Regulatory Role for a Novel Human Thioredoxin Peroxidase in NF-κB Activation

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Reduction-oxidation (redox) plays a critical role in NF-κB activation. Diverse stimuli appear to utilize reactive oxygen species (e.g. hydrogen peroxide) as common effectors for activating NF-κB. Antioxidants govern intracellular redox status, and many such molecules can reduce H₂O₂. However, functionally, it does appear that different antioxidants are variously selective for redox regulation of certain transcription factors such as NF-κB. For NF-κB, thioredoxin has been described to be a more potent antioxidant than either glutathione or N-acetylcysteine. Thioredoxin peroxidase is the immediate enzyme that links reduction of H₂O₂ to thioredoxin. Several putative human thioredoxin peroxidases have been identified using recursive sequence searches/alignments with yeast or prokaryotic enzymes. None has been characterized in detail for intracellular function(s). Here, we describe a new human thioredoxin peroxidase, antioxidant enzyme AOE372, identified by virtue of its protein-protein interaction with the product of a proliferation association gene, pag, which is also a thiol-specific antioxidant. In human cells, AOE372 defines a redox pathway that specifically regulates NF-κB activity via a modulation of IκB-α phosphorylation in the cytoplasm. We show that AOE372 activity is regulated through either homo- or heterodimerization with other thiol peroxidases, implicating subunit assortment as a mechanism for regulating antioxidant specificities. AOE372 function suggests thioredoxin peroxidase as an immediate regulator of H₂O₂-mediated activation of NF-κB.

Living organisms produce reactive oxygen species such as H₂O₂ during physiological processes and in response to external stimuli such as UV radiation. To cope with potentially destructive reactive oxygen species, cells have evolved antioxidant defenses (reviewed in Ref. 1). A delicate balance between oxidants and antioxidants is pivotally important for homeostasis. Several lines of evidence suggest that the regulation of intracellular redox, a process highly conserved in organisms ranging from bacteria to human, is a versatile control mechanism in signal transduction and gene expression (reviewed in Ref. 2). In mammalian cells, intracellular redox status has been linked to cellular differentiation, immune response, growth control, tumor promotion, and apoptosis, as well as activation of viruses, notably HIV, from latency (3, 4).

One redox-regulated protein is NF-κB. NF-κB is a member of the Rel family of transcription factors that exist ambiently in the cytoplasm via association with inhibitor protein, IκB (reviewed in Refs. 5 and 6). A wide variety of stimuli including tumor necrosis factor-α (TNF-α), phorbol ester, bacterial lipopolysaccharide, and virus infection can activate NF-κB. Studies have implicated reactive oxygen species (i.e. H₂O₂) as one common signal transducer for these diverse stimuli (7, 8). How H₂O₂ might affect cytoplasmic and nuclear events that lead to the activated function of NF-κB is an important issue that remains incompletely elucidated.

One pathway of NF-κB activation involves site-specific phosphorylation of IκB-α on serine residues 32 and 36. It has been suggested that serine phosphorylation targets IκB to the ubiquitin-proteasome pathway for degradation (9–11). IκB inactivation, without proteolytic degradation, has also been reported to occur as a consequence of tyrosine phosphorylation on residue 42 (12). In both instances, phosphorylation results in an unmasking of the NF-κB nuclear localization signal facilitating nuclear entry of protein. Thus, for stimuli such as oxidative stress, which potently and rapidly modulates the nuclear activity of NF-κB, IκB-α may represent a critical activation target (5).

Antioxidants govern intracellular redox status. Inside cells, glutathione, glutaredoxin, and thioredoxin (13) represent the major reducing agents. It is reasonable, although the evidence is not clear-cut, that different antioxidants should have preferential specificities for discrete redox pathways. A number of studies have suggested that thioredoxin is a specifically potent antioxidant for NF-κB activation (14–16). Thioredoxin reductase (TR), thioredoxin (Trx), and thioredoxin peroxidase (Tpx) are three linked components in a redox chain that couples peroxide reduction to NADPH oxidation (17). In such a scheme, within cells, Tpx is the immediate enzyme that detoxifies hydrogen peroxide.

Thioredoxin peroxidases are highly conserved in eukaryotes and prokaryotes (18–24). Their high degree of conservation suggests a biological importance of this type of enzyme. Some putative “Tpx” proteins have been identified by sequence alignment searches with yeast or bacteria enzymes. At present,

1 The abbreviations used are: HIV, human immunodeficiency virus; TNF, tumor necrosis factor; TR, thioredoxin reductase; Trx, thioredoxin; Tpx, thioredoxin peroxidase; bp, base pairs; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; SRE, serum response element; HTLV, human T-cell lymphotropic virus; LTR, long terminal repeat; α-Pag, anti-Pag antibody; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDC, paraquat dichloride.

