

# Lens Epithelium-derived Growth Factor Relieves Transforming Growth Factor- $\beta$ -induced Transcriptional Repression of Heat Shock Proteins in Human Lens Epithelial Cells\*

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Lens epithelium-cell derived growth factor (LEDGF) is a transcriptional activator. It protects the cells by binding to *cis*-stress response ((A/T)GGGG(T/A)), and heat shock (HSE; nGAAn) elements in the stress genes and activating their transcription. Transforming growth factor- $\beta$  (TGF- $\beta$ ) has been implicated in the control of tissue homeostasis, terminal differentiation, and apoptosis. Here we provide evidence that TGF- $\beta$ 1 down-regulates LEDGF expression and diminishes its affinity for DNA during TGF- $\beta$ 1-induced phenotypic changes and apoptosis in human lens epithelial cells. Surprisingly, TGF- $\beta$ 1 treatment for 48 h markedly decreased the LEDGF, Hsp27, and  $\alpha$ B-crystallin promoter activities with the decrease of abundance of LEDGF mRNA and protein. Deletion mutants of the LEDGF promoter showed that one TGF- $\beta$ 1 inhibitory element (TIE) like sequence nnnTTGGnnn (–444 to –433) contributed to this negative regulation. Mutation of TIE (TTGG to TATT) abolished the down-regulation of the LEDGF promoter. Gel mobility and supershift assays showed that LEDGF in the nuclear extracts of TGF- $\beta$ 1-treated human lens epithelial cells did not bind to stress-response elements and HSE. The TGF- $\beta$ 1-induced down-regulation of LEDGF, Hsp27, and  $\alpha$ B-crystallin promoters activity was reversed by cotransfection with a plasmid expressing LEDGF. Because overexpression of LEDGF was able to relieve TGF- $\beta$ 1 and/or stress-induced changes, it would be a candidate molecule to postpone age-related degenerating disorders.

Transforming growth factor  $\beta$  (TGF- $\beta$ )<sup>1</sup> is a multifunctional regulator of cell growth and differentiation. It was originally

identified for its ability to induce phenotypic transformation of fibroblasts in diverse cell types (1). Several isoforms of TGF- $\beta$  have been identified to date (2–4), each a product of a separate but closely related gene. This protein has also been found to be an important regulator of extracellular matrix synthesis and degradation, suggesting a potentially critical function for this growth factor in the regulation of tissue formation and repair. TGF- $\beta$  has been reported in the aqueous humor of human, pig, rabbits, cows, and mice (5–9) and implicated in the mechanisms of cataract formation. A variety of cells express the TGF- $\beta$  receptor. The presence of TGF- $\beta$  receptors (TGF- $\beta$  RI and RII) has been monitored in lens tissue (10). TGF- $\beta$ 1, which is present in the aqueous humor and vitreous (11), has been shown to induce cataracts at relatively high concentrations (12). Furthermore, McAvoy and co-workers (13) reported that TGF- $\beta$ 1 could induce cataractous changes in rat lens epithelial explants. These include the formation of spindle-shaped cells, accumulation of extracellular matrix, and cell death with features of apoptosis (14, 15). Some or all of these changes are typically found in the human subcapsular cataract, both the anterior and posterior forms and after the cataract (posterior capsular opacification). Two molecular markers for these forms of cataract,  $\alpha$ -smooth muscle actin and type I collagen, are induced (16).

Cloned lens epithelium cell-derived growth factor (LEDGF) is a novel growth and survival factor and transcriptional activator. It is found at low levels in most actively dividing cells. Cells expressing higher level of LEDGF survive remarkably well against a wide variety of stress (17–22). Notably, thermal and oxidative stresses up-regulate the LEDGF mRNA and protein expression (23). Based on cultured cell lines and our *in vivo* studies, overexpression of LEDGF gives selective survival advantage in growing cells by blocking death pathways (17–22, 24). The survival of many cell types seems to be due to the LEDGF activation of stress-associated genes such as Hsp27, -25, and -90, and  $\alpha$ B-crystallin (17, 19–21, 25), and these Hsps are now known to be negative regulators of apoptosis (26, 27). LEDGF binds to the stress-related element (STRE; consensus sequence ((A/T)GGGG(A/T)) and the heat shock element (HSE; consensus sequence nGAAn) present in the stress-associated genes and up-regulates their transcriptions (22, 25).

Progression of disease such as aging or cataractogenesis is driven by the progressive effects of environmental or physio-

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<sup>1</sup> The abbreviations used are: TGF- $\beta$ , transforming growth factor- $\beta$ ; LEDGF, lens epithelium-derived growth factor; TIE, transforming growth factor- $\beta$  inhibitory element; Hsps, heat shock proteins; CAT, chloramphenicol acetyltransferase; HSE, heat shock element; STRE, stress-response elements; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; EGFP, enhanced GFP; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; hLECs, human lens epithelial cells; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DAPI, 4,6-diamidino-2-phenylindole;

ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; TUNEL, terminal dUTP nick-end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase;  $\alpha$  sm-actin,  $\alpha$  smooth muscle actin.

logical stresses, including growth factors and their subsequent effect on sequential suppression of counteracting gene products, and at each stage in the progression, sequence suppression in growth regulatory genes and/or attenuation of antioxidant agents may occur. Thus, the unique pressure is being placed on lens epithelial cells that normally involve the induction of cataractogenesis by attenuating the function of survival factors. LEDGF is a survival factor, and its deactivation by UV or H<sub>2</sub>O<sub>2</sub> or TGF- $\beta$  may down-regulate Hsp27 and  $\alpha$ B-crystallin. Thus we hypothesized that during cell death and/or cataractogenesis, the LEDGF gene or its gene product is attenuated. TGF- $\beta$ 1 induced apoptosis in various cell types including hLECs (14, 28); hence we presumed the possible role of caspases in degradation of LEDGF that, in turn, may reduce the DNA binding property of LEDGF. Recently, it has been reported (29) that LEDGF contained three cleavage sites for caspase-3 and -7.

Moreover, earlier studies have documented that TGF- $\beta$  played a regulatory role in the modulation of the transcription of various genes (30–38), and some genes contained TIE (30–33, 39, 40). Several reports (30, 41–45) have demonstrated that TIE (nnnTTGnnn) is well conserved in various gene promoters of vertebrates. Kerr *et al.* (30) have shown that a 10-bp element in the transin promoter is required for the TGF- $\beta$ 1 inhibitory effects and that this sequence is conserved in the promoter regions of several other TGF- $\beta$ 1-inhibited genes. TIE specifically binds to a nuclear protein complex from TGF- $\beta$ 1-rat fibroblast, and this complex contained the *c-fos* proto-oncogene product, Fos, and induction of Fos expression was required for the inhibitory effect of TGF- $\beta$ 1 on gene expression (30, 39, 40). Because LEDGF gene expression is inhibited in hLECs treated with TGF- $\beta$ 1, we predict that LEDGF promoter bears TIE. An analysis of the LEDGF promoter revealed that it has various regulatory elements as follows: heat shock and stress-response elements, VDR/RXR (vitamin D receptor/retinoid X receptor), STAT, E2F, OCT1, GRE, SP1, GATA-1, IRF-1 and IRF-2, etc. (46, 47), including the TIE sites at -444 to -433 from the transcription start site.

In the studies presented here, we examined the regulation of LEDGF expression by TGF- $\beta$ 1 in hLECs, and we have shown it as a model to study the TGF- $\beta$ 1-mediated insults in hLECs or lenses that may lead to a type of cataractogenesis. We demonstrated that TGF- $\beta$ 1 induced phenotypic changes, expression of  $\alpha$  smooth muscle actin ( $\alpha$  sm-actin), suppression of Hsps in hLECs, and also apoptosis, and that these changes were associated with the repression of transcription and reduction of protein of LEDGF. Thus, this investigation addresses the mechanism by which TGF- $\beta$ 1 inhibits the LEDGF gene, and our findings add weight to the hypothesis that LEDGF plays a regulatory role in determining the fate of hLECs.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—Human lens epithelial cells (hLECs) (a gift of Dr. V. N. Reddy) were maintained routinely in our laboratory following the method as described elsewhere (48). Briefly, cells were cultured in a 75-mm tissue culture flask in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100  $\mu$ g/ml streptomycin, and 100  $\mu$ g/ml penicillin in a 5% CO<sub>2</sub> environment at 37 °C following the standard methods. Cells were trypsinized (0.25%) in phosphate-buffered saline (PBS) containing 1 mM EDTA-Na to separate them from the bottom of the flask and plated at a density of  $5 \times 10^5$  in 60-mm plates or  $1 \times 10^6$  in 100-mm plates depending on the experiment.

