J. Biochem. Mol. Biol. Vol. 30, No. 2, pp. 119~124 March 31, 1997

Transforming Growth Factor-β Enhances Tyrosine Phosphorylation of Two Cellular Proteins in HEL Cells

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(Received December 27, 1996)

Abstract : Transforming growth factor-β (TGF-β) is a multifunctional polypeptide that exerts biological roles including cell proliferation, differentiation, extracellular matrix deposition and apoptosis in many different cell types. TGF-β, although known as a negative growth regulator, has not been tested in human embryo lung (HEL) cells. This study attempts to understand the role of TGF-β on growth control of HEL cells in relationship to tyrosine phosphorylation pattern of cellular proteins. In density-arrested HEL cells treated with TGF-β, analysis of Western immunoblot showed induction of tyrosine phosphorylation of two major cellular proteins (15 kDa and 45 kDa). In normal proliferating HEL cells with different concentrations of serum, further analysis indicated that the increase in tyrosine phosphorylation of a 45 kDa protein was regulated in serum concentration-dependent manner. However, in proliferating HEL cells treated with TGF-β, tyrosine phosphorylation of 45 kDa was down-regulated. Calcium involvement in the regulation of tyrosine phosphorylation of 45 kDa and 15 kDa proteins was also examined. Tyrosine phosphorylation of 15 kDa protein but not of 45 kDa protein was regulated by exogenous calcium. The level of tyrosine phosphorylation of 15 kDa protein was low at reduced caclium concentration and high at elevated caclium concentration. TGF-β reversed the pattern of tyrosine phosphorylation of 15 kDa protein. These results suggest that tyrosine phosphorylation of 45 and 15 kDa proteins in HEL cells may be controlled depending on the physiological status of the cells, *i.e.*, low in arrested cells and high in proliferating cells. And the tyrosine phosphorylation of the two proteins appears to be down- or up-regulated by TGF-β.

Key words : calcium, human cytomegalovirus, transforming growth factor β, tyrosine phosphorylation.

Transforming growth factor-β is a member of a newly emerging large family of structurally related growth factors that elicit various responses including cell proliferation, differentiation, morphogenesis, apoptosis, and extracellular matrix (ECM) deposition (Massague 1990; Roberts and Sporn, 1990; Kingsley, 1994). In mammals, there are three TGF-β isoforms (1, 2 and 3) present. From here, TGF-β1 is referred to as TGF-β unless otherwise indicated. It is known that TGF-β acts through specific high affinity heteromeric receptor complex between TGF-β type I (TGF-βRI) and type II (TGF-βRII) receptors (Wrana *et al.*, 1992). TGF-βRI and TGF-βRII receptors are transmembrane serine/threonine kinases belonging to a novel receptor serine/threonine kinase family including Daf-1 and Daf-4 receptors (see a review by Georgi *et al.*, 1990; Lin *et al.*, 1992; Estevez *et al.*, 1993; Franzen *et al.*, 1993; Bassing *et al.*, 1994; Kingsley, 1994). Thus, these TGF-βRI and TGF-βRII receptors are referred to as the signal transducing receptors. However, the TGF-β type III receptor (TGF-βRIII) is a transmembrane proteoglycan/betaglycan which reveals no signaling motif in the cytoplasmic domain (Wang *et al.*, 1991). It is dominantly known that TGF-βRIII presents TGF-β isoforms, especially TGF-β2 to the signaling receptors (Lin and Lodish, 1993). Very recently, Wrana *et al.* (1994) determined the molecular mechanism of activation of the TGF-β receptors. They have shown that TGF-βRII autophosphorylates and is a constitutively active kinase regardless of the presence of TGF-β ligand. Then activated TGF-βRII recruits TGF-βRI to transphosphorylate the GS domain in the juxtamembrane region to activate the TGF-βRI, which is then functioning for downstream signaling (*e.g.*, gene responses and growth arrest at late G1, etc.).