2 We have recently suggested a new nomenclature for mammalian peroxiredoxins, Trx, that classifies them based on amino acid sequences and immunological reactivities (S. W. Kang, H. Z. Chae, H. J. Kim, K. T. Jeang; submitted for publication).

*a* This work was funded in part by the AIDS Targeted Anti-viral Program from the Office of the Director, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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however, the functions or properties of human thioredoxin peroxidase remain ill defined. One step toward better physiological understanding is to identify and characterize the intracellular activities of bona fide thiol-specific enzymes. With the idea that redox enzymes might be regulated through protein-protein interactions, we searched for human cellular partners of a known thiol-specific antioxidant, Pag (20, 25). Using a yeast two-hybrid approach, we isolated (as Pag partner) a cDNA that encodes a new prototype peroxiredoxin. This peroxiredoxin, designated antioxidant enzyme AOE372 (and classified in a new nomenclature as subfamily IV), is a human thioredoxin peroxidase. AOE372 defines a redox pathway that leads to NF-κB activation. The biological activity of AOE372 implicates a role for human thioredoxin peroxidase in modulating IkB-α phosphorylation in the cytoplasm.

EXPERIMENTAL PROCEDURES

Cloning and Sequence Analysis—AOE372 cDNA was isolated from a human HeLa S3 Matchmaker cDNA library (HL4000A1; CLONTECH). The 5' 95-bp sequence was obtained from the same library by the rapid amplification of cDNA ends procedure. Double-stranded AOE372 cDNA was sequenced on both strands by the dideoxy method using Sequenase 2.0 (U.S. Biochemical Corp.) as per the manufacturer's protocol. Nucleotide and peptide sequences were analyzed with the Wisconsin software package (version 8.1, Genetics Computer Group, Inc.). Multiple alignments of protein sequences were generated with a progressive pairwise algorithm (26). Phylogenetic analysis was based on a matrix of evolutionary distances, and the phylogeny was reconstructed using the UPGMA algorithm (27).

Yeast Two-hybrid Assay—Yeast two-hybrid screening was performed in yeast strain CG1495 according to the Matchmaker Two-hybrid System 2 protocol (CLONTECH). CG1495 was transformed with bait plasmids (pAS2-UPag) and then screened with a human HeLa S3 cell line Matchmaker cDNA library constructed in plasmid pGADGH (HL4000A1; CLONTECH). Positive clones were selected for expression of His3p and β-galactosidase. Plasmid DNA was recovered from CG1495 and electroporated into Escherichia coli. Reporter assays were performed as described previously (28).

Immunoprecipitation—Monolayer HeLa and HepG2 cells in a 100-mm Petri dish were harvested in 2 ml of immunoprecipitation buffer (PBS, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml aminomethyl benzene sulfonyl fluoride, and 1 μg/ml aprotinin) and disrupted by repeated aspiration through a 21-gauge needle. Cell debris was removed by centrifugation. One ml of cell extract was incubated with 2 μg of mouse anti-Pag monoclonal antibody for 1 h at 4 °C. 20 μl of protein A-Sepharose was added, and the mixture was rotated overnight at 4 °C. Immunoprecipitates were collected by centrifugation, washed three times with immunoprecipitation buffer, and resuspended in SDS gel loading buffer (60 μg Tris base, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol). Rabbit anti-Pag antiserum (a-Pag) was raised against a full-length recombinant Pag protein produced in E. coli.

Northern and Western Blot Analysis—Human multiple tissue Northern blots (CLONTECH) were probed individually with a 35P-labeled BamHI-HoxI fragment of human AOE372, a 900-bp EcoRI-HoxI fragment of human Pag, and a 2-kilobase pair human β-actin cDNA probe as per the CLONTECH protocol.

Protein samples were solubilized in SDS gel loading buffer, separated by 12% SDS-PAGE, and electrophoretically transferred onto Immobilon-P membranes (Millipore Corp.) using a semidry blotter (MilliBlot-SDE, Millipore). Blots were visualized by chemiluminescence (Western-Light, Tropix).