To examine the effect of TGF- $\beta$ 1 on hLECs, cells were plated ( $5 \times 10^5$ /well) in 6-well plates (Falcon) and cultured in complete medium (DMEM + 20% FBS). 24 h later, the cells were washed twice with DMEM without FBS and treated with TGF- $\beta$ 1 (R & D Systems, Minneapolis, MN) at different concentrations (5.0, 1.0, 0.5, and 0.25 ng/ml

in 0.2% bovine serum albumin) for 24 and 48 h. MTS, DAPI staining, and photomicrography of hLECs were performed after TGF- $\beta$ 1 treatment, and the results were recorded.

**Cell Survival Assay (MTS Assay)**—A colorimetric MTS assay (Promega) was performed as described by Barltrop and Owen (49). This colorimetric assay of cellular proliferation uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 to 4-sulfophenyl-2H-tetrazolium salt (MTS; Promega, Madison, MI), when added to medium containing viable cells, and MTS is reduced to a water-soluble formazan salt. The absorbance at 490 nm was measured after 4 h with an ELISA reader.

**DAPI Staining**—DAPI staining was done as described by Bonifacio *et al.* (50). Briefly, cells were grown in complete medium overnight, and the next day, cells were washed and treated with TGF- $\beta$ 1. Untreated culture was served as control. After treatment for 24 h, cells were washed once with PBS and were fixed in 70% ethanol. Cells were washed with PBS and thereafter incubated in DAPI solution for 30 min at room temperature, washed, and mounted. The morphology of the nuclei of the cell was observed under a fluorescent microscope at UV excitation at 350 nm. After photomicrography, apoptotic nuclei were identified by condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. Untreated culture was also photographed that served as control.

**TUNEL Staining**—TUNEL staining was carried out using *in situ* cell death detection kit, fluorescein (Roche Diagnostics) following the company's protocol. Briefly, TGF- $\beta$ 1-treated and -untreated cells were washed with PBS and fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.4), followed by incubation in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Cells were rinsed twice with PBS and incubate in TUNEL reaction mixture for 60 min at 37 °C in the dark. Cells were rinsed with PBS three times, and after mounting, samples were micro-photographed using a microscope (Nikon, ECLIPSE TE 300) and analyzed. The percentage of apoptotic cells per total cells (100 cells) in each 5 fields per slide was determined from two independent experiments.

**Western Blot Analysis**—Western analysis was performed following the methods of Singh *et al.* (17) and Hales *et al.* (16). Cells treated with or without TGF- $\beta$ 1 (1 and 5 ng/ml) were washed three times with PBS, harvested, and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture tablets (Roche Diagnostics). Cells were disrupted in a syringe fitted with a 21-gauge needle, and the cell debris was removed by centrifugation at  $10,000 \times g$  for 15 min at 4 °C. Protein samples were prepared in SDS-PAGE sample buffer. Equal amounts of protein were loaded onto a 10% SDS-PAGE and blotted onto polyvinylidene difluoride membrane (Immobilon™-P, Millipore Corp, Bedford, MA), and blocking was done with 5% nonfat dry milk in 0.1% Tween 20 in PBS and then incubated overnight at 4 °C either with mouse monoclonal antibody to LEDGF (BD Biosciences) as primary antibody at 1:1000 dilution or with mouse anti- $\alpha$  sm-actin at 1:400 dilution (Sigma). The filters were washed three times with PBS-T and incubated with anti-mouse IgG labeled with horseradish peroxidase (1:1500 dilution) as a second antibody. The specific protein band was then visualized by incubating the membrane with luminol reagent (Santa Cruz Biotechnology) and exposing the membrane to film (X-Omat, Eastman Kodak Co.). To ascertain comparative expression and equal loading of these protein samples, the membrane stained earlier with anti-LEDGF monoclonal antibody was stripped and re probed with  $\alpha$  sm-actin antibody.

**Construction of LEDGF in Eukaryotic Expression Vector**—A construct containing a green fluorescent protein (GFP) and LEDGF cDNA was generated with the "living color system" (Clontech, Palo Alto, CA) using the plasmid vector pEGFP-C1 (GFP-vector) for eukaryotic expression (17). This construct was used to generate stable eukaryotic cells that overexpressed LEDGF (GFP-LEDGF). Cells transfected with the empty vector served as a control.

**Reverse Transcription-PCR**—RT-PCR was conducted following the method as reported earlier (23). Briefly, we synthesized the primers specific to TGF- $\beta$  RI and RII and used them in this study. To perform RT-PCR, either total RNA or mRNA was extracted from hLECs after treatment with or without TGF- $\beta$ 1 (1 ng/ml). Total RNA was extracted using the single step guanidine thiocyanate/phenol/chloroform extraction method (Trizol Reagent; Invitrogen), and mRNA was isolated with the Micro-Fast Track™ kit (Invitrogen) from  $5 \times 10^5$  hLECs. The Micro-fast track 2 (Invitrogen), a cDNA synthesis kit for RT-PCR, was used to synthesize cDNA from mRNA. The resulting cDNA was used for different cycles in PCR. After denaturation for 2 min at 95 °C, 30 cycles of PCR amplification (denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and elongation at 72 °C for 3 min) were carried out,



TABLE I  
Primers used in engineering for LED-CAT constructs

Primers taken for the construction of sequential deletion mutants of LEDGF promoter. Restriction sites (*Mlu*I and *Nhe*I) and cleavage sites are shown for each primer. 3'-Primer was common in all the constructs.

Constructs	Forward Primer 5' $\rightarrow$ 3'	Position in gene
B-CAT	<i>Sac</i> I CTTTACCTGAGCTCCCAGGTAAAGC	-1461 to +35 <sup>a</sup>
C-CAT	<i>Mlu</i> I CCTTTTCCTACGCGTAATTTATAGCC	-1285 to +35 <sup>a</sup>
D-CAT	<i>Mlu</i> I GTTTTCTACCTACGCGTTTGTG	-482 to +35 <sup>a</sup>
E-CAT	<i>Mlu</i> I CTGCAACCCACGCGTGCTCCAATTTTC	-315 to +35 <sup>a</sup>
	Reverse primer GAGGAAGACGCTAGCGAAGAAG* <i>Nhe</i> I	

<sup>a</sup> Indicates position of reverse primer in LEDGF gene.

followed by a final extension for 7 min at 72 °C. The sequences of oligonucleotide probes were as follows: TGF- $\beta$  RI sense primer, 5'-CAC CTG GCC TTG GTC CTG TG-3', and antisense primer, 5'-CCT CAC GGA ACC ACG AAC GTT generating a 396-bp, TGF- $\beta$  RII sense primer, 5'-CAG AGA AGG ACA TCT TCT CAG GAC-3', and TGF- $\beta$  RII antisense primer, 5'-CTC CCA CTG CAT TAC AGC GAG ATG-3' generating a 484-bp fragment, were used.

**Northern Blot (RNA) Analysis**—Total RNA was extracted from hLECs with or without treatment of TGF- $\beta$ 1 for different time intervals using the single step guanidine thiocyanate/phenol/chloroform extraction method (Trizol Reagent; Invitrogen), and mRNA was isolated with the mRNA purification kit (Amersham Biosciences) according to the manufacturer's instructions. Approximately 10  $\mu$ g of poly(A)-RNA was fractionated by electrophoresis through a 1% agarose-formaldehyde gel. RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences) and hybridized using Rapid-hyb buffer (Amersham Biosciences) to an 846-bp *Eco*RI-*Xho*I restriction fragment from 3'-LEDGF cDNA. The probe was labeled by using the NEBlot® Kit using Random Primers (New England Biolabs, Beverly, MA). Prehybridization was performed at 65 °C for 1 h, and hybridization were conducted at 65 °C for 4 h following the company protocols. Membranes were washed and autoradiographed. Membranes were exposed for autoradiography on x-ray film (Fugi X-Ray) at -70 °C with an intensifying screen for 16 h. The filter from this experiment was stripped and used to reprobe with a p32-labeled GAPDH probe (Ambion).

**Preparation of Lens Epithelial Cell Nuclear Extract**—Human lens epithelial cell nuclear extract was prepared as described earlier (22) with certain modifications. Briefly, human LECs ( $1 \times 10^6$ ) were cultured in 100-mm culture plates. The cells treated with or without TGF- $\beta$ 1 (1 and 5 ng/ml) were washed gently with chilled PBS (pH 7.2). Cells were collected by centrifugation and resuspended in 5 pellet volumes of cytoplasmic extract buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, adjusted to pH 7.6). After a short incubation on ice, the cytoplasmic extract was removed from the pellet. Following careful washing with cytoplasmic extract buffer without detergent (Nonidet P-40), the fragile nuclei were resuspended in nuclear extract buffer (20 mM Tris-HCl, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2% EDTA, 1 mM phenylmethylsulfonyl fluoride, and 25% (v/v) glycerol, adjusted to pH 8.0). Salt concentration was adjusted to 400 mM using 5 M NaCl, and the extract was incubated on ice for 10 min with occasional vortexing. Finally, the extract was spun at 14,000 rpm for 30 min to pellet the nuclei. After dialysis, protein was estimated according to the Bradford method (51), and the extract was used for EMSA.

