21 h serum + TGF-β_1 4–21 h	5,394	26,281	0.20
21 h serum + TGF-β_1 8–21 h	4,077	19,294	0.21
21 h serum + TGF-β_1 13–21 h	8,344	20,237	0.41
Experiment III[3]			
Passage 19			
Starve	29	1,490	0.02
21 h serum	386	893	0.43
21 h serum + 50 pM TGF-β_1	46	1,090	0.04
21 h serum + 200 pM TGF-β_1	102	1,678	0.06
Passage 40			
Starve	95	1,979	0.05
21 h serum	1,613	1,685	0.95
21 h serum + 50 pM TGF-β_1	734	1,323	0.55
21 h serum + 200 pM TGF-β_1	1,128	2,317	0.49

*On the Western blots shown in Figures 2, 4, and 7, the bands representing the underphosphorylated (105 kDa) and highly phosphorylated (114–116 kDa) forms of pRb were analyzed by a video densitometer.
[1]These data are from a densitometric scan of the orginal Western blot shown in Figure 2.
[2]These data are from the Western blot shown in Figure 4.
[3]These data are from the Western blot shown in Figure 7.

autoradiograph (Fig. 2, right), it can be seen that very little ^{32}P is incorporated into this band. In extracts of serum-treated cells, about half of the material staining with antibodies to pRb now migrates as a band of 114–116 kDa. Using a video densitometer, we found that serum increased the intensity of the 114–116 kDa band relative to that of the 105 kDa band by more than tenfold (Table I, Experiment I). This slower migrating band coincides with an intense band of ^{32}P incorporation on the autoradiograph. Thus, after 21 h of serum treatment, there is a shift in the pool of pRb to the more highly phosphorylated state, and pRb is actively phosphorylated from 18–21 h of serum treatment. From Figure 1, it can be seen that the WB cells are in S phase at 18–20 h after serum treatment. Thus, our results showed that entry into S is associated with phosphorylation of pRb. TGF-β_1 not only reduced the incorporation of ^{32}P into pRb during the 3-h labeling period (Fig. 2, right), it also decreased the phosphorylation of the overall pool of pRb (Fig. 2, left). By densitometry, it was seen that TGF-β_1 decreased the relative intensity of the 114–116 kDa band by 75% (Table I).

TGF-β_1 Does Not Inhibit ^3H-Thymidine Incorporation or pRb Phosphorylation After WB Cells Enter S

In order to examine the temporal correlation between TGF-β_1 addition and inhibition of ^3H-thymidine in WB cells, we first examined the time-course of ^3H-thymidine incorporation in somewhat greater detail (Fig. 3A). ^3H-thymidine incorporation was measurable by 8 h after serum addition and reached a broad peak with a maximum at about 16 h. Using replicate cultures of these cells, we added TGF-β_1 at various times after serum addition and tested ^3H-thymidine incorporation from 18–20 h after serum