Confocal Microscopy—Monolayer HeLa cells were grown overnight on 18-mm diameter number 1 glass coverslips in 60 mm diameter tissue culture dishes with Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, and antibiotics. Cells were washed with PBS and fixed for 10 min at −20 °C with absolute methanol. Diluted antibodies were added in 60-μl volumes on tissue culture dishes, and the coverslips with fixed cells were inverted onto the antibodies. Antibodies were typically incubated for 1 h at room temperature. Fixative and excess antibodies were removed by several washes with fluorescein (Virotech International). Double label immunofluorescence experiments were performed by using primary antibodies from different species of animal (rabbit and mouse) and species-specific secondary antibodies conjugated to different fluorochromes (fluorescein and Texas Red).

Cell Fractionation—1 × 109 HeLa cells were harvested and washed twice with PBS. The cell pellet was resuspended in 400 μl extraction buffer (10 mM Hepes, pH 7.5, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mg/ml aminomethyl benzene sulfonyl fluoride, and 1 μg/ml aprotinin) and was incubated on ice for 15 min to swell cells. 25 μl of 10% Nonidet P-40 (or IGEPA CA-630 from Sigma) was added, and the mixture was vortexed for 10 min at 4 °C. Cells were then spun briefly at top speed to separate the cytosolic (supernatant) and the nuclear (pellet) fractions.

Protein Expression and Purification—GST-AOE372 fusion protein was expressed in E. coli from plasmid pGEX-4T-3 (PharMacia Biotech Inc.) with an insert coding for the full-length mature AOE372 protein as indicated in Fig. 1. Glutathione S-transferase fusion protein was purified through glutathione Sepharose 4B affinity columns using PharMacia's procedures.

Pag and the mature form of native AOE372 were expressed in E. coli from plasmid pKK223–3 (PharMacia) with a pag or AOE372 insert. Recombinant AOE372 and Pag proteins were purified by ammonium sulfate fractionation (40–60% saturation) and sequential HPLC on DEAE-Sephalac ion exchange and TSK heparin-5PW columns. Target proteins were followed by glutathione synthetase protection assay and by immunoblotting.

Antioxidant Assays—The glutathione synthetase protection assay, indirect peroxidase assay, and in vivo oxidant (i.e. paraquat dichloride or [r-buty]-hydroperoxide) resistance assay were performed essentially as described (17, 29, 30). Yeast cultivation and yeast transformation have also been described (28).

Gel Mobility Shift Assay—Probe labeling and gel mobility shift assay were performed as described previously (28). Two oligonucleotides 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3' and 5'-AGCTTGGGATTCGGCGGACTCGAGACAA-3' were used to produce a consensus κB site probe.

Reporter Assay—Calcius phosphate transfection of HeLa cells and the NF-κB assay were performed as described previously (31, 32). Radioactivity on TLC plates was quantitated with a Fuji BAS2000 phosphor imager. Reporter plasmids driven individually by HTLV-1 LTR and HIV 1 LTR (pU3RCAT and pBENNACT) have been described elsewhere (33, 34). Reporter plasmids containing synthetic NF-κB motif, Sp1 motif, SRE, and the HTLV-1 21-bp motif were based on pCAT-Basic (Promega). Oligonucleotides used to produce these motifs are as follows: NF-κB motif, 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3' and 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3'; Sp1 motif, 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3' and 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3'; SRE, 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3'; SRE, 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3'; and 5'-AGCTTGGGAGATCCCGGATGGACTCACA-3'.

RESULTS

AOE372 Is a Novel Human Thioredoxin Peroxidase—Peroxiredoxins are highly conserved in many mammalian species including human, mouse, rat, and bovine (18–25). The emerging importance of redox in various aspects of gene expression (2) prompted us to investigate the function(s) of human peroxiredoxins. An initial approach employing degenerate polymerase chain reaction was attempted but found to be unsuccessful. Subsequently, we used the yeast two-hybrid assay (26) to identify protein-protein partners for the human thiol-specific antioxidant, Pag (20, 25). From 109 individual transformants in a library containing Pag cDNA, we identified six putative interacting clones. Interestingly, two of the six were derivatives of pagA indicating that Pag protein can homodimerize. The other four clones all contained portions of the same cDNA. A full-length cDNA was constructed from clone 372 with the 5'-sequence repaired using 5'-rapid amplification of cDNA ends. In the intact cDNA, a Kazak sequence (GTGGTCATGG) (37) is present at the in