**Electrophoretic Mobility Shift Assay (EMSA)**—LEDGF physically and functionally binds to HSE and STRE (25). In the present study, we performed EMSA with nuclear extracts from hLECs with or without treatment with TGF- $\beta$ 1 to understand whether TGF- $\beta$ 1 attenuates the DNA binding property of LEDGF. For this, oligonucleotides having HSE (nGAAn) and STRE ((T/A)GGGG(A/T)) were synthesized chemically (Invitrogen). Sequences were annealed and end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (New England Biolabs, Inc.). The binding reaction was performed in 20  $\mu$ l of binding buffer containing 20 mM Tris-HCl (pH 8.0), 75 mM KCl, 5% glycerol, 50  $\mu$ g/ml bovine serum albumin, 0.025% Nonidet P-40, 1 mM EDTA, 5 mM dithiothreitol, and 1  $\mu$ g of poly(dI/dC). Five fmol (1000 cpm) of the end-labeled probe was incubated on ice for 30 min with 5  $\mu$ g of nuclear extract. Samples were then loaded on 5% polyacrylamide gel in 0.5 $\times$  TBE buffer for 2 h at 10 V/cm. The gel was dried and autoradiographed. In competition assays, 1000-fold molar excesses of cold probe were added. For the supershift assay, 1  $\mu$ l of rabbit anti-LEDGF antibody was added to the tube containing the probe and nuclear extract. The mixture was incubated

for an additional 30 min while the complexes formed, and then EMSA was performed as described earlier (22).

**Preparation of LEDGF Promoter-CAT Construct**—5'-Flanking region of LEDGF gene was isolated and sequenced. A construct of -5139 bp was prepared by ligating it to basic pCAT vector (Fig. 6A, construct B). Similarly, constructs of different sizes (Fig. 6A, construct B, -1461, C; -1285, D; -482, E; -315 to +35) of LEDGF promoter were prepared with appropriate sense primers bearing *Sac*I or *Mlu*I and antisense with *Nhe*I (see Table I) and ligated into pCAT-Basic vector (Promega, Madison, WI) using appropriate restriction enzymes. The plasmid was amplified and used for CAT assay.

**Site-directed Mutagenesis**—PCR-based site-directed mutagenesis was performed using the Quickchange™ Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) following the company's protocol. We made mutations in TGF- $\beta$  inhibitory element sites (TTGG changed to TATT). Briefly, the double-stranded LEDGF promoter construct D was used as template DNA with a pair of complementary primers used to mutate the LEDGF promoter construct with PCR. The primers used for mutation are as follows: 5'-CTT AAT AGT TCT ATT TGA TGC AAA TGC-3'; 3'-GCA TTT GCA TCA AAT AGA ACT ATT AAG-5'.

**Construction of Hsp27 and  $\alpha$ B-crystallin-CAT**—Hsp27-CAT and  $\alpha$ B-crystallin-CAT constructs were engineered as described previously (25). Briefly, the 5'-flanking region of the human Hsp27 gene was isolated with the genomic PCR kit (Clontech, Palo Alto, CA) using specific Hsp27 primers. Forward primer with the *Sac*I site (5'-GCGTC-GAGCTCTCGAATTCATTGCTT-3') and reverse primer with the *Xho*I site (5'-GCTCTCGAGGTCTGCTCAGAAAAGTGC-3') were used to generate the fragment and cloned between the *Eco*RI sites of TA vector (Invitrogen). Similarly a fragment of the 5'-flanking region of human  $\alpha$ B-crystallin promoter (a gift from Dr. Piatigorsky, NEI, National Institutes of Health) was prepared using specific primers. The forward primer having a *Sac*I site (5'-CTCTCTTCCAAGAGCTCACAAAG-3') and the reverse primer having an *Xho*I site (5'-ATGGTGGCTACTC-GAGAGTGA-3') were used to generate the above fragment and cloned between *Eco*RI sites of the TA vector. These Hsp27 and  $\alpha$ B-crystallin/TA constructs were digested with *Sac*I and *Xho*I (-214 to +21), and the promoter fragments were ligated with pCAT-Basic vector (Promega, Madison, WI) using appropriate restriction enzymes.

**Transfection and Chloramphenicol Acetyltransferase Assay (CAT Assay)**—CAT assay was carried out using a CAT-ELISA (Roche Diagnostics) kit. hLECs were cultured at a density of  $5 \times 10^5$  cells in 5 ml of DMEM containing 20% FBS per 60-mm Petri dish in a 37 °C incubator containing 5% CO<sub>2</sub>. 24 h later, the cells were washed with the same medium and transfected/co-transfected with LipofectAMINE PLUS Reagent (Invitrogen) with 2  $\mu$ g of promoter/CAT reporter construct and/or EGFP expression vector and 1  $\mu$ g of the pSV- $\beta$ -galactosidase vector. After 48 h of incubation, cells were harvested, and extract was prepared and normalized. CAT-ELISA was performed to monitor CAT activity following the company's protocol. The absorbance was measured at 405 nm using a microtiter plate ELISA reader. To examine the effect of TGF- $\beta$ , transfected and untransfected cells were treated with TGF- $\beta$ 1 at 1 ng/ml for 48 h.

## RESULTS

**TGF- $\beta$  RI and RII Present in Human Lens Epithelial Cell**—To investigate whether TGF- $\beta$  RI and RII receptors were present in human lens epithelial cells (48), we synthesized primers as stated under "Experimental Procedures," and RT-PCR was carried out. We found that both TGF- $\beta$  receptors were present in these cells. The bands of RI (396 bp) and RII (484 bp) could be seen in agarose gel as expected (Fig. 1, A and B).

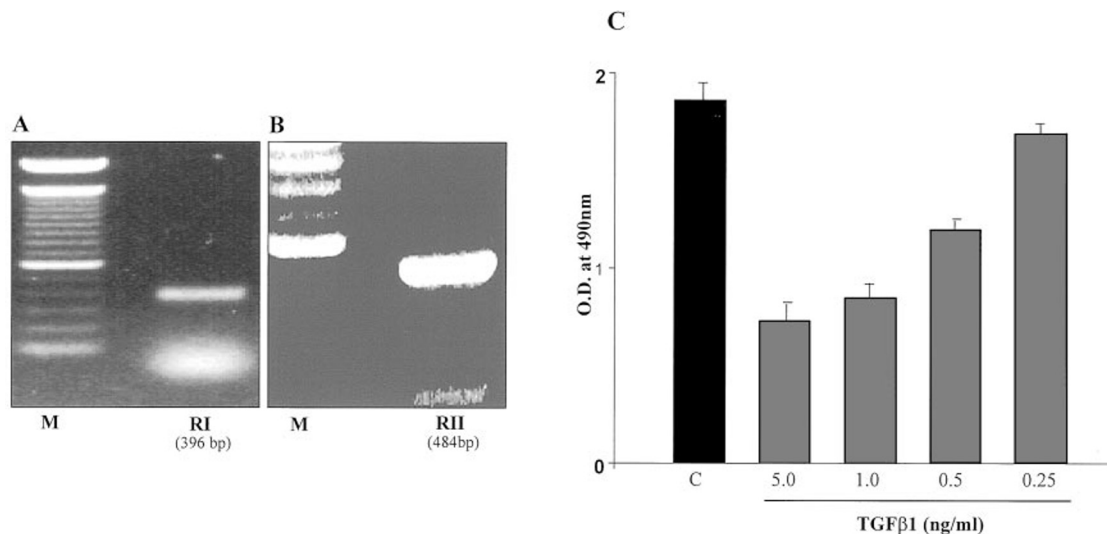


FIG. 1. *A* and *B*, RT-PCR showing the presence of TGF- $\beta$  RI (396 bp) and RII (484 bp) receptors in human lens epithelial cells. mRNA was isolated from hLECs and subjected to RT-PCR with primers specific to RI and RII (see "Experimental Procedures"). Resulting products were visualized on agarose gel with EtBr staining. *M* represents the 100-bp DNA marker (Invitrogen). *C*, MTS assay showing cell viability after TGF- $\beta$ 1 treatment. Human LECs were treated with different concentrations of TGF- $\beta$ 1 for 48 h, and cell viability was assessed by MTS assay. *Black* and *gray bars* represent the untreated control and hLECs treated with TGF- $\beta$ 1, respectively (*C*).