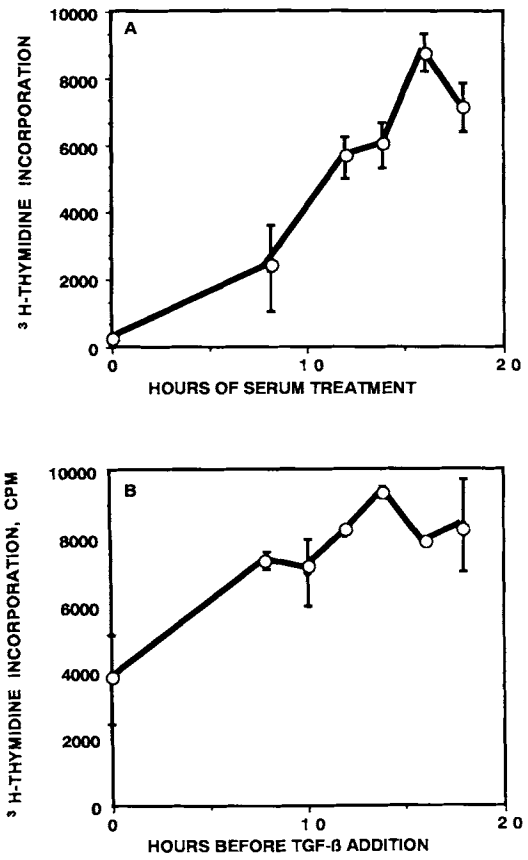

Fig. 3. TGF-β_1 does not inhibit DNA synthesis in WB cells after they enter S phase. **A:** WB cells were serum-arrested, then refed with serum for the indicated times. ^{3}H-thymidine was added during the last 2 h of each time period. **B:** WB cells were serum-arrested and refed with serum at time 0. TGF-β_1 was added at the indicated times, and the incubations were continued for a total of 18 h. ^{3}H-thymidine was added during the last 2 h of the incubation period. Values are means ± S.D. of triplicate determinations.

addition. If TGF-β_1 is added 8 h after serum, its effect on ^{3}H-thymidine incorporation is reduced by about 50%, compared to the effect found when TGF-β_1 is added at time zero. After the cells were exposed to serum for 14 h, the effects of TGF-β_1 were completely eliminated. The results suggest that in WB cells, the effects of TGF-β_1 are diminished once the cells enter S. Thus, there is a general correlation between the *onset of* ^{3}H-thymidine incorporation and the loss of response to TGF-β_1.

There was a similar time window for the ability of TGF-β_1 to inhibit phosphorylation of pRb. TGF-β_1 decreased the ^{32}P labeling of pRb when added 4 or 8 h after serum to about the same extent as when it was added simultaneously with serum (Fig. 4, right). When TGF-β_1 was added 13 h after serum, its effect on ^{32}P incorporation was substantially diminished. The time-related differences in the effects of TGF-β_1 seemed less pronounced when the overall pRb pool was examined in the Western blot (Fig. 4, left). The effects seen when TGF-β_1 was added 13 h after serum are not readily distinguishable from those seen when TGF-β_1 was added after 0–8 h of serum. However, if the intensity of the phosphorylated pRb bands on the Western blot were compared using a video densitometer, there were measurable differences (Table I, Experiment II). The relative intensity of phosphorylated pRb was equal in immunoprecipitates from serum-starved cells and from cells treated with serum plus TGF-β_1 at 0, 4, or 8 h. The intensity increased fourfold in cells treated with serum alone and twofold in serum plus TGF-β_1 at 13 h. Thus, the ability of TGF-β_1 to produce measurable changes in the total pool of phosphorylated pRb was diminished between 8 and 13 h of serum treatment.

When the immunoprecipitates from cells treated with serum alone were incubated with potato acid phosphatase, ^{32}P was completely removed from pRb (Fig. 4, right). The band corresponding to phosphorylated pRb also disappeared from the Western blot, and even the mobility of the underphosphorylated form of pRb appeared to increase (Fig. 4, left). This is consistent with our contention (and with the results of many others) that incorporation of phosphate retards the migration of pRb.

The Effects of TGF-β_1 on ^{3}H-Thymidine Incorporation and pRb Phosphorylation Are Diminished in Higher Passage WB Cells

It has been previously reported that as WB cells undergo serial passage, they become resistant to TGF-β_1 [20]. We also observed TGF-β_1 resistance in late passage WB cells. When passage 29 WB cells were refed with serum in the presence of 200 pM TGF-β_1, ^{3}H-thymidine incorporation was inhibited by only 35% at the peak time (Fig. 5A) compared to the 85% inhibition obtained in passage 15 cells (Fig. 1). This reduced sensitivity to TGF-β_1 persisted to at least passage 40 cells in which 200 pM TGF-β_1 inhibited ^{3}H-thymidine incorporation by 32% (Fig. 5B). In order to more fully characterize this TGF-β_1 resistance, we refed serum-starved WB cells of passage 20 or passage 39 in the presence of increasing doses of TGF-β_1 and measured ^{3}H-thymidine incorporation (Fig. 6). It can be