Kim, I. C. Baines, and S. G. Rhee, manuscript in preparation).
AOE372 is a novel 271-amino acid protein (apparent molecular mass of 31 kDa) that has motifs consistent with a peroxiredoxin. AOE372 has a distinctly hydrophobic region at its N-terminus, which is compatible with a signal peptide (38) that has putative cleavage sites at either Ser77 and Lys78 or Ala79 and Lys80. The protein sequence of AOE372 has 40–85% homology with known peroxiredoxins including yeast thiol-specific antioxidant (70% identity, 82% similarity) (30) and bacterial AhpC (alkyl hydroperoxide reductase; 51% identity, 67% similarity) (2). Notably, AOE372 conserves the two critical motifs found in all human peroxiredoxins (i.e. the cysteine-containing segments surrounding Cys47 (FFYPLDFT-FVCPTEI) and Cys848 (HGEVCPA)). These cysteine motifs have been implicated as being important for catalysis of peroxides (17, 21, 39, 40). At the same time, many residues in AOE372 (e.g. Thr67, Arg106, Tyr114, Lys186, Leu187, and Lys188) differ from consensus found for the other peroxiredoxins (Fig. 1). Because peroxiredoxin subfamilies diverge in sequence by approximately 5–10% (21, 24), AOE372 phylogenetically segregates from known peroxiredoxins into a new subfamily branch (Fig. 1C). (A new suggested nomenclature for the AOE372 subfamily is peroxiredoxin IV. By this convention, Pag, thiol-specific antioxidant/Tpx, and MER5 represent prototypes for the peroxiredoxin I, II, and III subfamilies, respectively.)

Antioxidant Activity of AOE372—We wished to assess the antioxidant activity of AOE372. We overexpressed full-length protein in E. coli and purified it to stainable single polypeptide homogeneity. Purified AOE372 was assessed by reducing SDS-PAGE (Fig. 6A) and was verified to be a single discrete band. Using this preparation, we assayed the antioxidant potential of AOE372.

Fig. 1. AOE372 is a new peroxiredoxin. A, nucleotide and deduced amino acid sequences of AOE372. The predicted N-terminal signal peptide is underlined. The sequence of the C-terminal synthetic peptide used to raise antiserum in rabbit is doubly underlined. AOE372 sequence is deposited in GenBank under accession number U25182. B, amino acid sequence alignment of five different human peroxiredoxins. The consensus sequence is highlighted by stars. The two most highly conserved blocks are shaded. GenBank accession numbers of sequences are as follows: natural killer cell enhancing factor B (NKEFB), L19185; thiolspecific antioxidant (TSA), Z22548; proliferation-associated protein (Pag), X67951; MER5 protein (MER5), D49396. The alignment was generated by the PILEUP program in the Wisconsin package of sequence analysis software. C, distance matrix tree relating AOE372 to other mammalian members in the peroxiredoxin family. The distance matrix was generated by DISTANCES in the Wisconsin package, and the tree reconstruction was performed with the GROWTREE program. GenBank accession numbers of sequences are as follows: human MER5 protein (MER5-human), D49396; mouse MER5 protein (MER5-mouse), M28723; bovine antioxidant protein and substrate protein for mitochondrial ATP-dependent protease SP22 (SP22-bovine), D59225; rat heme-binding 23-kDa protein (HBP23-rat), D30035; human natural killer cell enhancing factor A (NKEFA-human), L19184; rat thioredoxin peroxidase (Tpx-rat), U69099; human thioredoxin peroxidase (Tpx-mouse), U20611; human natural killer cell enhancing factor B (NKEFB-human), L19185; human thiolspecific antioxidant (TSA-human), Z22548; human ORF6 protein (ORF6-human), D14682.

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mine synthetase from inactivation by thiol-dependent metal-catalyzed oxidation (25, 29). This protection is conferred by a thiol peroxidase activity (17, 40). Here, thioredoxin serves as a bioactive hydrogen donor (17, 41). We performed two assays for glutamine synthetase protection and for thioredoxin-linked thiol peroxidase activity. In both assays, AOE372 was highly active (Fig. 2, A and B). Accordingly, recombinant AOE372 scavenged H$_2$O$_2$ in the presence of thioredoxin, thioredoxin reductase, and NADPH with reaction kinetics and potency virtually identical to a previously characterized peroxiredoxin, thiol-specific antioxidant (17). These results biochemically identify AOE372 as a functional human thioredoxin peroxidase.

