Transcriptional Regulation of the Antioxidant Protein 2 Gene, a Thiol-specific Antioxidant, by Lens Epithelium-derived Growth Factor to Protect Cells from Oxidative Stress*

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Antioxidant protein 2 (AOP2), a member of the newly defined family of thiol-specific antioxidant proteins, has been shown to remove H₂O₂ and protect proteins and DNA from oxidative stress. Here we report that LEDGF is one of the regulatory factors for the AOP2 gene. We found that LEDGF bound to the heat shock element and to stress-related elements in the AOP2 promoter. It trans-activated expression of AOP2-CAT in COS-7 cells and lens epithelial cells overexpressing LEDGF. Mutations in the heat shock element and stress-related elements of the AOP2 promoter reduced LEDGF-dependent trans-activation. Lens epithelial cells showed a higher level of AOP2 mRNA in the presence of LEDGF. Cells overexpressing LEDGF exhibited a higher level of AOP2 protein, the level of which was directly related to the increase in cellular protection. Thus, LEDGF, by activating the AOP2 gene, protected and enhanced the survival of cells under oxidative stress.

Lens epithelium-derived growth factor (LEDGF),1 a member of the hepatoma-derived growth factor family, is found at low levels in many actively dividing and long lived cells. Its gene yields two proteins, LEDGF/p75 and p52, by alternative splicing (1, 2). p52 is a general co-activator of transcription (3) and a co-activator of splicing of polymerase II-restricted mRNAs. LEDGF/p75 is known to be a weak co-activator of transcription (1). Recently, we reported that LECs and COS-7 cells overexpressing LEDGF exhibited a higher level of AOP2 protein, the level of which was directly related to the increase in cellular protection. Thus, LEDGF, by activating the AOP2 gene, protected and enhanced the survival of cells under oxidative stress.

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1 The abbreviations used are: LEDGF, lens epithelium-derived growth factor; AOP2, antioxidant protein 2; CAT, chloramphenicol acetyltransferase; LEC, lens epithelial cell; HSE, heat shock element; STRE, stress-related element; Hsp, heat shock protein; ROS, reactive oxygen species; GST, glutathione S-transferase; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; Ab, antibody; ELISA, enzyme-linked immunosorbent assay; MTs, (3-(6-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

EXPERIMENTAL PROCEDURES

Plasmid Construction, Expression, and Purification of GST-LEDGF—Most molecular subcloning techniques used in these experiments are found in Sambrook et al. (18). A fusion protein between LEDGF and glutathione S-transferase (GST) generated by inserting cells cultured in the presence of LEDGF survived well and expressed Hsp90 at higher levels than cells cultured without it (6). In an in vivo Lewis rat model in which intense light induced retinal photoreceptor cell damage, the intravitreal injection of LEDGF preserved normal retinal photoreceptor cell anatomy and significantly prolonged normal function (7).

LEDGF, a highly charged lysine-, arginine-, glutamic acid-, and aspartic acid-rich protein, binds to heparin and is rapidly translocated from the extracellular space into the nucleus (8). LEDGF also binds to DNA. In a previous report (9), we showed that LEDGF bound to heat shock (HSE; nGAAn, where n indicates any nucleotide) (10) and to stress-related (STRE; (T/A)GGG(A/T)) (11) elements in the promoters of Hsp27 and α-crystallin and activated expression of these genes. The consensus core nucleotide sequences to which LEDGF bound were GAA and AGG. LEDGF may bind to a single core sequence or to multiple core sequences, such as nGAAAttCtC. Furthermore, our results suggest that other stress-related genes are activated by LEDGF (9).

Although cells require oxygen for their survival, they may also suffer from the toxicity of reactive oxygen species (ROS). To reduce the adverse effects of ROS, cells have evolved efficient antioxidants that include enzymes (e.g. catalase), thiols (glutathione), and other substances that decompose peroxides and superoxide anions (12). Recently, several reports have emphasized the importance of antioxidant protein 2 (AOP2), a member of the thiol-specific antioxidant family of proteins in the removal of peroxides from liver, kidney, and brain (13, 14). AOP2 has a highly conserved cysteine at position 47 (15, 16). Furthermore, AOP2 protects various enzymes from inactivation by a nonenzymatic mixed function oxidation mechanism (13, 17). AOP2 also protects DNA from cleavage by ROS.