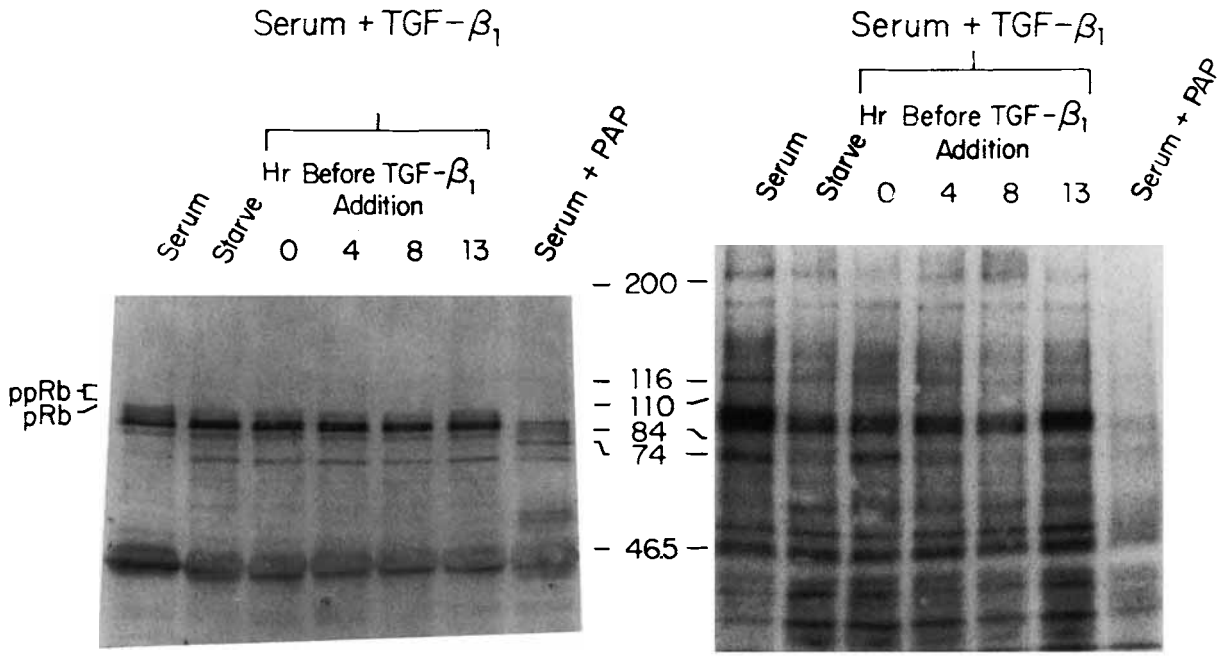

Fig. 4. The ability of TGF-β_1 to inhibit pRb phosphorylation is reduced after prolonged serum incubation. WB cells at passage 16 were serum-starved and refed and TGF-β_1 was added at the indicated times. The serum incubation was continued for a total of 18 h. The cells were incubated with ^{32}P-orthophosphate for an additional 3 h, then lysed and immunoprecipitated. Immunoprecipitates were applied to polyacrylamide gels, then transferred to nitrocellulose. Immunoblots are shown on the left and autoradiographs of the immunoblots are shown on the right.

seen that passage 39 cells require larger doses of TGF-β_1 than passage 20 cells to achieve significant inhibition of ^{3}H-thymidine incorporation. Substantial inhibition is obtained with 500 pM TGF-β_1 in the passage 39 cells, however. This "right-shift" in the dose response of the higher passage cells indicates a decreased sensitivity to TGF-β_1, rather than an outright loss of response.