In our original study, we sought to examine the effects of a typical negative growth regulator such as

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TGF-β on the regulation of immediate early gene expression of human cytomegalovirus (HCMV) in human embryonic lung fibroblast (HEL) cells. In our present study, we first investigated the responses of HEL cells, which are permissive to HCMV replication, to TGF-β to determine whether TGF-β is growth-inhibitory or growth-stimulatory in these cells. We further examined cellular response (*e.g.*, tyrosine phosphorylation) of cellular proteins in HEL cells upon TGF-β stimulation.

Materials and Methods

Cell culture

HEL cells were obtained from the Mogam Biotechnology Institute (Yongin, Kyunggido, Korea). HEL cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) in a moisture-laden atmosphere of 5% CO_2 in air at 37°C. Cells were subcultured with 1 to 2 ratio. For preparation of density-arrested cells, cells were grown to confluency and medium was changed with fresh EMEM supplemented with 2% FBS. For preparation of synchronously proliferating cells, cells were replated with 1 to 5 ratio and incubated with EMEM containing 10% FBS for 24 h and the medium containing 0.2% FBS was freshly added to the cultures for 5 h. Then fresh medium containing desired concentration of FBS was added. For preparation of cells for the analysis of cell cycle dependency, medium was changed with fresh medium containing different concentration of FBS ranging from 0.2% to 10%.

Porcine TGF-β1 (R&D Systems, Minneapolis, MN, USA) was used in these studies at a final concentration of 100 pM unless stated otherwise.

Cell proliferation assay

Cells were plated into 60 mm dishes at a density of 2.7×10^5 cells with complete growth medium. After 24 h incubation, the medium was changed with EMEM containing 5% FBS and then incubated for further 68 h. Cells were released from the dishes by trypsinization and counted using a haemocytometer. During this final incubation, the cultures received TGF-β ranging from 10 to 250 pM.

Sample preparation for Western immunoblotting

Cells were washed two times with phosphate buffered saline (PBS) (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and 1 ml of PBS was added to each 100 mm dishes. The cells were then collected by scraping, centrifuged at low speed and frozen at −70°C. The frozen pellets were resuspended in 400 μl of preboiled lysing solution (0.1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM phenylmethanesulfonyl fluoride; 10 mM Tris-HCl, pH 7.4, 1% SDS) and continued to boil for 5 min while resuspending them by pipetting up and down. Each sample was spun down at 12,000 rpm for 1 min. Fifty μl of each sample was removed for protein quantitation and 100 μl of each sample was taken for Western immunoblot and put into tube with the same volume of 2X SDS sample buffer, vortexed and stored at −70°C.

Protein quantitation

Fifty μl of total cellular extract of each sample was further mixed with 130 μl ddH_2O and 20 μl of 100% trichloroacetic acid. The samples were kept on ice for 30 min and spun at 12,000 rpm for 20 min at 4°C. Then they were washed with cold acetone, spun for 15 min and air-dried. To each sample for protein quantitation, 1 ml of ABC basic (2% Na_2CO_3, 0.02% sodium potassium tartarate, 0.01% $CuSO_4$) was added and followed by addition of 100 μl ddH_2O. After 10 min, 100 μl of phenol reagent (diluted 1:1 with ddH_2O) was added to each sample and OD at 700 nm was read after 30 min. The total amount of cellular proteins in each sample was comparatively estimated from standard curve made using bovine serum albumin (BSA).

Western immunoblotting

The samples were subjected to 15% SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinyl difluoride (PVDF) membrane filters (Amersham, Arlington Heights, IL, USA). The filters were blocked in TBS (25 mM Tris-HCl, pH 7.5, 0.5 M NaCl) containing 2% skim milk (DIFCO) overnight and incubated with primary anti-phosphotyrosine antibody (Promega, Madison, WI, USA) for 24 h. After washing four times (15 min with wash buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 0.5% BSA], 15 min with 1X TBS, 15 min with wash buffer, 15 min with 1X TBS), they were incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Amersham) for 1 h at room temperature. After being washed again, the filters were incubated in enhanced chemiluminescent (ECL) immunodetection reagents and exposed to Hybond-ECL film (Amersham), and developed.