Recently, we observed that COS-7 cells, and LECs overexpressing LEDGF, expressed elevated levels of AOP2 and survived longer under oxidative and thermal stress. In an earlier study (4), we showed that LEDGF up-regulated the expression of Hsp27 and α-crystallin in these same cells, so the finding of an additional, potent antioxidant protein, AOP2, prompted us to assess the role of LEDGF in activating the AOP2 gene. Ultimately, we were interested in understanding the relative importance of these stress proteins in determining survival in cells facing oxidative stress. In this series of investigations, we have identified LEDGF-responsive elements in the AOP2 gene promoter and ascertained if LEDGF exerts its protective, antioxidant effect via up-regulation of the AOP2 gene.

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Preparation of Lens Epithelial Cell Nuclear Extract—Human lens epithelial cell nuclear extract was prepared following the method of Sambrook et al. (18) with certain modifications. Briefly, human LECs \((1 \times 10^6)\) were cultured in 100-mm plates. The cells were washed gently with chilled phosphate-buffered saline (pH 7.2). Cells were collected by centrifugation using a microcentrifuge and resuspended in 5 pellet volumes of cytoplasmic extract buffer (10 mM HEPES, 80 mM KCl, 1 mM EDTA, 0.025% 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 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\(\text{\textsubscript{2}}\), 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20% (w/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, the extract was aliquoted, and individual aliquots were stored at \(-20^\circ\text{C}\) to avoid repeated freezing and thawing of the preparation. Protein was estimated according to the Bradford method (19), and extract was used for EMSA.

**Fig. 1.** Nucleotide sequence of the 5′-flanking region of the mouse AOP2 promoter linked to the bacterial CAT reporter gene. The nucleotide sequence between NheI and BglII sites is presented, and position +1 corresponds to the transcription start site. Underlining indicates the presumptive LEDGF binding sites (CS1 and CS2). Boldface lettering of FP1, FP2, FP3, FP4, and FP5 indicates the sequences protected by LEDGF from DNase I in the footprinting (FP) analyses (see Fig. 4). This nucleotide sequence was inserted into a pBS-CAT vector to generate a construct (AOP2-CAT) for the AOP2 promoter trans-activation assay. Lowercase letters indicate DNA linker sequences.

![DNA footprinting gel image](http://www.jbc.org/content/273/13/48900/F1.large.jpg)

**Fig. 2.** EMSA and supershift assays using the FP1 probe and purified GST-LEDGF or native LEDGF from HeLa cell and LEC nuclear extract. One \(\mu\)g of GST-LEDGF, 5 \(\mu\)g of HeLa cell nuclear extract, and 5 \(\mu\)g of LEC nuclear extract were incubated with the radiolabeled FP1 probe. This resulted in the formation of the Cm1 band (lanes 1, 4, and 7). When a LEDGF-specific antibody was added, the Cm1 band shifted to a higher molecular weight position (Ss1 band; lanes 2, 5, and 8). The addition of an unlabeled competitor at a 1000-fold molar excess eliminated the bands (lanes 3, 6, and 9).

**Construction of AOP2/TA Vector for DNase I Footprinting**—The 5′-flanking region of the mouse AOP2 gene promoter (containing NheI and BglII restriction sites) was isolated with the genomic PCR kit (CLONTECH Genomic) using AOP2-specific primers. The sense primer (5′-CGTTTCTCTCTCCATTGGTGAGAT3′) contained a NheI site; the antisense primer (5′-GAATTAGACCTACCTCCTGAGATCTTC-3′) contained a BglII site. The isolated fragment was cloned into the EcoRI sites of the TA vector (Invitrogen Corp., Carlsbad, CA) and sequenced following amplification. The sites created by PCR enabled us to obtain the desired segment of the AOP2 promoter for the footprinting experiments. NheI and BglII sites were created through PCR in 5′- and 3′-ends, respectively, to use for a second restriction mapping to make an end-labeled probe for footprinting. For footprinting, we made one end-
In analysis of the LEDGF binding sites on the DNase I digestion are indicated as FP1, sequences that LEDGF protected from lane 2, 10 ng in lane 2, 100 ng in lane 3, and 0 ng (bovine serum albumin (BSA)) in lane 4. A and B correspond to the 3’ and 5’-end-labeled fragments, respectively.