**TGF- $\beta$ 1 Treatment Induced the Phenotypic Changes, Apoptosis, and Expression of  $\alpha$  sm-actin**—HLECs were exposed for 48 h to the various concentrations (5.0, 1.0, 0.5, and 0.25 ng/ml) of TGF- $\beta$ 1 to induce the alterations in these cells, and then MTS assay was performed. A concentration of 1 ng/ml TGF- $\beta$ 1 was sufficient to inhibit the cellular growth (Fig. 1C) and induce phenotypic changes and apoptotic cell death. In this experiment we were able to reproduce the results as reported by others previously (41, 47).

Next, to confirm that TGF- $\beta$ 1 induced apoptotic cell death in hLECs, we performed DAPI staining of the cells incubated with or without TGF- $\beta$ 1. Cells were photomicrographed and analyzed (Nikon, ECLIPSE TE 300). A typical apoptotic nucleus (the fragmented and disintegrated nucleus) could be observed in TGF- $\beta$ 1-treated cells (Fig. 2C, *arrows* demonstrate the apoptotic bodies as well as the dense chromatin). We also demonstrated apoptotic cell death using the *in situ* Death Detection kit (see "Experimental Procedures"). It is evident from the analysis of Fig. 2, *D* and *E*, that TGF- $\beta$ 1 induced apoptotic cell death (~40%) in hLECs.

Phase contrast microscopy was performed to analyze the phenotypic changes after TGF- $\beta$ 1 treatment. Treated cells were clearly distinguishable from the untreated controls; they became elongated and fiber-like, formed cellular aggregates, and packed irregularly. They detached more frequently and manifested increased intercellular spaces (Fig. 3, *C* and *D*). The changes in the treated cells appeared after 24 h of treatment that became more apparent after culture. Here we present photomicrographs taken at 48 h. *Arrows* indicate dead cells (Fig. 3, *C* and *D*).

To verify that these cells expressed  $\alpha$ -smooth muscle actin following TGF- $\beta$ 1 treatment, proteins were extracted from untreated and treated (1 and 5 ng/ml) cells, and Western analysis was performed using an  $\alpha$  sm-actin-specific antibody. A protein band of 42 kDa could be detected in the treated cells (Fig. 4, *B*, lower panel, lanes 2 and 3, and *C*, lanes 2 and 3).

**TGF- $\beta$ 1 Down-regulates LEDGF Expression in Human Lens Epithelial Cells**—In order to test whether LEDGF plays a crucial role in the cellular changes induced by TGF- $\beta$ 1, we investigated the effect of TGF- $\beta$ 1, on LEDGF mRNA, and protein expression in hLECs. Northern blot analysis was performed with mRNA isolated from hLECs at different time

intervals (3, 12, 24, or 48 h) with or without treatment of TGF- $\beta$ 1. The intensity of hybridization of the labeled probe to LEDGF mRNA was significantly reduced in the cells incubated for longer times with TGF $\beta$ 1 (Fig. 4A, *upper panel*). Control tests showed no decrease in GAPDH mRNA after treatment with TGF- $\beta$ 1 (Fig. 4A, *lower panel*). Results indicated that TGF- $\beta$ 1 reduced the expression of LEDGF mRNA in hLEC, and the maximum reduction could be detected at 48 h following the treatment of TGF- $\beta$ 1 (Fig. 4A, *lane 4*).

Next, to determine whether LEDGF protein was affected by TGF- $\beta$ 1 treatment, we performed Western analysis. The extracted protein from treated and untreated cells was blotted onto nitrocellulose membranes following electrophoresis as described (see "Experimental Procedures"). To ascertain the equal loading and relative expression level of LEDGF and  $\alpha$  sm-actin protein, we continued with the same blotted membrane and re-probed with LEDGF monoclonal antibody and then immunostained. The results revealed reduced LEDGF protein expression (Fig. 4B, *upper panel*, lanes 2 and 3) after treatment and consistent with suppression of the LEDGF gene expression by TGF- $\beta$ 1 (Fig. 6). Furthermore, in another experiment we immunostained blotted membrane with  $\alpha$  sm-actin and anti-LEDGF monoclonal antibody simultaneously (Fig. 4C, *lanes 1–3*), and we found reduction in LEDGF protein level and increase expression of  $\alpha$  sm-actin protein (Fig. 4C) following treatment. Thus we confirmed from the above experiments that LEDGF protein is selectively and specifically reduced, because TGF- $\beta$ 1 enhanced the expression of  $\alpha$  sm-actin.

**TGF- $\beta$ 1-mediated Loss of DNA Binding Activity of LEDGF**—To determine whether TGF- $\beta$ 1-mediated alterations in hLECs resulted in the loss of DNA binding activity of LEDGF, nuclear extracts from untreated and TGF- $\beta$ 1-treated cells were examined on EMSA. Because LEDGF exerts its survival function by the activation of transcription of stress-related genes including  $\alpha$ B-crystallin and Hsp27 via binding through HSE (nGAAn) and STRE ((T/A)GGGG(A/T)), we commercially synthesized the oligomers having these sites (Fig. 5) and utilized them for DNA binding affinity of LEDGF in nuclear extract isolated from TGF- $\beta$ 1-treated and control cells. Nuclear extract from control cells forms a shifted band designated as Cm1 (Fig. 5, *A* and *B*, *lane 1*) with LEDGF consensus DNA-binding sequence having STRE. The Cm1 complex that appeared in the lanes was

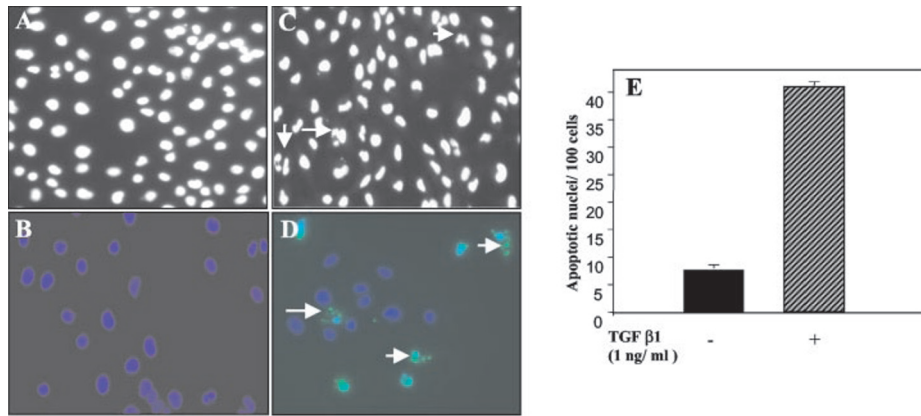


FIG. 2. **Detection of apoptosis by DAPI staining and TUNEL assay.** Human LECs treated with or without TGF- $\beta$ 1 (1 ng/ml) were incubated overnight and stained with DAPI (see "Experimental Procedures"). Parallel cultures were carried out and used for TUNEL assay. Photomicrographs of the stained cells were taken. Arrows point to apoptotic cells. A and C, DAPI staining. B and D, TUNEL staining. A and B, untreated controls. E, histogram showing the percentage of apoptotic nuclei in hLECs treated (striped bar) and untreated (black bar) with TGF- $\beta$ 1 (1 ng/ml).

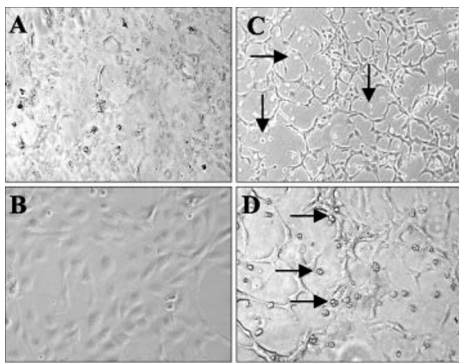


FIG. 3. **Photomicrograph of hLECs treated with TGF- $\beta$ 1 (1 ng/ml).**  $5 \times 10^5$  cells were cultured for 24 h in DMEM containing 20% FBS and then washed. The medium was changed to 0.2% bovine serum albumin in DMEM with or without TGF- $\beta$ 1 (1 ng/ml). A and B, untreated control; C and D, TGF- $\beta$ 1 treatment for 48 h. B and D are higher magnifications ( $\times 400$ ) of A and C. Arrows indicate dead cells.

supershifted and formed a band after the addition of LEDGF-specific antibody Ss1 (Fig. 5, A, lane 2, and B, lanes 2, 4, and 6). In contrast, LEDGF in nuclear extract from TGF- $\beta$ 1-treated cells did not bind (Fig. 5A, lanes 4 and 5) to the STRE probe. A band (Ns) appeared in all lanes (Fig. 5, A, lanes 1–5, and B, lanes 1–6) with approximately the same intensity suggesting as it is nonspecific. This band was used to show equal loading because it remains constant even after TGF- $\beta$ 1 treatment and to demonstrate that not all nuclear proteins in TGF- $\beta$ 1 treated cells have lost their DNA binding property. Additionally, the Cml band was out-competed by 1000-fold excess of cold probe (Fig. 5A, lane 3) demonstrated its LEDGF binding specificity. Similar results were obtained with HSE probe (Data not shown).

