Growth Arrest Induced by Transforming Growth Factor β1 Is Accompanied by Protein Phosphatase Activation in Human Keratinocytes*

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Protein phosphorylation and dephosphorylation are involved in regulation of cell growth. We tested the hypothesis that the growth inhibitory effect of transforming growth factor β1 (TGF-β1) involves activation of protein phosphatases. Exposure of human keratinocytes in culture to 400 pm TGF-β1 for 48 h led to 80% inhibition of DNA synthesis as measured by nuclear labeling. Incubation of cultured keratinocytes with 400 pm TGF-β1 rapidly activated (within 30 min) protein serine/threonine phosphatase, measured using phosphorylase as a substrate. Based on several criteria, including neutralization of activity with specific antibodies and inhibitor-2, TGF-β1-activated phosphorylase phosphatase was identified as protein phosphatase 1. TGF-β1 did not have rapid effects on protein serine/threonine phosphatase activity (type 2A) measured with histone phosphorylated by protein kinase C or on protein tyrosine phosphatase activity. However, protein tyrosine phosphatase was activated at 48 h, coincident with growth arrest. Differentiation, induced by the combination of TGF-β1 plus calcium or by serum, was not accompanied by further serine/threonine or tyrosine phosphatase activation. We conclude that induction of growth arrest in keratinocytes by TGF-β1 involves growth factor β1 (TGF-β1) plus protein phosphatase 1, while activation of protein tyrosine phosphatases may represent an additional mechanism for maintaining cells in a growth-arrested state.

Based on advances of the last several years, it appears that stimulation of cell proliferation may involve multiple protein phosphorylation cascades. The role of receptor-associated and non-receptor tyrosine kinases in the initiation of cell growth is established (1). Tumor-promoting phorbol esters stimulate cell growth via activation of the protein kinase C family (2). Signal transduction pathways also involve protein kinases such as ribosomal protein S6 kinase (3), raf (4), casein kinase II (5), and microtubule-associated protein kinase (6). In addition, the cell cycle-dependent protein kinase, cdc2 (7), appears to be directly involved in the control of DNA synthesis and mitosis. Finally, several lines of evidence point to involvement of protein phosphatases in cell cycle control (reviewed in Ref. 8).

The family of polypeptides designated by the term transforming growth factor beta (TGF-β) are ubiquitous regulators of cell growth, which have diverse, cell type-dependent biological effects (for reviews, see Refs. 9 and 10). In epithelial cells, including human keratinocytes, a member of the TGF-β family, TGF-β1, acts as a potent growth inhibitor (11). The mechanisms by which TGF-β1 exerts this effect are not known, although recent studies have demonstrated rapid effects on the expression of c-myc (12, 13) and on the phosphorylation state of the retinoblastoma gene product, RB (14). Given the important role of serine/threonine and tyrosine protein kinases in the stimulation of cell proliferation, we hypothesized that the inhibition of keratinocyte growth by TGF-β1 involves protein phosphatase activation. The present report describes studies on the effects of TGF-β1 on human keratinocyte protein phosphatase activities.

EXPERIMENTAL PROCEDURES

Culture of Human Keratinocytes—Early passage cultures of human keratinocytes were prepared from foreskin tissue as previously described (15). Cultures were established initially on feeder layers of irradiated mouse fibroblast 3T3 cells using Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 0.4 ug/ml hydrocortisone. After reaching confluence, the keratinocyte cultures were detached with trypsin and replated without a feeder layer in serum-free keratinocyte growth medium (KGM, Clonetics Corp., San Diego, CA) supplemented with 5 ug/ml insulin, 0.5 ug/ml hydrocortisone, 10 ng/ml epidermal growth factor, and 0.4% bovine pituitary extract (referred to as complete KGM).

Cells were plated at a density of 1 x 10⁴ cells/35-mm Petri dish (except where noted) in complete KGM. On the following day, medium was removed, and fresh KGM with or without 400 pm TGF-β1 (R&D Systems, Inc., Minneapolis, MN) was added. To monitor DNA synthesis, cultures were labeled for the last 24 h before harvesting with 5 μCi of [³H]thymidine and processed for autoradiography (16). The percent labeled nuclei was determined by counting at least 1000 cells in four high power fields.

Histochemical staining for keratin was performed as described by Ayoub and Skhilar (17). In this procedure, differentiated cells stain brilliant red and undifferentiated cells stain blue.