Results

TGF-β acts as a negative growth regulator for HEL cells

TGF-β has been shown to behave as either a positive or a negative regulator for growth of different types of cells (Massague, 1990; Roberts and Sporn,

1990). But it is largely known as a negative growth factor in a wide variety of animal cells (*e.g.*, epithelial, endothelial, neuronal, haematopoietic, lymphoid and some fibroblast cells). Since the effect of TGF-β on actively proliferating HEL cells is unknown, cell proliferation assay in the presence of TGF-β was performed. As shown in Fig. 1, proliferation of HEL cells was inhibited by TGF-β in a dose-dependent manner. The half maximal inhibition of proliferation was observed after 68 h treatment at a concentration of 250 pM TGF-β.

TGF-β induces tyrosine phosphorylation of two cellular proteins, 45 kDa and 15 kDa

In order to study the responsiveness of cellular proteins in the TGF-β action in connection with TGF-β-mediated regulation of tyrosine phosphorylation, we have used the HEL cells, which are permissive to human cytomegalovirus (HCMV) replication, and which have also been used in several experiments showing tumor suppressor protein p53-mediated irradiation-induced cell cycle arrest (Dulic *et al.*, 1994). We especially sought to analyze TGF-β-mediated cellular response in density growth-arrested HEL cells in the growth medium containing low serum content. As shown in Fig. 2, tyrosine phosphorylation of two cellular proteins was induced in HEL cells treated with TGF-β (lane 3 in Fig. 2), but not in cells in the absence of TGF-β (lane 1 in Fig. 2). In the range of 100 μg of total cellular lysates, 45 kDa and 15 kDa proteins were predominantly present. To see whether HCMV infection was also able to induce the similar patterns of tyrosine phosphorylation of cellular proteins, HEL cells infected with HCMV were analyzed for detection of tyrosine phosphorylated protein bands (lane 2 in Fig. 2).

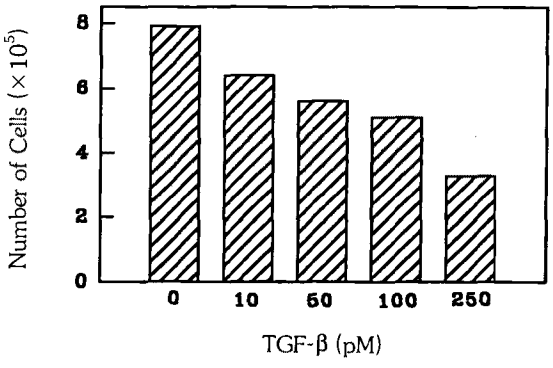

Fig. 1. TGF-β growth inhibition in HEL cells. HEL cells were seeded in 60 mm dishes at a density of 2.7×10^5 cells (25% confluent condition). Twenty four h later, cells were incubated with EMEM containing 0.2% FBS for 5 h. Then fresh EMEM supplemented with 5% FBS was added with different concentration of TGF-β ranging from 10 pM to 250 pM. After 68 h, the cells were released from the dishes and counted. The data are a representive of three independent experiments.

It was revealed that 45 kDa protein was less tyrosine-phosphorylated by HCMV infection compared to that of HEL cells treated with TGF-β. However, 15 kDa protein was much more phosphorylated on tyrosine in HEL cells infected with HCMV than in HEL cells in the presence of TGF-β.

TGF-β regulation of tyrosine phosphorylation of 45 kDa protein

As shown in Fig. 2, TGF-β induces tyrosine phosphorylation of two major cellular proteins in density-arrested HEL cells. To examine whether tyrosine phosphorylated proteins by TGF-β stimulation are regulated in cell cycle-dependent manner, the experiment shown