The sensitivity of pRb phosphorylation to TGF-β_1 inhibition was also decreased in higher passage cells. When passage 19 cells were refed with serum in the presence of 50 pM TGF-β_1, ^{32}P incorporation into pRb was effectively blocked (Fig. 7, right). In contrast, passage 40 cells refed with serum in the presence of 50 pM or 200 pM TGF-β_1 showed only slight decreases in the ^{32}P labeling of pRb. On the Western blot, similar results were observed on the relative effects of TGF-β_1 on the overall phosphorylation state of pRb in the two cell passages. The results of vidio-densitometric scans of these bands are presented in Table I (Experiment III). In passage 19 cells, 50 pM TGF-β_1 gave a substantial reduction in the intensity of the highly phosphorylated 114 kDa pRb band. In passage 40 cells, 50 pM and 200 pM TGF-β_1 reduced the intensity of this band by about 50%, relative to the intensity seen in the presence of serum alone.

DISCUSSION

In the rat liver epithelial cell line, WB, we observed a general correlation between the ability of TGF-β_1 to inhibit DNA synthesis and its ability to inhibit phosphorylation of pRb. In starved and serum-refed cells at low passage, TGF-β_1 inhibited ^{3}H-thymidine incorporation and pRb phosphorylation when added simultaneously with serum. If TGF-β_1 was added after the cells entered S, TGF-β_1 inhibited neither ^{3}H-thymidine incorporation nor pRb phosphorylation. At higher passages, the cells became less sensitive to TGF-β_1, and its effects on ^{3}H-thymidine incorporation and its effects on pRb phosphorylated were diminished. Thus, the correlation between these two actions of TGF-β_1 remained intact through two different experimental manipulations.

Serum refeeding of starved WB cells substantially increased phosphorylation of pRb at times when ^{3}H-thymidine incorporation was maximal, an observation consistent with those of many others [11–14] that pRb is phosphorylated in S. The serum-induced increase in ^{32}P incorpora-

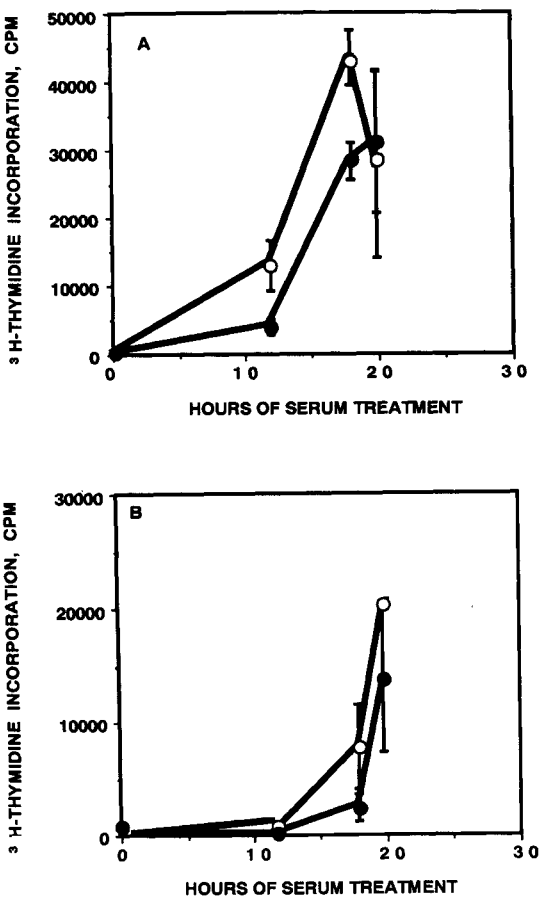

Fig. 5. The ability of TGF-β_1 to block DNA synthesis of WB cells is diminished at higher passages. WB cells at passage 29 (A) or passage 40 (B) were serum-starved and refed with serum for the indicated times in the presence (closed circles) or absence (open circles) of 200 pM TGF-β_1. ^{3}H-thymidine incorporation was measured during the last 3 h of serum treatment. Values are means ± S.D. of triplicate determinations.