To know whether cells overexpressing LEDGF could abolish TGF- $\beta$ 1-induced attenuation of DNA binding, nuclear extracts from the cells treated with TGF- $\beta$ 1 were used in EMSA with the same probe. Our results revealed that overexpressed LEDGF reversed the TGF- $\beta$ 1 effect (Fig. 5B, lanes 3 and 5). Cells overexpressed with LEDGF when treated with higher concentrations (5 ng/ml) of TGF- $\beta$ 1 could slightly reduced the DNA binding of LEDGF (Fig. 5B, lane 3).

**TGF- $\beta$  Down-regulates the Promoter Activity of LEDGF Gene in Human Lens Epithelial Cells**—Several reports (31, 39) have shown that TGF- $\beta$ 1 down-regulates gene expression and that reduced expression is mediated at the level of gene transcription. Here we wished to determine whether the TGF- $\beta$ 1 inhi-

bition of the LEDGF gene expression is induced by a distinct cis-acting transcriptional element. We engineered a series of mutation deletion constructs using PCR of the LEDGF promoter linked to a CAT bacterial reporter gene (Figs. 6 and 7). These constructs were transfected into hLECs, and cells were treated with TGF- $\beta$ 1 at a dose level of 1 ng/ml for 48 h. Cells were harvested and assayed for CAT activity. Transfection with constructs from –5139 to –315 bp of the LEDGF promoter showed inhibition in CAT activity by TGF- $\beta$ 1 (Fig. 6, cross-hatched bars). These data suggested that inhibition of the LEDGF gene by TGF- $\beta$ 1 is mediated by a distinct DNA element located in construct D (–482 to +35 bp). The sequence in the LEDGF promoter construct D was then analyzed and compared with the DNA sequences of other genes known to be inhibited by TGF- $\beta$ 1 (Table II). Each of these genes has been reported previously to be inhibited by TGF- $\beta$ 1. A comparison to these sequences suggested that the consensus sequence for the putative TGF- $\beta$ 1 inhibitory element (TIE) is nnnnTTGGnnn (–444 to 433). These results suggest that the mechanism of repression of LEDGF gene expression may be similar as reported previously by other investigators for other genes (Table II). To confirm whether the putative TIE (–444 to –433) sequence in the LEDGF promoter is indeed responsible for the inhibition of LEDGF transcription, we performed site-directed mutagenesis to alter the TTGG site to TATT, and we performed CAT assay.

**TGF- $\beta$ 1 Down-regulates LEDGF Promoter Activity via TIE**—To understand the contribution of TIE, if any, in transcriptional repression of LEDGF, we altered TTGG to TATT. We found that the mutated construct abolished the TGF- $\beta$ 1 effect on LEDGF gene transcription (Fig. 8, hatched and dotted bars). These results revealed that the repressive effect of TIE could be ablated in the mutant constructs following TGF- $\beta$ 1 treatment and suggested that the TIE present in the LEDGF promoter was indeed functional and that TGF- $\beta$ 1 acted through TIE.

**Down-regulation of Hsp27 and  $\alpha$ B-crystallin Is Largely Derived from Reduction of DNA Binding of LEDGF**—In the present study, it is clear from the results that 48 h of treatment with TGF- $\beta$ 1 decreased LEDGF mRNA expression (Fig. 4A, lane 4) and the promoter activity (Fig. 6). Surprisingly, it also affected the DNA binding of LEDGF protein (Fig. 5). Because LEDGF up-regulated stress-response genes such as Hsp27 and  $\alpha$ B-crystallin (25), we hypothesized that expression of the stress-response gene may also be down-regulated by TGF- $\beta$ 1 treatment. To test this hypothesis, we transfected hLECs with



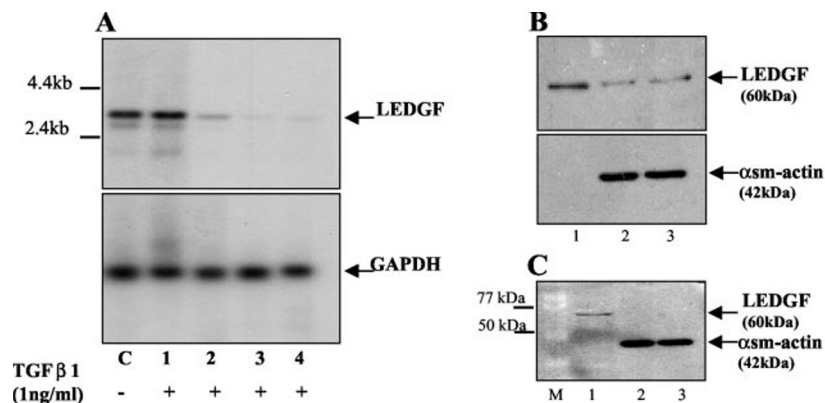


FIG. 4. A, Northern blot hybridization of mRNA from untreated (lane C) and TGF- $\beta$ 1-treated (lanes 1-4) hLECs. hLECs were cultured with or without TGF- $\beta$ 1 (1 ng/ml) for variable time periods (3 h, lane 1; 12 h, lane 2; 24 h, lane 3; and 48 h, lane 4), and mRNA was isolated as described under "Experimental Procedures." Northern hybridization experiment was performed with LEDGF 3'-cDNA fragment or GAPDH (Ambion) probe and autoradiographed. Upper panel, mRNA hybridized with p32-labeled LEDGF 3'-cDNA fragment probe; lower panel, p32-labeled GAPDH probe. The size of RNA markers is indicated (in kb) on the left. B, protein blot analysis showing reduced levels of LEDGF and increased levels of  $\alpha$ -sm-actin by TGF- $\beta$ 1. hLECs were incubated at 37 °C in the presence or absence of TGF- $\beta$ 1. After 48 h, cultures were terminated, and whole cell lysate was prepared using the RIPA buffer for protein blot analysis (see "Experimental Procedures"). Equal amount of protein was loaded on each well, and Western analysis was performed using  $\alpha$ -sm-actin monoclonal antibody. B, lane 1, untreated control; lane 2, TGF- $\beta$ 1 1 ng/ml; lane 3, 5 ng/ml. A 42-kDa band was detected (lower panel; lanes 2 and 3 but not in lane 1). The Immobilon-P membranes immunostained with  $\alpha$ -sm-actin monoclonal antibody were stripped and reprobed with monoclonal anti-LEDGF antibody (BD Biosciences); a 60-kDa of LEDGF band was present in TGF- $\beta$ 1-untreated (B, upper panel, lane 1) which was significantly reduced in TGF- $\beta$ 1-treated cells (B, lower panel, lane 2, 1 ng/ml, and lane 3, 5 ng/ml). C, membrane immunostained with  $\alpha$ -sm-actin and anti-LEDGF monoclonal antibodies simultaneously (M, marker; lane 1, untreated control; lanes 2 and 3, treated with TGF- $\beta$ 1 at 1 and 5 ng/ml, respectively).