Preparation of Keratinocyte Lysates—Cells were lysed with 0.5 ml/35-mm well of a buffer containing 50 mM HEPES, 5 mM EDTA, 2% Triton X-100, 250 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, pH 7.4. Following scraping, cells were broken by several strokes in a ground glass homogenizer. The resulting homogenate was diluted 1:2 (v/v) in assay buffers appropriate for measurement of protein serine/threonine phosphatase (18) and protein tyrosine phosphatase (19) activities. An aliquot of the undiluted homogenate was retained for measurement of protein phosphatase heat-stable inhibitor activity (see below) and protein determination using bicinchoninic acid (Pierce Chemical Co.). Samples were frozen in liquid nitrogen.

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1 The abbreviations used are: TGF-β, transforming growth factor β; KGM, keratinocyte growth medium; HEPES, N-2-hydroxylethylpiperazine-N’-2-ethanesulfonic acid; inhibitor-2; RCM, reduced carboxymethylated lysozyme; MBP, myelin basic protein; MOPS, 3-(N-morpholino)propanesulfonic acid.
and stored at −70 °C until analysis 1–3 days later.

For all experiments parallel incubations were carried out so that cell counts could be done on duplicate wells. Cells were removed from the substratum using trypsin digestion and counted on a hemocytometer.

**Determination of Protein Phosphatase Activities**—Phosphorylase phosphatase was measured as the inactivation of rabbit skeletal muscle phosphorylase a (18). Protein serine/threonine phosphatase activity was also measured as the release of 32P from histone type III-S (Sigma), which was phosphorylated by partially purified rat liver protein kinase C. This represents a modification of our previously described method which used casein as substrate (20).

Protein tyrosine phosphatase activity was measured as the release of 32P from Tyr(P)-reduced carboxamidomethylated lysozyme (Tyr(P)-RCML) or Tyr(P)-myelin basic protein (Tyr(P)-MBP). These substrates were phosphorylated on tyrosine using partially purified human placental insulin receptors (19). Protein tyrosine phosphatase activity was measured as 32P released from the Tyr(P) substrates as described previously (19). Assignment of the activities measured with Tyr(P)-RCML lysozyme and Tyr(P)-MBP to protein tyrosine phosphatases, as opposed to acid or alkaline phosphatases, was supported by sensitivity to vanadate, zinc, and poly(glutamic acid-tyrosine) (21). In addition, the activity of protein tyrosine phosphatases is insensitive to EDTA, an inhibitor of acid and alkaline phosphatases (21) and a constituent of our protein tyrosine phosphatase assay buffer.

For all protein phosphatase activity determinations, samples were diluted so that substrate consumption was less than 50%. In this range, the relationship between substrate consumed and time is linear.

The inhibition of phosphorylase phosphatase by heat-stable proteins, a measure of inhibitor-2 (12) activity, was determined using rabbit skeletal muscle protein phosphatase 1 catalytic fragment as described previously (18). For these analyses, undiluted homogenates were placed in a boiling water bath for 5 min and then centrifuged for 5 min at 12,000 × g to remove denatured proteins. One unit of I2 activity is defined as that amount which produces 50% inhibition of the standard amount of phosphorylase phosphatase activity (15 milliunits) included in the assay. Purified I2 used to characterize protein phosphatase activity was prepared from rabbit skeletal muscle (18). Antibodies against rabbit skeletal muscle protein phosphatase 1 (22, 23), also used to characterize lysate phosphorylase phosphatase activity, were prepared as described previously by ammonium sulfate precipitation followed by dialysis with 20 mM MOPS, 150 mM NaCl, pH 7.4 (22).

**Statistical Analyses**—The significance of differences from control data was determined using the Rank Sum Test with the Bonferroni correction for multiple comparisons (24). Significance was defined as p < 0.05.

**RESULTS**

**Effect of TGF-β1-induced Growth Arrest on Phosphorylase Phosphatase Activity**—We found that TGF-β1 induced a reversible state of growth arrest in rapidly proliferating, early passage human keratinocytes. This effect was maximal within 48 h after the addition of 400 pm TGF-β1, as indicated by an 80% decrease in labeled nuclei detected by autoradiography (data not shown). To determine if growth inhibition of human keratinocytes was accompanied by changes in the activity of protein serine/threonine phosphatases, we measured activity using phosphorylase a as a substrate in keratinocyte lysates at various time points after the addition of 400 pm TGF-β1 in serum-free complete KGM. Phosphorylase phosphatase activity increased significantly within 30 min after treatment of subconfluent keratinocyte monolayers with TGF-β1 (Fig. 1). This activation increased progressively for up to 48 h, by which time, a 3–4-fold increase in phosphorylase phosphatase activity was observed.