**Construction of AOP2/CAT Reporter Vector and CAT Assay**—The above mentioned AOP2-TA construct was digested with NheI and BglII, and the promoter fragment was ligated into the pCAT Basic vector (Promega, Madison, WI) after digestion with the same enzymes. The plasmid was amplified and used for the CAT assay after co-transfection of cells with constructs. The CAT assay was carried out using CAT-ELISA (Roche Molecular Biochemicals). In an incubator containing 5% CO2, COS-7 and LECs were cultured at 37°C in Dulbecco’s modified Eagle’s medium containing 10% serum in 60-mm Petri dishes at a density of 1 × 105 cells/ml. Twenty-four h later, the cells were washed with the same medium and co-transfected using the calcium phosphate method with three constructs: 1) 5 µg of GFP-LEDGF expression vector, 2) 10 µg of promoter/CAT reporter construct, and 3) 5 µg of the pSV-β-galactosidase vector. After 72 h of incubation, cells were harvested, and the extract was prepared and normalized to the soluble protein level. CAT-ELISA was performed according to the manufacturer’s protocol. The absorbance was measured at 405 nm using a microtiter plate ELISA reader. All CAT values were normalized to β-galactosidase activity (21).

**Site-directed Mutagenesis**—PCR-based site-directed mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s protocol. We made specific mutations in the AOP2 promoter at the LEDGF binding sites. Briefly, the double-stranded pCAT-AOP2 construct was used as template DNA and three pairs of complementary primers were used to mutate DNA and three pairs of complementary primers were used to mutate AOP2-CAT. The plasmid was amplified, and the mutation was confirmed by sequencing. These mutated plasmids were linked to the CAT reporter, and CAT activities were compared with those of wild type AOP2-CAT. 

**DNase I Footprinting Analysis**—DNase I footprinting was performed as described by Sambrook et al. (18). Briefly, the AOP2/TA construct containing the 220-bp promoter fragment with the EcoRI sites at both ends was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. This was digested with BglII to get a 5’-end-labeled probe and with NheI to get a 3’-end-labeled probe. This probe was bound to LEDGF in a volume of 50 µl of binding buffer containing 8 % Tris acetate (pH 8.0), 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.1% Triton X-100, 2 mM CaCl2, 8% glycerol, and 0.5 µg of poly(dI/dC) for 30 min at room temperature. The protein-DNA complexes were treated with 0.33 units (final concentration) of DNase I for 1–5 min, and the reaction was stopped by adding 140 µl of stop buffer (768 mM sodium acetate, 128 mM EDTA, 0.56% SDS, and 256 µg/ml yeast RNA) followed by phenol and ethanol precipitation. The samples were then run on 8% polyacrylamide gel containing 7 M urea followed by autoradiography of the dried gel.

**Construction of LEDGF-antisense and AOP2-antisense**—We subcloned LEDGF cDNA into a pCDNA3 vector in reverse orientation to make a full-length LEDGF antisense construct. We used a human LEC cDNA library to isolate AOP2 cDNA having a full-length open reading frame. A full-length AOP2-antisense construct was made by subcloning of AOP2 cDNA into a pcDNA3.1/NT-GFP-TOPO vector in reverse orientation.

**Elevated Level of AOP2 mRNA in Cells Overexpressing LEDGF (RT-PCR)**—To assess the level of the AOP2 transcript in LECs overexpressing LEDGF (GFP-LEDGF) and in cells transfected with the empty vector, we synthesized a pair of sense (5’-AGCGTCACCACTGCCGAGCCG-3’) and antisense (5’-GTACTGGATGTGCAGATGCAGCC-3’) AOP2-specific primers. These primers covered the full-length 698-bp open reading frames of AOP2. To perform RT-PCR, mRNA was isolated with the Micro-Fast Track™ kit (Invitrogen, Carlsbad, CA) from 5 × 10^5 LECs transiently transfected with GFP-LEDGF or empty vector. The Micro-Fast Track 2 (Invitrogen, Carlsbad, CA), a cDNA synthesis kit for RT-PCR, was used to synthesize cDNA from mRNA. The resulting cDNA was used as a template for amplification of the AOP2 transcript with the specific primers mentioned above. The following cycling conditions for the PCR were used for 7 min for final extension: 94°C for 3 min; 15, 25, or 35 cycles of 94°C for 1 min; 55°C for 2 min; and 72°C for 3 min.

**Western Blot Analysis and Protection Assay (MTS Assay)**—Normal cells and cells overexpressing LEDGF were transfected with AOP2-antisense and LEDGF-antisense. Protein was extracted by lysing the cells in cold radioimmunoprecipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS in phosphate-buffered saline) 24, 48, and 72 h after transfection, and then protein was estimated (19). Equal concentrations of protein were applied to SDS-PAGE and immunostained with anti-LEDGF antibody (1:10,000 dilution), anti-GFP antibody (1:4000), or anti-AOP2 antibody (1:2000 dilution) to monitor LEDGF, GFP-LEDGF, or AOP2 bands.