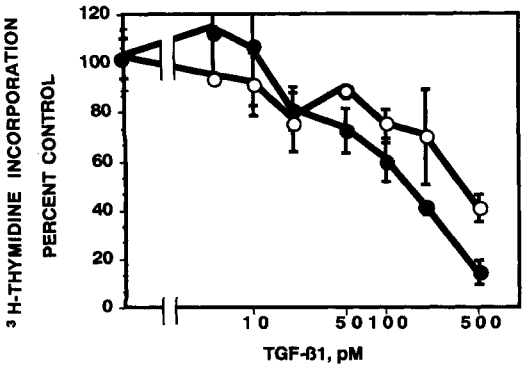

Fig. 6. The dose-response for TGF-β_1 inhibition of DNA synthesis is shifted in WB cells at higher passages. WB cells at passage 20 (closed circles) or passage 39 (open circles) were serum-starved, then refed in the presence of the indicated concentrations of TGF-β_1. ^{3}H-thymidine incorporation was measured at 18–21 h after addition of serum.

tion in this time period (18–21 h following serum addition) indicated that active phosphorylation of pRb was taking place. Simultaneous addition of TGF-β_1 and serum abolished both the shift in the phosphorylation state of pRb observed on the Western blot and the active phosphorylation observed by ^{32}P incorporation. The mechanism by which this occurs is not clear. Since TGF-β_1 does not appear to stimulate dephosphorylation of pRb once S phase has begun (cf. Fig. 4 and [4]), it is tempting to speculate that the effect of TGF-β_1 is to inhibit the pRb kinase rather than to stimulate a pRb phosphatase. The resolution of this question, however, will await the identification of these enzymes, and a determination of the effects of TGF-β_1 on their activities.

When WB cells were exposed to serum for increasing times before the addition of TGF-β_1 its effects on both ^{3}H-thymidine incorporation and pRb phosphorylation were diminished (Figs. 3, 4). This correlation was not absolute, however. When TGF-β_1 was added after 8 h of serum retreatment, ^{3}H-thymidine incorporation at 16–18 h was only slightly inhibited. TGF-β_1 did, however, inhibit pRb phosphorylation when added 8 h after serum. In fact, the effect of TGF-β_1 on pRb phosphorylation was the same whether it was added simultaneously with serum or 8 h following serum. At 13 h postserum, TGF-β_1 had no effect on ^{3}H-thymidine incorporation and a diminished effect on pRb phosphorylation. Thus, the cells appear to lose the ability to decrease pRb phosphorylation in response to TGF-β_1 after they lose the ability to decrease ^{3}H-thymidine incorporation. This suggests that the effects of TGF-β_1 on these two processes are not rigidly coupled.

It has been previously reported that TGF-β_1 arrests WB cells at two different points in the cell cycle: in early G_1 and at the G_1/S border [20]. If the cells serum-arrest in early G_1 in our experiments, then TGF-β_1 may keep the cells arrested somewhere in G_1 when added simultaneously with serum. With longer periods of serum exposure, the cell population would move past the first point of TGF-β_1 arrest (early G_1), so that TGF-β_1 could arrest the cells only at the G_1/S border. The fact that TGF-β_1 inhibits pRb phosphorylation more efficiently than it inhibits ^{3}H-thymidine incorporation when added after 8–13 h of serum exposure could imply that it must act in early G_1 to arrest DNA synthesis, but can act at the G_1/S border to arrest pRb phosphoryla-