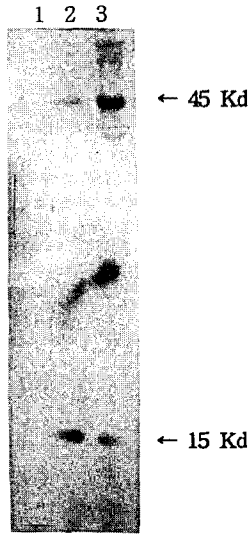

Fig. 2. Induction of tyrosine phosphorylation of cellular proteins by TGF-β treatment. HEL cells in lanes 1, 2, and 3 were grown nearly confluent. The medium was changed with fresh EMEM containing 2% FBS. One hundred g of cell lysates were subjected to reducing SDS-PAGE (15%) and immunoblotted using an anti-phosphotyrosine antibody. Lane 1: mock-infected HEL cells; lane 2: HEL cells infected with HCMV (multiplicity of infection=3 PFU/cell) for 24 h; Lane 3 : HEL cells treated with 100 pM TGF-β for 24 h.

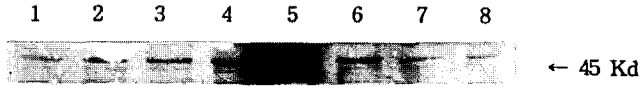

Fig. 3. TGF-β regulates tyrosine phosphorylation of 45 kDa in a cell cycle dependent manner. Proliferating HEL cells were growth arrested with EMEM containing 0.2% FBS for 6 h. Then fresh medium containing different concentration of serum was added with or without 100 pM TGF-β. After 24 h stimulation, cell lysates were prepared and 200 g of cell lysates was subjected to reducing 15% SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody was done. Lanes 1, 2, 3, and 4 were treated with EMEM containing 0.2%, 2%, 6%, and 10% FBS, respectively. Lanes 5, 6, 7, and 8 were treated as those (lanes 1, 2, 3, and 4) in the presence of TGF-β.

in Fig. 3 was performed. The result shown in Fig. 3 indicates that tyrosine phosphorylation of 45 kDa protein was gradually upregulated with increment of serum concentration from 0.2% to 10% in the absence of TGF-β (lanes 1, 2, 3, and 4). However, tyrosine phosphorylation of 15 kDa by TGF-β stimulation was not detectably changed by the differing serum concentration in the medium (data not shown). Surprisingly, the pattern of tyrosine phosphorylation of 45 kDa was contrasted by the presence of TGF-β in the medium. In the presence of TGF-β, phosphorylation of the 45 kDa on tyrosine was by slow degrees decreased as serum concentration increased (Fig. 3, lanes 5 to 8).

To determine the kinetics of TGF-β-regulated-tyrosine phosphorylation of 45 kDa protein, TGF-β was added to proliferating HEL cultures, and the pattern of tyrosine phosphorylation of 45 kDa was determined over the next 36 h period. Relatively similar level of tyrosine phosphorylation was observed for the 6 h and 12 h time points following TGF-β stimulation (data not shown). Level of tyrosine phosphorylation of 45 kDa reached a peak at 24 h post-stimulation, and become undetectable by 36 h.

Tyrosine phosphorylation of 15 kDa protein is regulated by both TGF-β and calcium

To date, a growing body of evidence has shown that calcium is involved in the regulation of cell proliferation as one of the predominant intracellular second messengers. Calcium has been implicated in the proliferative cycle of the quiescent cells as well as for transiting the G1/S, G2/M and metaphase/anaphase boundaries of the cell cycle (Whitaker and Patel, 1990). So it was interesting to determine if tyrosine phosphorylation of 45 kDa and 15 kDa by TGF-β was dependent on different concentration of exogenous calcium. As shown in Fig. 4, 15 kDa was regulated for its tyrosine phosphorylation pattern by higher exogenous calcium concentration. However, tyrosine phosphorylation of 45 kDa was not affected by the exogenous cacium (data not shown). In addition, tyrosine phosphorylation of 15 kDa protein was synergistically induced by low concentration of exogenous calcium (1 mM) in the presence of TGF-β. This may be due to the separate signaling pathways between a TGF-β-mediated signaling pathway resulting in the induction of tyrosine phosphorylation of 15 kDa and a calcium-dependent pathway leading to increase in the tyrosine phosphorylation of 15 kDa protein. When exogenous calcium was increased from 1 mM to 3 mM (lane 3 in Fig. 4) in the absence of TGF-β, upregualtion in tyrosine phosphorylation of 15 kDa was observed. In contrast, when cells were treated with TGF-β in addition to high calcium concentration (3 mM), tyrosine phosphorylation of 15 kDa protein was completely abolished (lane 6 in Fig. 4).