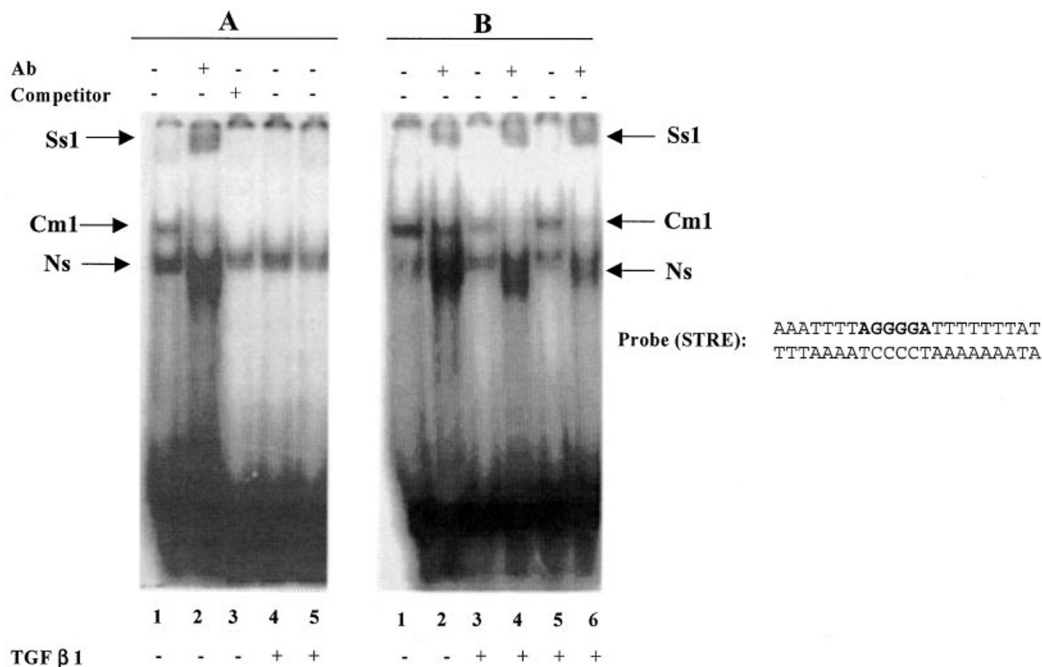


FIG. 5. Gel shift and supershift assays using hLEC nuclear extract with or without treatment of TGF- $\beta$ 1. Five  $\mu$ g of nuclear extract from normal cells (A) or cells overexpressing LEDGF (B) was incubated with the radiolabeled probe having STRE ((A/T)GGGG(A/T))-formed protein-DNA complex (Cm1 band in A and B, lane 1). When an LEDGF-specific antibody was added, the Cm1 band shifted to a higher molecular weight position shown by the Ss1 band (A, lane 2; B, lanes 2, 4, and 6). The addition of unlabeled self-competitor at 1000-fold molar excess eliminated the bands (A, lane 3). Nuclear extract isolated from hLECs treated with TGF- $\beta$ 1 did not bind to the probe: A, lanes 4 and 5. These results show that TGF- $\beta$ 1 reduced the DNA binding of LEDGF to HSE and STRE. In contrast, cells overexpressing LEDGF treated with TGF- $\beta$ 1 showed a Cm1 band (B, lanes 3 and 5) that supershifted to the Ss1 band (B, lanes 4 and 6). Results indicate that overexpression of LEDGF restores TGF- $\beta$ 1-mediated reduction of DNA binding.

these constructs (Hsp27 and  $\alpha$ B-crystallin linked to CAT reporter gene) (25), and cells were treated with TGF- $\beta$ 1 at 1 ng/ml for 48 h. Analysis of CAT activity showed that TGF- $\beta$ 1 strongly suppressed promoter activity of Hsp27 and  $\alpha$ B-crystallin (Fig. 9A). We concluded that reduction of DNA binding activity of LEDGF was responsible for down-regulation of Hsp27 and  $\alpha$ B-crystallin transcription.

To demonstrate that overexpression of LEDGF could restore the down-regulation of the stress-response gene, we

used hLECs stably transfected with LEDGF, and these cells were cotransfected with Hsp27 and  $\alpha$ B-crystallin promoter constructs followed by 48 h of treatment with TGF- $\beta$ 1 at 1 ng/ml. As shown in Fig. 9B, overexpression of LEDGF in these cells counteracted the TGF- $\beta$ 1 effect. We conducted a parallel experiment with higher concentrations of TGF- $\beta$ 1 (5 ng/ml); however, at this concentration, the promoter activity of Hsp27 and  $\alpha$ B-crystallin was slightly reduced (Fig. 9B).

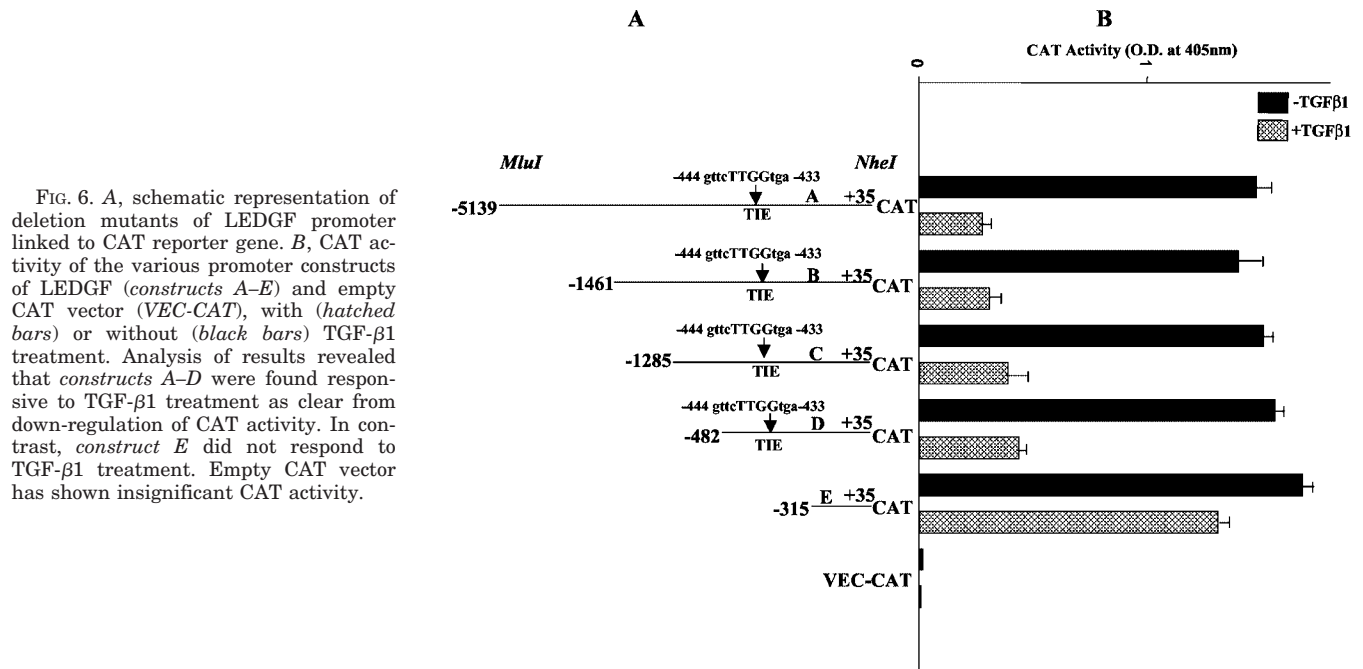


FIG. 6. A, schematic representation of deletion mutants of LEDGF promoter linked to CAT reporter gene. B, CAT activity of the various promoter constructs of LEDGF (constructs A–E) and empty CAT vector (VEC-CAT), with (hatched bars) or without (black bars) TGF- $\beta$ 1 treatment. Analysis of results revealed that constructs A–D were found responsive to TGF- $\beta$ 1 treatment as clear from down-regulation of CAT activity. In contrast, construct E did not respond to TGF- $\beta$ 1 treatment. Empty CAT vector has shown insignificant CAT activity.

#### Putative TGF $\beta$ inhibitory element of the 5'-flanking region of LEDGF promoter

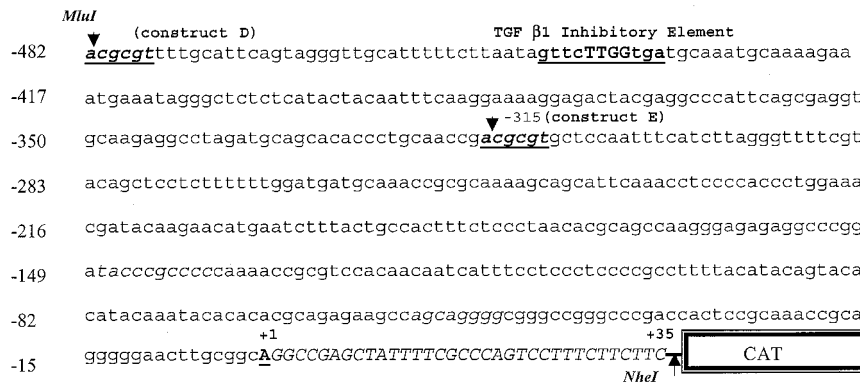


FIG. 7. Sequence reveals that construct D consists of TIE (**boldface underlined letters**), and this construct was used for site-directed mutagenesis. Disruption of the TIE site is shown in Fig. 9. Genes bearing similar sequences (TIE) have been reported earlier by others and have been depicted in Table II.