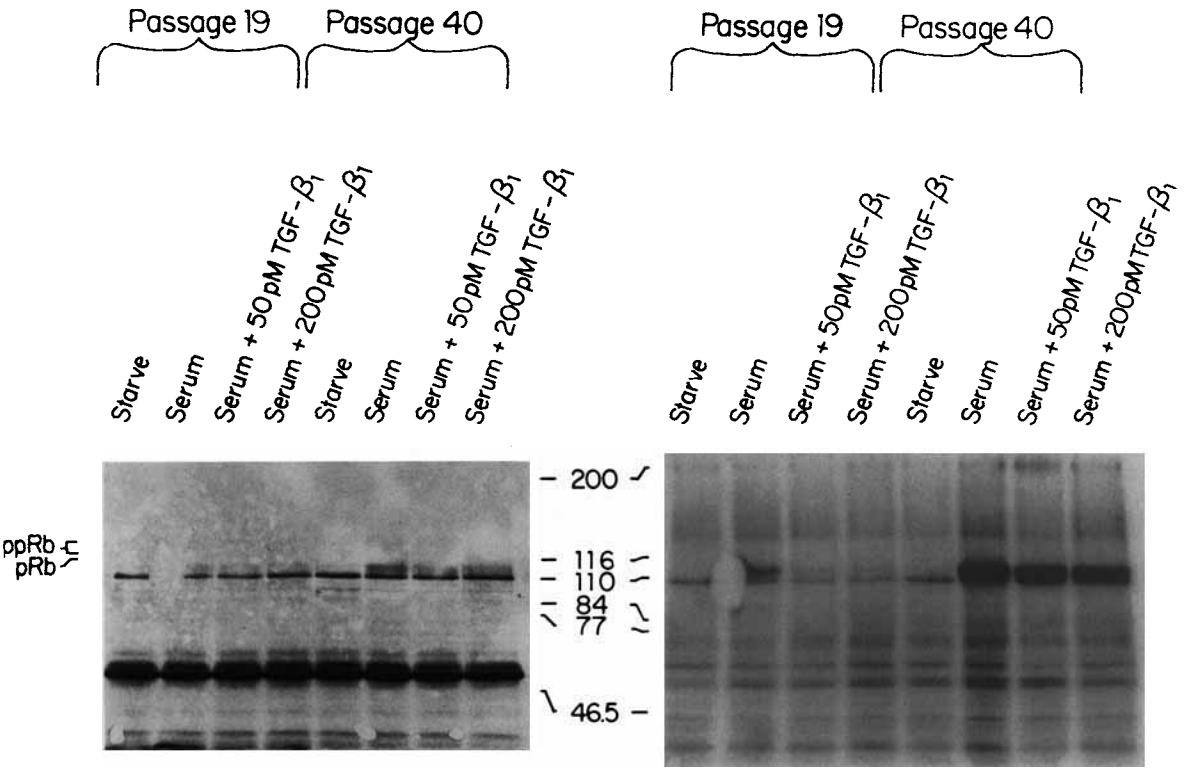

Fig. 7. The effects of TGF-β_1 on pRb phosphorylation are diminished in WB cells at higher passages. WB cells of passage 19 and passage 40 were serum-starved, then refed for 18 h in the presence of the indicated concentrations of TGF-β_1. The cells were then incubated for 3 h with ^{32}P-orthophosphate, then immunoprecipitates were prepared from cell lysates. The immunoprecipitates were applied to polyacrylamide gels and immunoblots were prepared. The immunoblots are shown on the left and autoradiographs are shown on the right.

tion. This would indicate that the effects of TGF-β_1 on ^{3}H-thymidine incorporation are not coupled to its effects on pRb phosphorylation and that DNA synthesis can begin without the phosphorylation of pRb. This runs counter to the widely accepted view that pRb phosphorylation accompanies entry into S [11–14]. This issue might be resolved if we could identify the point in the cell cycle at which WB cells are arrested when TGF-β_1 and serum are added simultaneously. This might be done by adding serum and TGF-β_1 to the starved cells simultaneously, incubating for 20 h, then releasing the TGF-β_1 block and following the time course for entry into S. If the cells arrest at G_1/S, then the onset of DNA synthesis should be faster and more concerted than that observed upon the addition of serum to starved cells.