Expression of phosphorylase phosphatase activity per unit of protein yielded similar results for incubation periods up to 6 h. After 24 and 48 h of exposure to TGF-β1, protein content per unit of cell number increased 1.5–2.5-fold, such that the apparent increase in phosphatase activity was proportionately less. These findings are consistent with those of Wikner et al. (25), who observed a 2-fold increase in protein synthesis in human keratinocytes exposed to TGF-β1 for 22 h. However, these authors described different rates of increased production for various proteins. Since increased protein synthesis in response to TGF-β1 involves specific proteins and protein content did not vary during early time points in our experiments, we have expressed phosphatase activities per unit of cell number.

Due to TGF-β1-induced growth arrest, cell densities in treated and untreated cultures differed significantly by the end of each experiment. To determine if the TGF-β1-associated increase in phosphorylase phosphatase activity was independent of cell density, we examined phosphatase activity in keratinocytes plated at varying densities (0.5, 1.0, and 3.0 × 10^5 cells/35-mm well) and exposed to TGF-β1 for 48 h. Comparison of activities in wells with comparable cell numbers showed TGF-β1-associated activation of phosphorylase phosphatase regardless of cell density (data not shown).

**Characterization of Protein Serine/Threonine Phosphatase Activity**—In cell extracts, phosphorylase phosphatase activity is predominantly, if not exclusively, due to protein phosphatases 1 and 2A (26). We studied the sensitivity of phosphorylase phosphatase activity to the protein phosphatase regulatory protein, I2, which selectively blocks protein phosphatase 1 (26). Using keratinocyte lysates obtained before and after exposure to TGF-β1, phosphorylase phosphatase activity was inhibited by approximately 50% in the presence of 2 ng of I2 and was maximally inhibited at concentrations of 10–100 ng/assay tube (approximately 17–170 nM I2 (21)). The I2-resistant activity (measured in the presence of 100 ng of I2) did not change with 2- or 48-h exposure to 400 pm TGF-β1 (Fig. 2). Therefore, the increase in total phosphorylase phosphatase activity in response to TGF-β1 represented an increase in protein phosphatase 1 activity that was sensitive to I2. Phosphorylase phosphatase activity measured after trypsin activation (Fig. 2) also increased after exposure to TGF-β1. Trypsin treatment may expose protein phosphatase 1 catalytic activity by removing associated inhibitors or regulatory subunits including I2 (see "Discussion").

Phosphorylase phosphatase activity that was insensitive to I2 may represent protein phosphatase 2A. However, it is possible that this I2-resistant activity was due to a form of protein phosphatase 1 catalytic subunit that was associated with regulatory subunits and therefore resistant to inhibition.
Protein Phosphatase Activity in the Presence of TGF-β1

We have demonstrated previously that the presence of antibodies against rabbit skeletal muscle protein phosphatase 1 while having no effect on the activity of purified protein phosphatase 1A at varying dilutions for cells exposed to TGF-β1. Lysates of cells harvested at 0, 2, and 48 h after the addition of TGF-β1 to culture medium were assayed in the presence of varying concentrations of antibody (Fig. 3). Neutralization was nearly complete in the 0- and 2-h lysates. However, activities in all three lysates were not sufficient to inhibit phosphatase activity completely in the 48-h lysate. The maximum concentration of antibody was insufficient to inhibit phosphatase activity completely in the 48-h lysate. However, activities in all three lysates were neutralized in parallel. These results are most consistent with the conclusion that the keratinocyte lysate phosphorylase phosphatase activity is nearly completely accounted for by protein phosphatase 1. A portion of this activity is sensitive to I2. After exposure to TGF-β1, I2-resistant protein phosphatase 1 activity remains constant, while I2-sensitive activity decreases.

We employed a second substrate, histone phosphorylated by rat liver protein kinase C, as an additional probe for protein serine/threonine (type 2A) phosphatase activity. TGF-β1 had no effect on protein phosphatase activity measured with phosphorylase (data not shown). Activities ranged from 1.5-2.1 pmol/min/10^6 cells in samples obtained before and after 48-h exposure to TGF-β1. I2 activity, measured in heat-treated lysates, was unchanged after either acute (30 min to 6 h) or 48-h exposure to TGF-β1. Activities in all samples ranged from 15 to 36 units/10^6 cells.