**Tissue Distribution and Subcellular Localization of AOE372**—Expression of AOE372 mRNA in human tissues and cell lines was analyzed by Northern blotting. AOE372 transcript has an apparent size of 1.0 kilobase pair (Fig. 3, upper panel). AOE372 mRNA is ubiquitously expressed in all tested human cell lines including promyelocytic leukemia HL60, HeLa S3, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, Burkitt’s lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361 (Fig. 3C). However, the expression levels vary significantly between different tissues, with pancreas > liver > heart, (skeletal) muscle, colon (mucosal lining) and testis > ovary > placenta, lung, kidney, and prostate > (small) intestine, thymus,
spleen, and brain (Fig. 3, A and B). By Northern blotting, AOE372 mRNA was not detected in peripheral blood leukocytes. To check that peroxiredoxins could be differentially regulated at the level of tissue-specific expression, we also probed for the expression profile of pag mRNA. We found that the AOE372 and pag expression patterns contrasted distinctly (compare Fig. 3, top panels, AOE372; with Fig. 3, middle panels, pag). In the same blots, whereas pag was preferentially abundant in spleen, thymus, and brain, AOE372 was more highly expressed in tissues such as pancreas (Fig. 3). Thus, there exist tissue-distinct expression profiles for different peroxiredoxins.

To characterize AOE372, we raised rabbit antiserum (372-1) to a keyhole limpet hemocyanin-conjugated C-terminal AOE372 peptide (see Fig. 1 for sequence of synthetic peptide). Antibody 372-1 reacts specifically with either recombinant GST-AOE372 fusion protein (55 kDa) or native HeLa cell AOE372 protein (23 kDa) (Fig. 4, A and B). We used this antibody to assess the protein expression of AOE372 by Western blotting. As shown in Fig. 4C, the protein patterns (pancreas > liver > heart > spleen and thymus) are generally consistent with the mRNA profiles (Fig. 3, A and B).

Because the location of a protein inside cells can provide important information toward function, we stained human cells to determine the subcellular locale(s) for AOE372. AOE372 was found predominantly in the cytoplasm (Fig. 5, A, panel 2). Control stainings performed with either preimmune serum (Fig. 5A, panel 1) or 372-1 antibody first neutralized with an excess of immunizing peptide (Fig. 5A, panel 3) verified the specificity of the stained signals. The cytoplasmic staining of AOE372 is consistent with a similar compartmentalization for Pag (Fig. 5A, panel 4). The immunostainings were verified biochemically by parallel detergent fractionations of cells. Fig. 5B shows that AOE372 and Pag co-fractionated with β-tubulin (a well characterized cytoplasmic protein) and were separated distinctly from NuMA (nuclear/mitotic apparatus protein, a well characterized nuclear marker) (42).

Homo- and Heterodimerization of AOE372—AOE372 was identified initially as a Pag partner in a yeast two-hybrid assay. Previous studies have suggested that thiol antioxidants could be bridged through interchain disulfides (21). These observations led us to consider whether human peroxiredoxins might form homo- and heterodimers and whether this type of protein-protein complex might mechanistically contribute toward the regulation of functional specificity. To test this hypothesis, we genetically analyzed in yeast three human peroxiredoxins, AOE372, Pag, and MER5 in reciprocal combinations. We queried for two-way interactions between the three proteins (Table I). While all three peroxiredoxins exhibited self-dimerization, only AOE372 and Pag heterodimerized in yeast.

Dimerization between purified recombinant AOE372 and Pag was further examined biochemically using native PAGE (Fig. 6, A and B). In nonreducing gels, bands consistent with

Fig. 4. Analysis of AOE372 protein expression. A and B, verification of the specificity of anti-AOE372 serum, 372-1. Cell lysates from E. coli overexpressing GST-AOE372 fusion protein (A) or from HeLa cells (B) were prepared. Externs containing equal amounts (20 μg for HeLa and 8 μg for E. coli) of protein were separated by 12% SDS-PAGE. Immunoblotting was performed separately with preimmune serum (Fig. 5A, panel 2) or 372-1 antibody first neutralized with an excess of immunizing peptide (Fig. 5A, panel 3) to verify the specificity of the stained signals. The cytoplasmic staining of AOE372 is consistent with a similar compartmentalization for Pag (Fig. 5A, panel 4). The immunostainings were verified biochemically by parallel detergent fractionations of cells. Fig. 5B shows that AOE372 and Pag co-fractionated with β-tubulin (a well characterized cytoplasmic protein) and were separated distinctly from NuMA (nuclear/mitotic apparatus protein, a well characterized nuclear marker) (42).