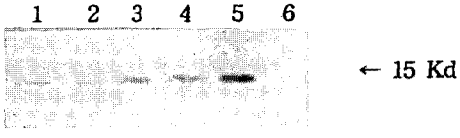

Fig. 4. Induction of tyrosine phosphorylation of 15 kDa protein is regualted by calcium and TGF-β. HEL cells were grown to near confluency and fresh medium containing 2% FBS was added. At the same time, 100 pM TGF-β and different concentration of calcium were added for 24 h. Cell lysates were loaded onto 15% SDS-PAGE and immunoblotting was done using anti-phosphotyrosine antiboty. Lane 1: control HEL cells with normal calcium (1.8 mM $CaCl_2$); lane 2: HEL cells treated with low calcium (1 mM $CaCl_2$); lane 3: HEL cells treated with high calcium (3 mM $CaCl_2$); lane 4: HEL cells treated with 100 pM TGF-β; lane 5: HEL cells treated with 100 pM TGF-β and 1 mM $CaCl_2$; lane 6: HEL cells treated with 100 pM TGF-β and 3 mM $CaCl_2$.

Discussion

Normal cellular proliferation is a complex multiple process which requires the coordinated integration of both positive and negative regulatory growth factors. A unique example in this regard is TGF-β since it is able to stimulate or inhibit cell growth depending upon cell types of animal cells tested (Massague, 1990; Sporn and Roberts, 1990). Very recently the molecular mechanism of activation of TGF-β receptors upon TGF-β ligand binding leading to growth arrest and cellular gene responses has been demonstrated (Wrana *et al.*, 1994).

In this report, we show that TGF-β inhibits proliferation of HEL cells. This result indicates that proliferation of HEL cells treated with TGF-β was less potently inhibited than that of mink lung epithelial (Mv 1Lu) cells, which are much more sensitively growth-inhibited by the same dose of TGF-β (Tucker *et al.*, 1984; Carcamo *et al.*, 1994). Complete inhibition of DNA synthesis of Mv1Lu cells is obtained with 10 pM TGF-β in medium with low serum content and accompanied by an enlarged and flattened cell shape (Boyd and Massague 1989; Laiho *et al.*, 1990). In the majority of cell types tested until now, 100 pM TGF-β concentration was potent more than the half maximal effects (that is, over 50% growth inhibition). This result further reveals that TGF-β may similarly arrest rapidly proliferating HEL cells in the late G1 phase as dominantly described in Mv1Lu cells (Shipley *et al.*, 1986). In density-arrested HEL cells, TGF-β stimulates tyrosine phosphorylation of two cellular proteins (45 kDa and 15 kDa) in the range of 100 μg of total cellular extracts

(Fig. 2). These results are somewhat interesting because the data show a link between a tyrosine protein kinase pathway and a TGF-β signaling pathway. Notably HCMV infection of HEL cells also induced tyrosine phosphorylation of the same sized proteins as those by TGF-β treatment. However it is possible that these two proteins may not be the same proteins.

Serum concentration in the medium was a factor upregulating tyrosine phosphorylation of 45 kDa in the absence of TGF-β. By contrast, in TGF-β-treated cells, TGF-β downregulates tyrosine phosphorylation of 45 kDa proportionally to increasing concentration of serum. One explanation for this could be that TGF-β signaling pathways leading to growth arrest indirectly through induction of tyrosine phosphorylation of 45 kDa are interferred in cells growing in medium containing high serum concentration. In contrast, in the absence of a negative growth regulator such as TGF-β, 45 kDa would rather function as a helper protein in the cell cycle progression or in the cellular action, suggesting its dual functions depending upon the growth condition or cell types tested. But it does not exclude involvement of other unknown functional proteins which might be related to a TGF-β signal transduction pathway, either directly or indirectly regulated by other signaling pathways synergistically or antagonistically. Surprisingly, 15 kDa phosphorylation on tyrosine was not affected by serum concentration in the medium. Thus, we speculate that one of cellular TGF-β targets leading to growth inhibition in HEL cells may be through downregulation of 45 kDa protein phosphorylation on tyrosine. However, future identification of 45 kDa protein will answer our present speculation.