**Fig. 3.** DNase I footprinting (FP) analysis of the LEDGF binding sites in the AOP2 promoter fragment. In this promoter fragment, the DNA sequences that LEDGF protected from DNase I digestion are indicated as FP1, FP2, FP3, FP4, and FP5 and are shown on the right and left. Lane 1 indicates the A + G chemical sequencing reaction. In lanes 2–4, varying amounts of GST-LEDGF have been used: 10 ng in lane 2, 100 ng in lane 3, and 0 ng (bovine serum albumin (BSA)) in lane 4. A and B correspond to the 3’- and 5’-end-labeled fragments, respectively.
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The same batch of cells (24, 48, and 72 h after transfection) were treated with 100 μM H₂O₂ for 2 h, and a colorimetric MTS assay (Promega) was performed after a 1-h recovery period to monitor the number of surviving cells in each group. The OD at 490 nm was measured after 4 h with an ELISA reader (5).

Construction of Prokaryotic Expression Vector and Purification of AOP2 Protein—The AOP2 cDNA isolated from the lens epithelial cell library was expressed as a histidine fusion protein (His tag AOP2). PCR was performed using sense (5'-ATGCCGGCGTGCTGCTTCTCCGGG-3') and antisense (5'-AATTGGCGAGCTGACATCCTCTGGCTC-3') primers, and the resultant amplified product was cloned in PCR® T/TNT T7/TOP. The plasmid was used to transform BL2 (DE3) pLysS cells (Invitrogen, Carlsbad, CA). The His tag AOP2 fusion protein was purified from the bacterial lysate with metal-chelating chromatography. The protein was purified using a nickel-chelating resin (Invitrogen) under nondenaturing conditions according to the manufacturer's protocol. Pure protein was eluted with low pH (4.0) imidazole and dialyzed against phosphate-buffered saline containing 10 mM EDTA and 5 mM DTT. Polyacrylamide gel electrophoresis and immunoblots analyses were carried out to monitor the purification of AOP2 protein. Anti-His antibody was used to visualize the AOP2 protein in immunoblots. Also, an antibody to AOP2 was used to visualize AOP2 protein in cell extracts.

H₂O₂ Removal Assay—For the H₂O₂ removal assay, we followed the method of Lim et al. (22). Briefly, a reaction mixture containing different concentrations of AOP2 protein in reaction buffer with 1 μM HEPES and 10 mM DTT in a final volume of 100 μl was incubated at 37 °C for 30 min. After incubation, H₂O₂ was added at 100 μM to the reaction mixtures and incubated at 37 °C for an additional 30 min. The reaction was stopped by adding 750 μl of a trichloroacetic acid solution (12.5%) followed by the addition of a mixture of 300 μl of 10 mM Fe(SH)₂SO₄ and 200 μl of 2.5 M KSCN, which reacted with the remaining H₂O₂ to form a purple complex. We monitored the removal of H₂O₂ by AOP2 by measuring the decrease in absorbance at 490 nm. The percentage removal was derived from the change in absorbance obtained with AOP2 compared with that without AOP2. Bovine serum albumin was used as a control for this peroxidase assay.

RESULTS

Multiple HSE and STRE in the AOP2 Promoter Bound to GST-LEDGF—There are multiple HSEs (GAA) and one STRE (TGGGGGT) in the mouse AOP2 promoter (20) (Fig. 1, indicated by underlining or boldface letters). We have reported these sequences to be LEDGF binding consensus core sequences (5). We identified five potential LEDGF-binding sites (FP1–FP5) in the selected fragment of AOP2 promoter and confirmed LEDGF binding to these sites with EMSA. Binding of the fusion protein, GST-LEDGF, to double-stranded 12-mer probes each containing a consensus core element (FP1, FP2, FP3, FP4, or FP5) produced bands indicating the GST-LEDGF complex for each sequence. The addition of 10–100-fold molar excess of unlabeled self-competitor reduced the amount of the complex and the intensity of the band, and a 1000-fold molar excess eliminated complex formation completely with each sequence. These results were similar to those published in Ref. 9 and are not reproduced here.

To confirm that the Cm1 complexes were composed of LEDGF and DNA, an Ab supershift assay was performed with an Ab to LEDGF (9). Initially, we complexed purified GST-LEDGF with the radiolabeled FP1 probe (see Cm1 band in Fig. 2, lane 1). We added anti-LEDGF Ab to the mixture and noted on the gel that the Cm1 band had shifted to a higher molecular weight position (see the Ss1 band in Fig. 2, lane 2). These results showed that anti-LEDGF Abs bound to LEDGF in the GST-LEDGF-FP1 complexes and formed a larger complex.