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Fig. 5. Subcellular localization of AOE372. A, confocal microscopy. HeLa cells were seeded onto coverslips and propagated overnight. Cells were washed and fixed as described under “Experimental Procedures.” Coverslips were stained individually with a 1:200 dilution of preimmune rabbit serum (1), a 1:200 dilution of rabbit 372-1 (2), a 1:200 dilution of rabbit 372-1 preincubated with 6 μg of immunizing peptide (3), and a 1:200 dilution of rabbit α-Pag (4). The nuclei were counterstained with a 1:60 dilution of mouse monoclonal anti-NuMA (Matritech, clone 107–7), Texas Red-conjugated goat antibody to rabbit IgG and fluorescein-conjugated goat antibody to mouse IgG (Cappel) were used as secondary antibodies. The primary and secondary antibodies were diluted in PBS with 3% bovine serum albumin. Images were obtained on a Zeis Axiohot inverted microscope with a ×64 objective lens. The red (representing 372-1 and Pag) and the green (representing NuMA) fluorescent signals were overlaid by computer assistance. B, detergent fractionation. Total cell extracts and nuclear and cytoplasmic fractions from 10^6 cells were prepared and separated by 12% SDS-PAGE. Immunoblotting was performed with anti-NuMA, anti-α-tubulin (Sigma, clone B-5–1-2), 372-1 and anti-Pag.

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AOE372 and Pag homodimers could be observed (Fig. 6, A, lanes 1 and 2, B, lane 2). However, because AOE372 and Pag have very similar molecular sizes, formation of AOE372-Pag heterodimers was difficult to distinguish from homodimers based on electrophoretic migration in native PAGE (Fig. 6B, lane 1). Hence, to check for Pag-AOE372 interaction, immunoprecipitations followed by Western blotting with specific antisera (Fig. 6, C and D) were performed. Accordingly, HeLa and HepG2 cells were transiently transfected in combinations with plasmids expressing Gal4-tagged Pag (pMPag), Gal4-tagged AOE372 (pM372), or Gal4-tag alone (pM). Extracts from these cells were first immunoprecipitated with a mouse anti-Gal4 antibody. The immunoprecipitates were then resolved by SDS-PAGE, transferred to filters, and probed with either rabbit anti-AOE372 (372-1) or rabbit α-Pag. Accordingly, we found that AOE372 co-precipitated with Gal4-tagged Pag (Fig. 6C) and that Pag co-precipitated with Gal4-tagged AOE372 (Fig. 6D). These findings are consistent with AOE372 and Pag existing as heterodimers in addition to their respective ability to homodimerize.

### Table I

| Gal4bd fusion | Gal4ad fusion | LacZ filter assay | LacZ CPRG units |
|---------------|---------------|------------------|-----------------|
| Gal4bd-AOE372 | Gal4ad-AOE372 | +                | 34              |
| Gal4bd-Pag    | Gal4ad-Pag    | +                | 26              |
| Gal4bd-MER5   | Gal4ad-MER5   | +                | 25              |
| Gal4bd-AOE372 | Gal4ad-Pag    | +                | 51              |
| Gal4bd-Pag    | Gal4ad-AOE372 | +                | 46              |
| Gal4bd-MER5   | Gal4ad-MER5   | +                | <1              |
| Gal4bd-Pag    | Gal4ad-MER5   | -                | <1              |
| Gal4bd-Pag    | Gal4ad-MER5   | -                | <1              |
| Gal4bd-p53    | Gal4ad-LT (SV40) | + | >1000 |
| Gal4bd-Tax    | Gal4ad       | +                | 18              |

FIG. 6. Dimerization of AOE372. A, PAGE analysis of purified recombinant AOE372. AOE372 cDNA was engineered to remove the sequence coding for signal peptide (Fig. 1), cloned into pKK223-3 (Pharmacia), and expressed as a nonfusion protein in E. coli. Purified AOE372 protein (5 μg, lanes 1 and 3; 6.25 μg, lanes 2 and 4) was separated by nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) PAGE, and the gels were stained with Coomassie Blue R250. Dimeric (star) and monomeric (arrow) forms of AOE372 are evident in the nonreducing gel, while only the monomeric form (23 kDa) is present in the reducing gel. Relative migration positions of molecular weight markers are indicated. B, Coomassie-stained gel of purified recombinant Pag and Pag plus AOE372. pag cDNA was inserted into pKK223-3 and expressed in E. coli. C and D, AOE372 heterodimerizes with Pag in vivo. HeLa and HepG2 cells were transfected with plasmids p Mustang (CLONTECH), pMPag or pM372. Cleared cell lysates were immunoprecipitated using mouse anti-Gal4 antibody (α-Gal4; RR5C1 from Santa Cruz Biotechnology, Inc.). Blots were probed with 372-1 (C) and α-Pag (D). Bands representing co-immunoprecipitating AOE372 and Pag by α-Gal4 are indicated.