In any case, we must be cautious about the interpretation of our time course data, since the WB cells were not in complete synchrony after refeeding with serum. The time course of ^{3}H-thymidine incorporation after serum refeeding (Fig. 3A) suggests that the cells were not well synchronized. The peak of ^{3}H-thymidine incorporation was rather broad, beginning at 8 h postserum and continuing for more than 20 h. In addition, we never observed a complete shift in pRb to the higher phosphorylated states, suggesting that some cells were not in S when the peak of ^{3}H-thymidine was maximal for the population. Our attempts to resolve these issues using cell sorting techniques were thwarted by the fact that the starved cells could not be trypsinized efficiently and failed to sort properly. It is possible that the lack of cell synchrony may account for the apparent uncoupling of the effects of TGF-β_1 on pRb phosphorylation and ^{3}H-thymidine incorporation.

At higher passages, WB cells lost the capacity to be growth-arrested by TGF-β_1 and this, too, correlated with the loss of inhibition of pRb phosphorylation. The mechanism by which the cells lose sensitivity to TGF-β_1 is not known. It does not appear that the high passage cells serum-arrest at a different point in the cell cycle

from lower passage cells. In both cases, serum-arrested cells had virtually 100% of pRb in the underphosphorylated state, characteristic of G_1. Moreover, loss of growth inhibition by TGF-β_1 has been demonstrated under logarithmic growth conditions in high passage WB cells [20].

It appears that some TGF-β_1-resistant cells were present even in the earliest passage cells we used. A previous report [20] indicated that a measurable loss of TGF-β_1 sensitivity (as shown by a right-shift in dose response) occurred from passage 9 to passage 20 in WB cells. This is consistent with the presence of TGF-β_1-resistant cells in our cultures at passage 15. By passage 40, it appears that a majority of the WB cells were TGF-β_1 resistant. It is not clear whether the culture conditions select for TGF-β_1-resistant cells, or sensitive cells convert to the resistant state with continued passage.

Another change we observed in the higher passage cells was a decreased doubling time (data not shown). It also appeared that serum stimulated incorporation of ^{32}P in pRb to a greater extent in passage 40 than in passage 19 cells (Fig. 7). It is not clear whether a greater sensitivity to serum is related to TGF-β_1 resistance in the higher passage cells.

In some experiments (cf. Fig. 4), we resolved two bands of the underphosphorylated form of pRb. It has been reported that in HL-60 cells undergoing terminal differentiation a low molecular weight species of pRb appears [13]. It was suggested that this might result from synthesis of a truncated pRb by use of an internal ATG as the initiation codon. It is not clear if a similar phenomenon is occurring here.

The results we report here are consistent both with the idea that underphosphorylated pRb acts as a block to entry into S and the idea that TGF-β_1 inhibits cell division by keeping pRb in this underphosphorylated state. As has been pointed out by Roberts et al., however, these results are equally consistent with the idea that TGF-β_1 arrests cells in G by a different mechanism, and inhibition of pRb is the consequence rather than the cause of arrest in G_1 [25]. Correlation of cell growth arrest by TGF-β_1 with inhibition of pRb phosphorylation has also been reported in mink lung epithelial cells and murine keratinocytes [4]. In those studies, experiments with SV40 large T strengthened the case that TGF-β_1 acts through pRb. In mink lung epithelial cells transfected with large T antigen, TGF-β_1 inhibited pRb phosphorylation, but did not inhibit cell growth [4]. Transfection with a mutant of large T which did not bind pRb did not counteract the effects of TGF-β_1 on cell growth. Since SV40 large T binds only to the underphosphorylated form of pRb, these results suggested that large T removed the block for entry into S by inactivating pRb, and thus made the effects of TGF-β_1 on pRb phosphorylation irrelevant. Although this would seem to strongly favor a direct role for pRb in TGF-β_1 action, it has been pointed out that SV40 large T binds other cellular proteins as well. Moreover, it has been shown that two breast cancer cell lines are growth-inhibited by TGF-β_1, but do not express functional pRb [26]. It remains a possibility that TGF-β_1 inhibits cell growth differently in different cell types. We have shown that TGF-β_1 inhibits the growth of human Hep 3B hepatoma cells in a novel manner that causes them to lose attachment to tissue culture flasks [27]. TGF-β_1 does not appear to inhibit pRb phosphorylation in this cell type (Whitson, unpublished results). In the work presented here, we have shown a good but imperfect correlation between the effects of TGF-β_1 on ^3H-thymidine incorporation and pRb phosphorylation in WB liver epithelial cells. Together with the results of these other studies, our results suggest that TGF-β_1 affects events in the transition from G1 to S which control both DNA synthesis and pRb phosphorylation, however it is not clear that pRb mediates the effects of TGF-β_1 on cell division.