To ascertain whether native LEDGF in a cell also bound to these sequences, we incubated the FP1 probe with a HeLa cell nuclear extract. An antibody supershift assay in combination with EMSA was performed to confirm that the probe bound to native LEDGF. Fig. 2 shows that a specific Cm1 band was generated between the LEDGF present in the HeLa cell (Fig. 2, lanes 4–6) and LEC nuclear extracts (Fig. 2, lanes 7–9). The addition of unlabeled competitor resulted in a significant decrease in complex formation (Fig. 2, lanes 6 and 9). Furthermore, this band was shifted to a position of higher molecular weight when Ab was added (Ss1 band in Fig. 2, lanes 5 and 8). These results indicated that LEDGF in HeLa and LEC nuclear extracts bound specifically to FP1 probe. HeLa cell and LEC nuclear extracts contained LEDGF as reported earlier (3, 9).

DNase I Footprinting Showed That LEDGF Bound to the STRE and HSE in the Promoter of AOP2 Gene—To confirm that LEDGF bound to the HSE and STRE, we performed DNase I foot printing analyses with an AOP2 promoter fragment. The end-labeled probe was incubated with bovine serum albumin and 0, 10, and 100 ng/ml GST-LEDGF, and then the mixtures were treated with DNase I. The regions protected from DNase I were FP1 (~11 to ~20), FP2 (~84 to ~94), FP3 (~136 to ~144), FP4 (~147 to ~154), and FP5 (~163 to ~172) (see Fig. 3). These protected regions contained sequences (AGAA or TGGGGT) that are found also in HSE and STRE, respectively. Although we expected to find two more LEDGF binding sites (CS1 and CS2), we were not able to demonstrate LEDGF protection of these sites in the DNase I footprinting experiments.

Next EMSA was performed using synthetic oligonucleotides whose sequences corresponded to those of the protected regions. A double-stranded 26-bp fragment of region 1 (Fig. 4, FP1) was selected and synthesized (5'-TTAGGAGCTTGGGGATAGCCCTC-3'). LEDGF had bound to the underlined sequence and had protected DNA in that region from DNase I in the footprinting experiment. The boldface lettering indicates potential LEDGF binding sites; in fact, LEDGF bound well to this probe. Mutating STRE (from TGGGGT to TGATGT, Mut1-1) resulted in a significant reduction in LEDGF binding. Another fragment, FP3—4, contained two AGAA sequences (CAATAAGAAGAACCTTTTGGTG), and this also showed strong LEDGF binding in EMSA. When one site AGAA was mutated to AAAA (Fig. 4, Mut3-1), the binding was reduced comparatively, but when both sites were mutated (Fig. 4, Mut3-2) LEDGF binding was reduced significantly. The fragment FP3 contained the AGAA sequence, and LEDGF bound well to it; however, when we mutated it from AGAA to TTTT, LEDGF bound weakly (Fig. 4, Mut5-1). These results confirmed that LEDGF bound to HSE (nGAAn and (A/T)GGGG(A/T)) and that the consensus sequences (nGAAn and (A/T)GGGG(A/T)) were directly related to the binding affinity.

Next, we investigated whether LEDGF trans-activated the AOP2 promoter. A construct between nucleotides –194 and +23 in the AOP2 promoter was fused to a CAT reporter gene to form AOP2-CAT (Figs. 1 and 5). The selected region contained a number of elements sufficient to activate the AOP2 promoter (20). We transfected the construct into COS-7 cells (Fig. 5A) and mouse LECs (Fig. 5B). Prior to this experiment, the COS-7 cells and LECs had been stably transfected with a plasmid construct having GFP-LEDGF or empty vector. A high CAT value was seen in these stably transfected COS-7 cells and LECs (Fig. 5, compare columns 1 and 2). These results indicated that LEDGF bound to HSE and STRE of the AOP2 promoter and activated them.