Activity of AOE372 on NF-κB Activation—As a cytoplasmic antioxidant, AOE372 has a likely function in balancing intracellular redox. Conceivably, in this fashion, AOE372 impacts signal transduction and gene transcription. To shed additional light on AOE372 function, we asked whether its overexpression in HeLa cells might modulate, through redox, NF-κB-mediated signaling. By Western blotting (Fig. 7A, top part) and by immunofluorescent staining (data not shown), AOE372-expressing plasmid pSV372 conferred a 3–6-fold overexpression of AOE372 in transiently transfected HeLa cells. We thus transfected pSV372 into cells and subsequently checked for NF-κB activity by gel mobility shift assays. An NF-κB-specific band was evident when nuclear extracts from HeLa cells were incubated with labeled probe containing κB sites (Fig. 7A, lane 1; Fig. 7B, lane 2). When no extract was added, this band was not seen (Fig. 7B, lane 1). The same binding activity was erased by the addition of a 50-fold excess of unlabeled specific-oligonucleotide competitor (Fig. 7B, lane 3). This NF-κB-specific signal fades progressively if one mixes in nuclear extracts from AOE372-overexpressing cells harvested at different times (Fig. 7A, lanes 2–4; see also Fig. 7B, lane 4). Thus extracts from AOE372 overexpressing cells are significantly depleted in NF-κB binding activity. Consistent with this in vitro finding, overexpression of AOE372 also suppressed TNF- and TPA-dependent intracellular activation of NF-κB (Fig. 7B, compare lane 5 with lane 7 and lane 6 with lane 8). Hence, AOE372 activities mirror those previously described for thioredoxin (43), suggesting that the former is the linked effector of the latter.

Exactly how oxidants and antioxidants influence NF-κB activation is incompletely understood. Our results indicate thioredoxin peroxidase (i.e. AOE372) as an additional component in the multisteped redox-sensitive pathway that regulates NF-κB. To understand better the molecular basis for this activity, we asked how AOE372 affects the status of nuclear

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**Human Thioredoxin Peroxidase and NF-κB Activation**

NF-κB p65, p50, and cytoplasmic IκB-α. We treated HeLa cells with TPA and assayed p65, p50, and IκB-α by Western blotting. In AOE372-overexpressing HeLa cells, we found coordinated changes in nuclear p65, p50, and phosphorylated cytoplasmic IκB-α (Fig. 7, C and D). For example, upon TPA treatment, nuclear p50 was increased 2.9-fold, and nuclear p65 was increased 3.5-fold (Fig. 7C, lane 2). When AOE372 was overexpressed, the amounts of p50 and p65 were reduced to a relative level of 0.4–0.5 (Fig. 7C, lane 3) compared with the base line (Fig. 7C, lane 1). Similarly, when one assesses the ratio of phosphorylated/total IκB-α (relative ratios are 1:3.3:0.4 for lanes 1–3, Fig. 7D), one finds a significant diminution of the phosphorylated species in the AOE372 sample. While there could be many explanations, one interpretation is that AOE372 affects the phosphorylation of IκB-α, thus activating p65 and p50.

To check for specificity of AOE372 action, we stimulated cells using different agents. We found that NF-κB-dependent expression normally activated by tumor necrosis factor, TPA, or HIV-1 Tat was repressed when AOE372 was overexpressed (Fig. 8A). Controls (Fig. 8B) showed that this effect was NF-κB-specific, since Sp1-dependent expression (Fig. 8B, compare lanes 1 and 2) was unaffected by AOE372 (compare lanes 1 and 3). The responsiveness of additional enhancers to AOE372 was also assessed. HeLa cells were separately co-transfected with AOE372-expressing plasmid (pSV372) paired with CAT-reporters under the control of various enhancers. Results from five different enhancer-containing reporters (Sp1, SRE, HTLV-1 21-bp motif, NF-κB, and HIV-1 LTR) demonstrated that NF-κB and the NF-κB-dependent HIV-1 LTR were selectively affected by AOE372 (Fig. 8C).