Effect of TGF-β1-induced Growth Arrest on Protein Tyrosine Phosphatase Activity—Growth arrest following 48 h of exposure to TGF-β1 was associated with a 3-4-fold increase in protein tyrosine phosphatase activity measured with Tyr(P)-RCML (Fig. 4A). However, the kinetics of protein

Fig. 2. Phosphorylase phosphatase activity measured in the presence of I2 and following activation by trypsin. Keratinocyte lysates obtained after 0-, 2-, and 48-h exposure to 400 pM TGF-β1 were used to measure phosphorylase phosphatase activity in the presence of 100 ng of I2 (open bars), without pretreatment or additions (hatched bars), and following digestion by trypsin (20 μg/ml for 10 min at 30 °C, solid bars). Measurements were made in triplicate using lysates from separate wells. Error bars represent 1 standard deviation.

Fig. 3. Neutralization of phosphorylase activity by protein phosphatase (PP-1) antibodies. Keratinocyte lysates (20 μl) from cells exposed to 400 pM TGF-β1 for 0 h (circles), 2 h (squares), or 48 h (triangles) were incubated with protein phosphatase 1 antibody (10 μl) at varying dilutions for 30 min at 30 °C. The mixture was then assayed for phosphorylase phosphatase activity.

Fig. 4. Effect of TGF-β1 and cell density on protein tyrosine phosphatase (PTPase). Panel A shows keratinocyte lysate protein tyrosine phosphatase activity measured with Tyr(P)-RCML before (hatched bar, n = 10) and after (solid bar, n = 9) exposure for 48 h to TGF-β1. Error bars represent 1 standard deviation. The difference between the means was significant (p < 0.001). Panel B shows protein tyrosine phosphatase activities in lysates of cells harvested before (solid circles) and 48 h after (open circles) exposure to 400 pM TGF-β1. Results are expressed as a function of cell density determined at the end of the experiment.

Fig. 5. Effect of keratinocyte differentiation on protein phosphatase 1 (PP-1) and protein tyrosine phosphatase (PTPase). Protein phosphatase 1 and protein tyrosine phosphatase activities were measured in cells treated with various combinations of 400 pM TGF-β1, 1.8 mM calcium, and 10% fetal bovine serum (see "Experimental Procedures"). Results are expressed as a percentage of control activity. Error bars represent 1 standard deviation. Asterisks denote a significant difference from the corresponding control (no additions) activity. Control and TGF-β1 results are compiled from six experiments, while other data represent three combined experiments.
tyrosine phosphatase activity differed markedly from activation of protein phosphatase 1, with no change in protein tyrosine phosphatase activity occurring until 24 h of exposure (data not shown). Unlike protein phosphatase 1, the increase in protein tyrosine phosphatase activity seen at 48 h was affected by cell density (Fig. 4B). The greatest increase induced by TGF-β1 was seen at the lowest cell densities. In contrast, cells cultured without TGF-β1 appeared to have increased activity at higher densities. Longer culture times in the absence of TGF-β1 (>72 h), which resulted in the highest cell densities we studied (7–14 × 10^6 cells/35-mm well), were associated with no further increase in protein tyrosine phosphatase activity.

Protein tyrosine phosphatase activities were also measured with a second substrate, Tyr(P)-MBP. Protein tyrosine phosphatase activities measured with 1 μM Tyr(P)-RCML and Tyr(P)-MBP were approximately equal in multiple keratinocye lysates prepared from cells in various stages of growth arrest and differentiation (see below). Activity measured with both substrates was inhibited 50% by 105 μM Zn²⁺ and was fully inhibited at 250 μM. Protein tyrosine phosphatase activity was also sensitive to vanadate (50% inhibition at 250 μM) and poly(glutamic acid-tyrosine (4:1)) (50% inhibition at 1.3 μM).

Effects of Keratinocyte Differentiation on Protein Phosphatase Activities—Growth arrest in human keratinocytes by TGF-β1 is not accompanied by terminal differentiation, even after prolonged (5–12 days) exposure (27). Differentiation, however, can be induced in cells exposed to TGF-β1 within 2 days by the addition of 1.8 mM Ca²⁺. In the present studies, we confirmed that growth arrest of keratinocytes induced by TGF-β1 was not accompanied by differentiation, as indicated by morphologic evidence of stratification as well as histochemical staining for keratin. The addition of 1.8 mM calcium to nonproliferating cells cultured in the presence of 400 pM TGF-β1 for 48 h produced differentiation without DNA synthesis (as determined by autoradiography). In contrast, the addition of 1.8 mM calcium to proliferating cultures produced minimal signs of differentiation and no growth arrest. Treatment of cells with 10% fetal bovine serum led to full differentiation while proliferation continued.