Mutations in the HSE or STRE Reduced LEDGF-dependent Trans-activation of AOP 2 Promoter—To prove that trans-activation of the AOP2 promoter by LEDGF is HSE- and STRE-dependent and that other elements are not involved, HSE and STRE were mutated from AGAAGTTGTTG to AAAATGTTTG (Mut1), AAGAAA to AAAAAA (Mut2), TTTCCTTTTG to ATTTTTTTG (Mut3), AGAGGG to ATACGG (Mut4), and ACCCA to ACACCA (Mut5) as shown in Fig. 6, using site-
directed mutagenesis. If mutations Mut1 Mut2 were present together in the same construct, there was a 40% loss of CAT activity. If mutations Mut1, Mut2, and Mut3 were present together in the same construct, there was a 60% loss of CAT activity, and if mutations Mut1, Mut2, Mut3, Mut4, and Mut5 were present together in the construct, there was an 80% loss of CAT activity (Fig. 6). Each mutation of HSE or STRE resulted in a decrease of some transcriptional activity, and this gradual reduction suggested that each consensus element had made a contribution to the total trans-activation of the AOP2 promoter. When we mutated five binding sites, we noted an 80% loss in promoter activity. The other LEDGF-dependent sites in the AOP2 promoter (FP2 and CS2; Fig. 1) may be responsible for the remaining 20% activity.

**Fig. 4.** Relative binding of LEDGF to wild type and mutant AOP2 promoter regions. We prepared FPR1, FPR3→4, and FPR5 (footprint region) probes. Each of three probes was mutated and presented as Mut1-1, Mut3-1, and Mut5-1. EMSA showed that the 26-mer probes and GST-LEDGF generated the Cm1 band. Dot, a position of mutation; boldface lettering, the consensus sequences. Underlining indicates that the nucleotides were protected in the DNase I footprinting experiment. FPR1 has STRE, and FPR3→4 and FPR5 have HSE.

**Fig. 5.** Trans-activation of AOP2-CAT in COS-7 cells and LECs. The cells were transiently transfected with AOP2-CAT construct or empty pCAT vector. After 72 h, the CAT activity was assayed. Prior to these experiments, these cells had been stably transfected with a GFP-LEDGF or empty vector. CAT value in cells transfected with the GFP-LEDGF construct was significantly higher than in cells transfected with the empty vector.
AOP2 proteins was reduced significantly (Fig. 8, B-1 and B-2). These results clearly indicate that the level of LEDGF controls the level of AOP2, but the level of AOP2 does not control the level of LEDGF. Furthermore, these results confirm that expression of AOP2 was indeed regulated by LEDGF.

We also monitored how the level of AOP2 and LEDGF proteins affected the ability of cells to resist oxidative stress (Fig. 9, A and B). Having monitored LEDGF and AOP2 protein levels by Western blots in cells transfected with sense or antisense constructs (Fig. 8), we then exposed LECs to 100 μM of H2O2 to assess the relative roles of AOP2 and LEDGF in the antioxidant protection mechanism. The viability assay revealed that cells overexpressing LEDGF resisted oxidative stress (Fig. 9, black column). Cells transfected with the LEDGF-antisense construct succumbed more easily to oxidative stress than cells transfected with AOP2-antisense construct (Fig. 9, compare empty and gray columns). In other words, LEDGF has more protective antioxidant potency than AOP2 alone. LEDGF activates multiple stress-related genes (9), and AOP2 is one of the stress proteins protecting cells against oxidative stress. The data in Fig. 9 suggest that AOP2 provides ~60% of the total protective response caused by LEDGF. Results presented in Fig. 9B show that normal cells transfected with LEDGF-antisense and AOP2-antisense constructs are more susceptible than normal cells to oxidative stress. Comparing the black and gray columns in Fig. 9B shows the functional contribution of endogenous LEDGF.

Concentration-dependent Removal of H2O2 by AOP2—We also studied thiol-specific antioxidant property of AOP2 by monitoring its ability to remove H2O2 in an in vitro system. The results showed that AOP2 can reduce H2O2 in the presence of DTT in a concentration-dependent manner (Fig. 10). This means that AOP2 itself plays a major role in the cells’ complex multifactorial antioxidant mechanisms.

**DISCUSSION**

In this study, we found that LEDGF bound to several sites in the HSE or STRE of the AOP2 promoter and transactivated
expression of AOP2 gene products. Mutation in HSE and STRE sequences diminished LEDGF-dependent trans-activation. The cells overexpressing AOP2 acquired a strong resistance to H2O2 insult. Elevation of AOP2 by LEDGF might be important in preventing cell death. To our knowledge, this is the first report that the AOP2 gene is regulated by LEDGF, and it suggests that LEDGF-dependent trans-activation of AOP2 is significant in the survival of many cells including skin cells, COS-7 cells, and LECs.