Eblen *et al.* (1994) have shown that $p34^{cdc2}$ was present in high level in contact-growth-arrested Mv1Lu cells which were essentially unaffected by TGF-β. This was due to a reflection that TGF-β acted mainly in the late G1 phase of the cell cycle. Similar results were seen if *Jun* protein synthesis was examined in serum-starved quiescent cells restimulated to initiate cell cycling (Eblen *et al.*, 1994). TGF-β effect on *Jun* synthesis was not seen and this is consistent with the fact that TGF-β acted at a late G1 phase. In our study, since tyrosine phosphorylation of 45 kDa was initially observed in density-arrested HEL cells (Fig. 1), we assumed that in proliferating HEL cells, its regulation of phosphorylation on tyrosine might also be affected by TGF-β if the 45 kDa protein played a role in normal growth regulation of HEL cells. Our results (Fig. 3) suggest that the 45 kDa protein might be a TGF-β responsive growth-regulatory protein and might not necessarily be regulated by a late G1 action of TGF-β.

As discussed earlier, calcium plays pivotal roles in cell proliferation, especially in the reentry of quiescent cells to cell cycling of normal cells (Whitaker and Patel, 1990). In our present study, as shown in Fig. 4, tyrosine phosphorylation of 15 kDa protein was highly upregulated by TGF-β treatment in the presence of low calcium concentration (1 mM) compared to control (lane 1 in Fig. 4) or cells receiving low calcium only (lane 2 in Fig. 4). However, it is not clear how 15 kDa protein band phosphorylated on tyrosine is abolished when cells receive high exogenous calcium (3 mM) in the presence of TGF-β. Taken together, although the mechanisms regulating this difference are not apparent at the present time, it may not be a simple reflection of the differing cell passage numbers and cell culture conditions used. It is conceivable that over certain level (1 mM) of calcium, the TGF-β signaling pathway either directly or indirectly leading to upregulation of tyrosine phosphorylation of 15 kDa may interfere with the calcium dependent signaling pathway via working through other signaling pathways and vice versa.

One of the important issues that should be considered is the protein synthesis level rather than just the level of tyrosine phosphorylation of cellular proteins. We don't know the level of protein synthesis of 45 kDa and 15 kDa phosphorylated on tyrosine in intact cells upon TGF-β administration. However, for instance, in contact-growth arrested cells, thymidine kinase (TK) gene expression is under transcriptional control, whereas in proliferating cells, its expression is primarily regulated posttranscriptionally (Eblen *et al.*, 1994). As in this case, it is probable that different levels of tyrosine phosphorylation of 45 kDa and 15 kDa depending upon the assay conditions may not be due to the different protein synthesis levels but to the different quantity of phosphorylation on tyrosine in these proteins. Considering altogether the pivotal role that 45 kDa and 15 kDa may play in cell growth, it would be expected that regulation of 45 kDa and 15 kDa phosphorylation on tyrosine would be much more complex than we thought.

Our hypothesis provides the first demonstration, in an intact cell, of TGF-β-stimulated tyrosine phosphorylation of two major cellular proteins. Identification of two tyrosine phosphorylated proteins should elucidate the relationship between these two proteins and the mechanism of TGF-β signaling and also help understand the global aspects of TGF-β targets of action.

Acknowledgements

This work was supported by a research grant from KOSEF (961-0525-018-2) to C. H. Lee. We thank Drs. Quae Chae, Soo-Young Choe, Seung-Taik Kim for their advice and comments on the manuscript.

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