ACKNOWLEDGMENTS

The authors would like to thank Faith Sorensen for her assistance in the preparation of this manuscript. This work was supported, in part, by the IDEN Foundation.

REFERENCES

1. Roberts AB, Sporn MB: Adv Cancer Res 51:107–145, 1988.
2. Barnard JA, Lyons R, Moses HL: Biochem Biophys Acta 1032:79087, 1990.
3. Shipley GD, Pittelkow MR, Wille JJ, Scott RE, Moses HL: Cancer Res 46:2068–2071, 1986.
4. Laiho M, DeCaprio JA, Ludlow JW, Livingston DM, Massagué J: Cell 62:175–185, 1990.
5. Boyd FT, Massagué J: J Biol Chem 264:2272–2278, 1989.
6. Lee W-H, Bookstein R, Hong F, Young L-J, Shew J-Y, Lee EY-H: Science 235:1394–1399, 1987.
7. Friend SH, Bernards R, Rogeli S, Weinberg R, Rapaport JM, Albert DM, Dryja TP: Nature 323:643–646, 1986.
8. T'Ang A, Varley J, Chakraborty S, Murphree AL, Fung Y-KT: Science 242:263–266, 1988.

9. Lee W-H, Shew J-Y, Dong FD, Serg TW, Donoso LA, Young L-J, Bookstein R, Lee EY-H: Nature 329:642–645, 1987.
10. Huang H-JS, Yee JK, Shew J-Y, Chen P-L, Bookstein R, Friedmann T, Lee W-H: Science 242:1563–1566, 1988.
11. Buchkovich K, Duffy LA, Harlow E: Cell 58:1097–1105, 1989.
12. Chen P-L, Scully P, Shew J-Y, Wang JYJ, Lee W-H: Cell 58:1193–1198, 1989.
13. Mihara K, Cao X-R, Yen A, Chandler S, Driscoll B, Murphree AL, T'Ang A, Fung Y-KT: Science 246:1300–1303, 1989.
14. Ludlow J, DeCaprio JA, Huang C-M, Lee W-H, Paucha E, Livingston DM: Cell 56:57–65, 1989.
15. Ludlow JW, Shon J, Pipas JM, Livingston DM, DeCaprio JA: Cell 60:387–396, 1990.
16. DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, Huang C-M, Livingston DM: Cell 58:1085–1095, 1989.
17. Schlegel R: Semin Virol 1:297–306, 1990.
18. Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weiberg RA, Harlow E: Nature 334:124–129, 1988.
19. Whyte P, Williamson NM, Harlow E: Cell 56:67–75, 1989.
20. Lin P, Liu C, Tsao M-S, Grisham J: Biochem Biophys Res Commun 143:26–30, 1987.
21. O'Farrell PH: J Biol Chem 250:4007–4021, 1975.
22. Towbin H, Staehelin T, Gordon J: Proc Natl Acad Sci USA 76:4350–4354, 1979.
23. Massagé J: J Biol Chem 259:9756–9761, 1984.
24. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB: J Biol Chem 263:10783–10789, 1988.
25. Roberts A, Kim SJ, Sporn MB: Cancer Cells 3:19–21, 1991.
26. Ong G, Sikora K, Gullick WJ: Oncogene 6:761–763, 1991.
27. Whitson RH, Wong WL, Itakura K: J Cell Biochem 47:31–42, 1991.