We reported earlier that LEDGF bound to consensus core sequences (e.g. tandem or inverted AGG or GAA repeats) and to consensus STRE and/or HSE (9). The (A/T)GGGG(T/A) sequence is known to be a consensus element of STRE (11), and the nGAAnTTCn sequence is a well characterized HSE. The consensus core elements of HSE were confirmed as nGAAn (for reviews, see Refs. 23 and 24). In this study, we have shown that DNA fragments containing TGGGGT, AGAA, AGAAG, and AA-GAAAA sequences bound strongly to the LEDGF. Thus, the consensus core AAG or GAA and TGGGGT in the AOP2 promoter are important for LEDGF binding. This result is consistent with our previous observation that LEDGF bound to these same sequences found in the Hsp27 and αB-crystallin promoters (9).

Between nucleotides –194 and +1 of the AOP2 promoter, there are seven presumptive LEDGF binding sites (shown in Fig. 1). DNase I footprinting experiments showed that LEDGF bound to five of these sites (FP1–FP5). CS1 and CS2 contain sequences identical to sites where LEDGF binds selectively, so we presume that CS1 and CS2 are also LEDGF binding sites. We could not, however, demonstrate this in the footprinting experiment. Sequential mutations in the LEDGF binding sites were clearly associated with a decrease in the AOP2 promoter activity that was approximately proportional to the number of mutations at FP1–FP5 in the promoter. It was interesting to observe, however, that even in the construct with the highest number of mutations, 20% of the total promoter activity remained. This finding suggested that LEDGF binding at the CS1 and CS2 sites could be contributing to the total activation of the AOP2 promoter. These findings suggest that all of the defined LEDGF binding sites participated in the regulation of AOP2 promoter.

We have shown that external stimuli (e.g. H2O2 (25) and UV)2 activated the expression of LEDGF in LECs and COS-7 cells (25) and that the elevated levels of LEDGF activated the expression of AOP2 protein as described in this paper. We do not know how oxidative signals activate expression of LEDGF. We do know, however, that LEDGF binds to promoter elements in the AOP2 gene and activates it directly. Oxidative signals

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**Fig. 8. The expression of LEDGF and AOP2 proteins in LECs transfected with their sense and antisense constructs.** The protein levels of GFP-LEDGF (A-1), LEDGF (B-1), and AOP2 (A-2 and B-2) in normal LECs and LECs transfected with GFP-LEDGF construct having sense (LEDGF-S) or antisense orientations (LEDGF-AS) and/or GFP-AOP2 construct with antisense orientations (AOP2-AS) were visualized after 48 h of transfection with immunostaining using anti-GFP, anti-LEDGF, or anti-AOP2 Abs. Anti-GFP Abs will not react with native LEDGF. Cells overexpressing LEDGF (A-1, striped columns) showed higher levels of AOP2 (Fig. 8, A-2, striped column). AOP2 levels diminished in cells transfected with LEDGF antisense (A-2 and B-2, gray columns). Cells overexpressing LEDGF (LEDGF-S) and transfected with AOP2 antisense (AOP2-AS) expressed high levels of GFP-LEDGF (A-1) but low levels of AOP2 (A-2) (empty column). Cells transfected with AOP2 antisense (AOP2-AS) expressed high levels of native LEDGF (B-1, empty column) but low levels of AOP2 (B-2, empty column). The relative densities of the scanned bands in the Western blots are presented as bar graphs in B-1 and B-2. In A-1 and A-2, however, the bar graphs represent the relative densities in relation to the expression levels in normal cells (B-1 and B-2).
Activation of AOP2 Gene by LEDGF

Fig. 9. AOP2-mediated protection of cells by LEDGF. Cells overexpressing LEDGF and normal cells were transfected with LEDGF-antisense or AOP2-antisense constructs. The cells were exposed to 100 μM H₂O₂ for 2 h at 24, 48, and 72 h after transfection. After a 1-h recovery period, the MTS assay was performed. A, results of 48 h indicate that cells overexpressing LEDGF were well protected (black columns). The percentage protection was reduced in the cells transfected with LEDGF-antisense and AOP2-antisense constructs (A, gray and empty columns). The protective effect was directly related to LEDGF-mediated AOP2 protein level in the cells as quantified by Western blots (see Fig. 8). B, normal cells were susceptible to the oxidative stress (black column); however, these cells were more susceptible to stress after transfection with AOP2 and LEDGF-antisense constructs (empty and gray columns).