TABLE II  
Comparison of promoter regions from TGF- $\beta$ 1-inhibited genes

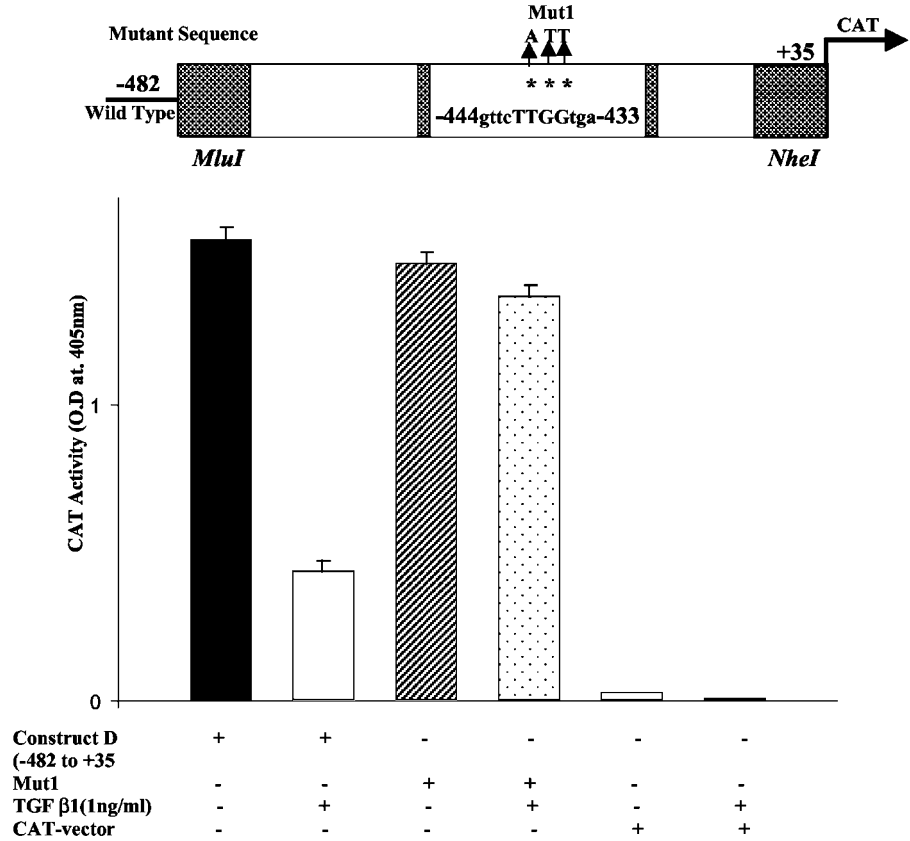
The promoter regions of various genes demonstrated to be inhibited by TGF- $\beta$ 1 were compared, and this region was reported to contain TGF- $\beta$  inhibitory element (TIE) responsible for TGF- $\beta$ 1-induced repression of gene promoters. We used MatInspector Genomatix analysis to spot putative TIE in LEDGF promoter that is present at position -444 to -433 in construct D as shown in Fig. 7.

Promoter	Species	Position	Sequence	Refs.
Transin	Rat	-709	GAGTTGGTGA	52
Urokinase	Human	-572	GTGTTGGTGA	64
Elastase	Mouse	-331	GAGTTGGTGA	81
Collagenase	Human	-246	GAGTTGGAGA	2
MRP/proliferin	Mouse	-1004	GCCTTGGTGT	14
c-myc	Human	-87	GGCTTGGCGG	5
LEDGF	Human	-444	GTCTTGGTGA	This work
Consensus			NNNNTTGGNNN	

**Resistance to TGF- $\beta$ 1-induced Changes by the Cells Overexpressed with LEDGF**—To determine whether cells overexpressing LEDGF could counteract TGF- $\beta$ 1-mediated adverse effects, we compared the cells stably transfected with EGFP-LEDGF to those transfected with empty vector. These cells were treated with TGF- $\beta$ 1. Results showed that LEDGF prevented TGF- $\beta$ 1-mediated adverse effects (Fig. 10, compare C with D). We verified the expression of EGFP-LEDGF by West-

ern blot analysis (data not shown). Furthermore, we quantified the apoptotic cell death per 100 cells either stably transfected with EGFP-LEDGF or with empty vector treated with TGF- $\beta$ 1, in the different fields of each slide following DAPI and TUNEL staining (data not shown), and apoptotic index was determined (Fig. 10E). Overexpressed cells treated with TGF- $\beta$ 1 were indistinguishable from the control cells. We concluded that the overexpression of LEDGF abolished the effect of TGF- $\beta$ 1.

**FIG. 8. Point mutation at TIE of LEDGF promoter abolished the effect of TGF-β1.** Site-directed mutagenesis was conducted as stated under “Experimental Procedures” using construct D (–482 to +35) and a pair of complementary primers. Untreated control (black bar) and TGF-β1-treated cells (open bar) showed repression of the LEDGF promoter. The mutated construct (dotted bar) did not show TGF-β1-induced transcription repression suggesting that disruption of the TIE (TTGG) element is responsible for TGF-β1-mediated suppression of the promoter activity of LEDGF.



DISCUSSION

TGF-β1 produces a variety of effects in a number of tissues and cell lines and is responsible for inducing extracellular matrix proteins (52). This protein is an important regulator of apoptosis in a number of tissues including liver, mammary gland, prostate gland, and lens epithelial cells (13, 53–62). Furthermore, regulation of cell shape is an important process that controls tissue morphogenesis, cell migration, differentiation, proliferation, and survival, probably by regulating expression of genes central to these processes (63–65). Thus, it is possible that TGF-β contributes to the differentiation and modulation of gene expression affecting hLECs physiology. Recently it has been shown that TGF-β induced cataract-like changes in lens epithelial cell explants and whole cultured lenses (12, 13, 16). TGF-β is present in ocular media (11), and various levels of TGF-β activity have been reported in aqueous and vitreous forms from human and other species (6, 7, 66). Our present studies focus on the molecular mechanisms underlying TGF-β1 induction of LECs phenotype and apoptosis; specifically, we investigated TGF-β1 regulation of LEDGF gene expression and its association to Hsp27 and αB-crystallin expression.

We observed morphological changes and apoptotic cell death of hLECs and expression of α-smooth muscle expression in these cells. We found 1 ng/ml TGF-β was sufficient to induce these changes in hLECs cultured *in vitro* (Fig. 1C). The changes were similar to the phenotypic changes observed in lens explants and whole lens culture *in vitro* (10). TGF-β acts on cells by binding to TGF-β type I and type II receptors, which are transmembrane serine and threonine kinases (67). We verified the presence of TGF-β receptors I and II in hLECs. We found both receptors in the hLECs (Fig. 1, A and B). These result suggest that hLEC and TGF-β1 system can be used to understand the role and fate of LEDGF in the hLECs exposed to TGF-β1.

LEDGF is a survival factor and protects the cell from physiological and/or environmental stresses (17, 18). In addition, it is an essential regulator of various stress-associated genes, for instance Hsp25, Hsp 27, Hsp90, αB-crystallin, AOP2, and expression of these proteins is required to confer the resistance against various stresses. This protein is also inducible to oxidative and heat stress (52). Interestingly, overexpression of LEDGF overcomes TGF-β1-induced phenotypic changes and apoptosis in hLECs (Fig. 10), and LEDGF is frequently highly expressed in cells facing stress. Based on the above observation it is pertinent to question how LEDGF can play the roles of cell death and cell survival. Although we do not yet know the complete answer to this question, increasing evidence indicates that it does so by up- or down-regulation of stress-associated genes. Next, the ability of LEDGF to play multiple regulatory roles can also be seen in its response to different signal-transducing agents such as UV, oxidative stress, heat stress, 12-O-tetradecanoylphorbol-13-acetate, all of which stimulate LEDGF expression (52, 68)<sup>2</sup>; by contrast, we found in the present study that LEDGF gene transcription and protein expressions were down-regulated in hLECs treated with TGF-β1 (Fig. 4, A and B, and Fig. 6). These results indicated that there may be two possible mechanisms for TGF-β1 inhibition of LEDGF expression. First, TGF-β1 may act at the TIE site through *c-fos* (30) and may dominantly repress the positive signal. Closer analysis of this gene revealed the presence of the TIE element, nnnnTTGGnnn (Fig. 7 and Table II). Our results showed that transcription of LEDGF was repressed, because interruption of the sequence nnnnTTGGnnn found at position –444 to –433 in the LEDGF promoter (construct D) resulted in the loss of TGF-β1 inhibition (Fig. 8). This finding demonstrated the negative regulation of LEDGF gene by TGF-β1 might be related to the presence of TIE in the LEDGF gene.

<sup>2</sup> D. Prabhakaran, personal communication.