The greatest increase above control levels in protein phosphatase 1 activity (Fig. 5, top panel) was seen in growth-arrested, undifferentiated cells (400 pM TGF-β1 for 48 h). Induction of differentiation in growth-arrested cells (i.e. TGF-β1 alone for 48 h) did not lead to a further increase in protein phosphatase 1 activity. Neither the addition of 1.8 mM calcium to proliferating cells nor the differentiation of proliferating cells with fetal bovine serum plus 1.8 mM calcium resulted in changes in protein phosphatase 1 activity from control levels.

Protein tyrosine phosphatase activity (Fig. 5, bottom panel) increased only in growth-arrested, undifferentiated keratinocytes (exposure to TGF-β1 alone). Neither exposure to 1.8 mM calcium nor differentiation in proliferating or nonproliferating cells led to a change in protein tyrosine phosphatase activity from control levels. Similar results were obtained using Tyr(P)-RCML and Tyr(P)-MBP as substrate.

**DISCUSSION**

Our data support the hypothesis that the induction of growth arrest in keratinocytes by TGF-β1 involves activation of protein phosphatases. More specifically, a rapid increase in type 1 protein serine/threonine phosphatase activity was observed following the addition of TGF-β1 to human keratinocyte cultures. All the activity that increased following exposure to TGF-β1 was sensitive to inhibition by 12. This has been used as a primary criterion for classification of phosphatase activity as type 1 (28). To further confirm that increases in phosphorylase phosphatase activity following TGF-β1 were due to protein phosphatase 1, the activity was neutralized with protein phosphatase 1-specific antibodies. The conclusion that TGF-β1 led to rapid activation of protein phosphatase 1 and not protein phosphatase 2A was also supported by the finding that protein phosphatase activity measured with histone phosphorylated by protein kinase C did not change with exposure to TGF-β1. The latter substrate has been used for the selective measurement of protein phosphatase 2A activity (29).

A direct relationship between TGF-β1 and regulation of protein tyrosine phosphatase is less clear. Although exposure to TGF-β1 did lead to increased protein tyrosine phosphatase activity, this effect was only observed at 24 and 48 h. TGF-β1 arrests keratinocyte growth during progression from G1 to S phase (11). It is possible that the increased protein tyrosine phosphatase activity associated with TGF-β1-induced growth arrest is a function of the point in the cell cycle at which the cells are arrested rather than direct involvement in TGF-β1 signal transmission. We originally considered a role for protein tyrosine phosphatase activation during keratinocyte differentiation. Such an effect has been seen with monocytic differentiation of HL-60 leukemia cells (30). However, calcium-induced keratinocyte differentiation was not accompanied by protein tyrosine phosphatase activation. Indeed, treatment with 1.8 mM calcium following growth arrest with TGF-β1 was associated with a return of protein tyrosine phosphatase activity to levels seen in proliferating, undifferentiated cells. This result is consistent with the findings of Filvaroff et al. (31), who observed that differentiation of mouse keratinocytes in response to calcium was accompanied by protein tyrosine phosphorylation and that tyrosine kinase inhibitors blocked differentiation.

The above studies on protein tyrosine phosphatase regulation utilized two substrates, Tyr(P)-RCML and Tyr(P)-MBP. Multiple classes of protein tyrosine phosphatase have now been described based on molecular cloning. We utilized the protein tyrosine phosphatase substrate, Tyr(P)-MBP, as a probe for the transmembrane class of protein tyrosine phosphatases (M, ~200,000), of which the leucocyte common antigen, CD45 (32), and LAR (leukocyte antigen-related (33)) are members. Our own findings using rat liver membranes (34) indicate that Tyr(P)-MBP is a better substrate than Tyr(P)-RCML for a protein tyrosine phosphatase resembling LAR, while it is a poor substrate relative to Tyr(P)-RCML for a rat liver homologue of the M, ~50,000 enzyme which is termed protein tyrosine phosphatase 1B (35). Our protein tyrosine phosphatase measurements would include the activities of either LAR-related or protein tyrosine phosphatase 1B-related enzymes, or both. It is of interest that the structure of the extracellular domain of LAR has characteristics of cell adhesion molecules (33). The effect of cell density on protein tyrosine phosphatase activity may reflect changes mediated via the cell-surface domain of a transmembrane protein tyrosine phosphatase.

In summary, our results show the rapid activation of protein phosphatase type 1 in human keratinocytes treated with TGF-β1. This finding is consistent with a role for protein phosphatases in the mechanism of action of TGF-β1. It seems reasonable to speculate that protein phosphatase activation might lead to growth arrest via dephosphorylation of multiple sub-

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2 L. Braun and R. Mikulko, unpublished observations.
strates, possibly including the anti-oncogene product RB, which is dephosphorylated in response to TGF-β1 (14).

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REFERENCES