Fig. 10. Concentration-dependent removal of H₂O₂ by AOP2. The thiol-specific antioxidant property of AOP2 was demonstrated by monitoring the ability of AOP2 to reduce H₂O₂ in an in vitro system (23). The reduction was monitored as a decrease in the absorbance at 490 nm (A₄₉₀). The percentage of removal was calculated on the basis of the change in A₄₉₀ obtained with AOP2 relative to that achieved without AOP2. The reaction mixture, which contained different concentrations of protein (1.0, 0.5, 0.25, and 0.125 mg/ml), DTT (10 mM), and HEPES (100 mM), was incubated for 30 min and further incubated for 30 min after the addition of H₂O₂ at 100 μM concentration.

Also are known to stimulate expression of heat shock factors (10), and a trimer of the heat shock factor binds to HSE and activates expression of Hsp genes directly (23, 24). The mechanisms by which cells protect themselves from oxidative stress are not simple; several transcription factors participate in up- and down-regulating stress-related proteins. For example, oxidative stress activates two transcriptional activators (Msn2p and Msn4p) that bind to STRE and activate expression of stress-related genes (26–28). Activation of the AOP2 gene undoubtfuly involves multiple transcription factors and regulatory elements. We have shown that LEDGF is one of these factors.

AOP2 is a protective protein, a member of a novel family of antioxidant proteins (29) first isolated from Saccharomyces cerevisiae (17). These proteins protected cells from oxidative damage through a thiol-specific pathway (e.g. DTT/Fe⁺/O₂). They did not protect through ascorbate/Fe⁺/O₂ (14, 17). Since the isolation of yeast thiol-specific antioxidant, other related genes have been identified in E. coli, mice, rats, pigs, and humans (31). Since thiol-specific antioxidants act as a thio-redoxin-dependent manner, they are also referred to as peroxiredoxins. It is reported that peroxiredoxins can degrade H₂O₂ with electrons provided by dithiothreitol and alkyl hydroperoxides and influence the differentiation, proliferation, survival, and activity of a number of cell types (32–34). The upstream region of the AOP2 promoter contains biologically significant transcription factor binding sites like STRE and HSE that are involved in oxidative stress. One member of the AOP2 family of proteins is secreted from cells (35); it may capture the ROS in the extracellular compartment prior to their attacking the cells. In this paper, we have shown that LEDGF binds specifically to stress-response genes, like AOP2 and up-regulates its activity.

Various organs/tissues in the living organism are under continuous oxidative stress from oxidants generated intracellularly by chemical (e.g. H₂O₂) or photochemical mechanisms (e.g. from UV radiation). To cope with potentially destructive ROS, cells have evolved antioxidant defenses. A delicate balance between oxidants and antioxidants is important for homeostasis. Several lines of evidence suggest that the mechanisms regulating intracellular redox systems are highly conserved in organisms ranging from bacteria to humans and involve signal transduction and gene expression systems.

Recent reports indicate that although many antioxidant proteins are ubiquitous in mammalian cells, their amounts vary widely with tissue type (17, 36–40). Under stress, elevated expression of these proteins is observed in tissues such as lens, skin, brain, liver, and kidney. In the present report, we have shown that LEDGF interacts with the gene promoter of AOP2 and increases the expression of AOP2 protein. This interaction and increased expression is directly correlated with enhanced protection of cells against oxidative stress.
With antisense experiments, we estimated the contribution of AOP2 protein to the total protective effect of LEDGF. The results revealed that AOP2 protein played a major role in the cellular defense against oxidative stress, a defense that involves multiple stress proteins (9). The presence of this protein in lens and its regulation by LEDGF may be relevant to the rate and risk of age-related cataractogenesis, a degenerative process in which oxidative stress plays an important role (41, 42). By enhancing resistance to oxidative stress, LEDGF and/or AOP2 may offer opportunities for intervention to slow progression of age-related cataractogenesis. In LECs, antioxidative enzymes that destroy free radicals (superoxidases, superoxide dismutase, GSH peroxidase, thioltransferase, and catalase) (30). ROS can cause mitochondrial swelling, DNA fragmentation, loss of LEC viability, and eventual cataract formation (42). Despite higher levels of GSH in the lens, survival of lens epithelial cells depends upon other antioxidants, since GSH cannot be transported from lens fiber cells to lens epithelial cells.

We have emphasized the roles of LEDGF and AOP2 as defense mechanisms in cells under oxidative stress, but this antioxidant defense mechanism may have broader implications for cell survival in skin, brain, kidney, and other tissues.

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Transcriptional Regulation of the Antioxidant Protein 2 Gene, a Thiol-specific Antioxidant, by Lens Epithelium-derived Growth Factor to Protect Cells from Oxidative Stress

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