The TIE in the LEDGF gene is also found in several other genes known to be inhibited by TGF- $\beta$ 1. Oligonucleotides based on the TIE consensus sequence in the collagenase, *c-myc*, and transin promoters bound to protein from TGF- $\beta$ 1-stimulated cell extracts (30, 39, 69). nnnnTTGGnnn TIE consensus sequence is present in LEDGF. This suggests that the TIE sequence may also be functional in TGF- $\beta$ 1 inhibition of LEDGF gene. However, these sequences are conserved among other TGF- $\beta$ 1 inhibited genes (Table II). The presence of this element in other TGF- $\beta$ 1-inhibited genes suggests this may be a general mechanism for TGF- $\beta$ 1-induced inhibition of gene expression. We did not monitor the TGF- $\beta$ 1-mediated expression of *c-fos* in

the present study as it appeared from the literature survey that the TGF- $\beta$ 1 inhibitory effect on LEDGF gene was through the TIE, where *c-fos* expression and/or activation play major role (30), and a similar mechanism is involved in the repression of LEDGF gene transcription. The role of Smad in the repression of LEDGF transcription is another possibility. Recently, it has been reported (70) that TGF- $\beta$  inhibits collagenase gene transcription through the participation of Smad3. An analysis of the LEDGF promoter revealed that it is less likely that the Smad pathway is involved in its regulation.

Second, there was a possibility that DNA binding property of LEDGF was diminished either due to lower abundance of LEDGF protein or its attenuation. Because this protein protects the cell via transactivation of various stress-associated genes (25, 22) by binding to stress-response elements ((A/T)GGGG(A/T)) and heat shock response element (nGAAn) for elevation of stress gene transcription, diminution of DNA binding may paralyze the survival function of LEDGF. In the present study Western analysis data revealed diminished expression of LEDGF protein in the TGF- $\beta$ 1-treated cell (Fig. 4B), and this may be either associated with repression of transcription or degradation of protein. TGF- $\beta$ 1 induced apoptosis in various cells including hLECs (14, 60, 61, 71–73), and during apoptosis cells remain overwhelmed with caspases (61, 74–77). Interestingly, we found LEDGF-DNA binding affinity is reduced (Fig. 5). It was evident from gel shift assay when oligonucleotide consisting of either (A/T)GGGG(A/T) or nGAAn consensus sequence did not bind to the LEDGF in nuclear extract isolated from hLECs treated with TGF- $\beta$ 1 but did bind to nuclear extract from untreated cells (Fig. 5). Next we utilized the transfection system to monitor the potency of the transcriptional activation of LEDGF in the presence and the absence of TGF- $\beta$ 1. We performed CAT assay using Hsp27 and  $\alpha$ B-crystallin constructs (25). The hLECs were transfected with the construct and treated with TGF- $\beta$ 1. We found that CAT activity of the promoter was significantly reduced (Fig. 9). Structural analysis of the LEDGF protein revealed that it is vulnerable to caspase-3 and -7. Wu *et al.* (29) have reported that LEDGF was cleaved during apoptosis into fragments of 65 and 58, and this protein has three sites: DEVPD30 G, DAQD486 G, and WEID85. They further emphasized that the apoptotic cleavage of LEDGF may contribute to the pathogenesis by abrogating its pro-survival function. They observed that LEDGF belongs to a subset of intracellular proteins that are cleaved during apo-

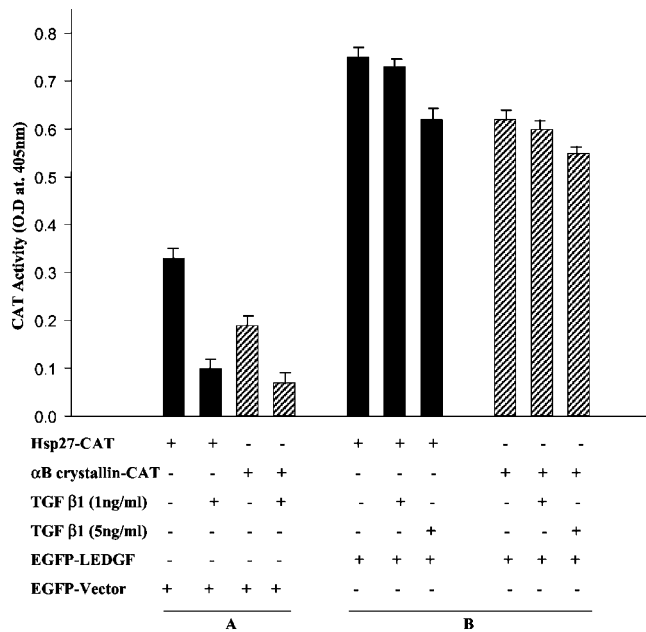


FIG. 9. Human lens epithelial cells were co-transfected with  $\alpha$ B-crystallin or Hsp27 CAT promoter constructs either with EGFP-LEDGF or with EGFP empty vector and treated with or without TGF- $\beta$ 1. After 72 h, the CAT activity was assayed. A, cells co-transfected with EGFP vector and Hsp27-CAT (black bars) or with  $\alpha$ B-crystallin-CAT construct (striped bars) showed down-regulation of promoter activity by TGF- $\beta$ 1. B, cells co-transfected with EGFP-LEDGF either with Hsp27 (black bars) or with  $\alpha$ B-crystallin-CAT construct (striped bars) with or without TGF- $\beta$ 1 treatment at different concentrations. Cells overexpressing LEDGF overcome the adverse effect of TGF- $\beta$ 1.

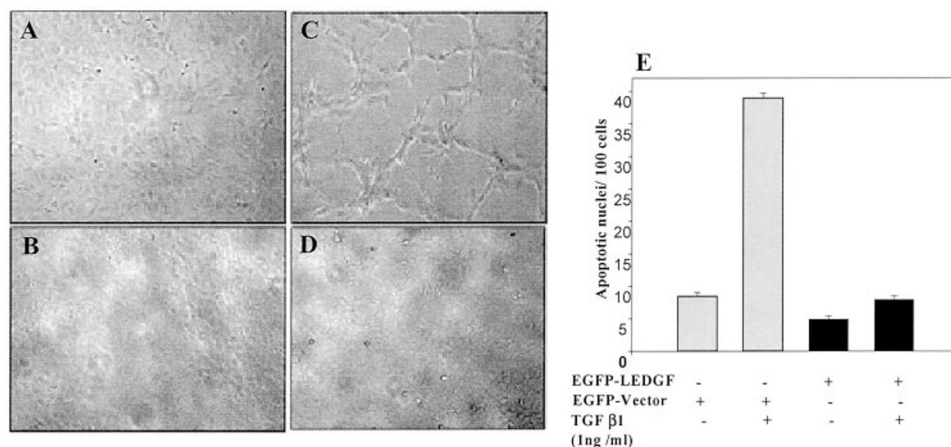


FIG. 10. Photomicrograph of hLECs overexpressing LEDGF with or without treatment with TGF- $\beta$ 1 (1 ng/ml). Cells stably transfected with either EGFP-Vector (A and C) or with EGFP-LEDGF (B and D) were treated with TGF- $\beta$ 1. A, control cells transfected with empty vector. B, control cells overexpressing LEDGF. C, vector-transfected cells showing phenotypic changes after TGF- $\beta$ 1 treatment. D, LEDGF overexpressing cells treated with TGF- $\beta$ 1 was indistinguishable from untreated control (B). E, histogram showing percentage of apoptotic nuclei in the hLECs with empty vector, treated with TGF- $\beta$ 1 (right shaded bar) and untreated (left shaded bar), and in hLECs overexpressing LEDGF (EGFP-LEDGF)-treated (right black bar) and untreated (left black bar).

ptosis and undergo a second wave of proteolysis as apoptosis progresses into secondary necrosis. In the present study TGF- $\beta$ 1-induced apoptosis in hLECs and similar observations have also been reported previously (60, 61, 78, 79). It appears that instead of suppression of LEDGF protein in TGF- $\beta$ 1-treated cells, another possibility of diminution of LEDGF DNA binding affinity could be due to its degradation by the caspases that result in repression of the stress-response gene such as  $\alpha$ B-crystallin and Hsp27 (80). Notably,  $\alpha$ B-crystallin and Hsp27 play major roles in cellular survival including hLEC (25, 28, 81).

In conclusion we have described a novel mechanism of negative regulation of LEDGF and LEDGF-dependent stress-associated gene expression by TGF- $\beta$ 1. Taken together, the findings of this study showed that TGF- $\beta$ 1 inactivated LEDGF by the repression of its transcription and attenuation of its DNA binding. It appears that the repression of LEDGF is a critical event in TGF- $\beta$ 1-induced changes. Because LEDGF could reverse the TGF- $\beta$ 1 insult of hLECs, this protein may be a novel candidate to postpone the age-related degenerating disorders including cataractogenesis.

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