Productive replication of HIV is influenced by NF-κB (44–46). A biologically important corollary of AOE372 activity on NF-κB is to document an effect on HIV-1 infection. We thus searched for the influence of AOE372 on HIV expression and the influence of HIV infection on AOE372 expression. In Fig. 9A, we show that AOE372 protein is poorly detectable in T cell lines (C81 and MT2) that are acutely infected with high titers of HIV-1 NL4–3 under conditions where essentially 100% of cells showed cytopathic effects (lanes 2 and 5). Similarly, AOE372 expression was also significantly reduced in T-cells, chronically infected with HIV-1 (C81; Fig. 9A, lane 4). These findings are consistent with a suggestion that infection by HIV modulates the expression of AOE372. Conversely, forced overexpression of AOE372 in cells transfected with pNL4–3 substantially reduced the expression of viral proteins as assayed by either p24 or viral reverse transcriptase (Fig. 9B). These findings are compatible with a reciprocal regulation of AOE372 and HIV-1, with the former probably exerting an effect on the latter through NF-κB.

**Protein-Protein Complexes between Different Antioxidants Impact Activity**—There are five forms of human thioredoxin while lane 1 was mock-treated. Each lane contains 8 μg of nuclear extract. Approximately 7-fold reduction of p50 and p65 was observed in AOE372-overexpressing cells (compare band intensity in lanes 2 and 3), while the amount of NuMA is constant in three lanes. D, effects on IκB-α phosphorylation. Cells in lanes 1–3 were transfected and treated as in panel C. Cytoplasmic fractions were prepared and resolved by 12% SDS-PAGE. Blot was probed with mouse monoclonal anti-α-tubulin (Sigma, clone B-5-1-2) or rabbit polyclonal anti-IκB-α (Santa Cruz Biotechnology). Each lane contains 10 μg of the cytoplasmic fraction. Positions of hypophosphorylated or phosphorylated IκB-α (IκBα and IκBβ, respectively) are indicated. Approximately 8-fold reduction of IκBα was observed in AOE372-overexpressing cells (compare IκBα/IκBβ + IκBβ ratios for lanes 2 and 3). Phosphatase inhibitor (300 μM calyculin A) and proteasome inhibitor (40 μM ZLLLH) were used to pretreat cells for 60 min before harvesting to prevent dephosphorylation and proteolysis of IκB-α.
human peroxiredoxins. We provide the first evidence that the AOE372 class of peroxiredoxins functions through cytoplasmic IkB-dependent regulation. Overexpression of AOE372 and Pag shows a similar protection to toxicity from an oxidant, paraquat dichloride (PDC). In parallel, overexpression of Pag showed a similar protection. Interestingly, when AOE372 and Pag were expressed together, total amounts stoichiometrically equivalent to each expressed alone, cooperativity (38–58% increase in resistance) was observed (Fig. 10A). Cooperation between AOE372 and Pag was also found in a related assay with a different oxidant, t-butyl hydroperoxide (24–30% increase in resistance at 2–8 h; Fig. 10B). We also transfected AOE372- and/or Pag-expressing plasmids (pSV372 and/or pSVPag) into HeLa cells and assayed the expression of NF-κB-dependent reporters. Fig. 10C shows that AOE372 and Pag act synergistically in inhibiting NF-κB activation to a degree greater than either alone (-fold inhibition: AOE372 = 2.5, Pag = 3, AOE372 plus Pag = 9). These results suggest that the specific activity of antioxidants can be regulated through either homo- or heterodimer formation. They provide the first evidence that intracellular subunit assortment between different forms of thioredoxin peroxidases affects potency and perhaps specificity of action.

**DISCUSSION**

**AOE372 Is a Novel Peroxiredoxin—Peroxiredoxins are evolutionarily conserved in all organisms. The ubiquity and structural conservation of peroxiredoxins suggest that they serve fundamentally important functions. Until now, studies, based in part on recursive sequence searches/alignments using yeast and bacteria proteins, have suggested the existence of four human peroxiredoxins (17, 40, 41). Here, we demonstrate the existence of a fifth human peroxiredoxin, AOE372. We provide the first evidence that the AOE372 class of peroxiredoxins functions through cytoplasmic IkB-α to regulate nuclear activity of NF-κB. One nomenclature divides mammalian peroxiredoxins into

**Fig. 9.** AOE372 and HIV infection. A. Western blot analysis of AOE372 protein expression in HIV-infected cells. Lanes 1–5, uninfected C8166-45 cells, C8166-45 acutely infected with pNL4-3 (C81/pNL4-3), uninfected MT2, MT2 chronically infected with HIV-1 (MT2/C1), and MT2 acutely infected with pNL4-3 (MT2/pNL4-3), respectively. 30 μg of cell extracts were separated by 12% SDS-PAGE, and the blot was probed as in Fig. 4. A 23-kDa monomeric AOE372 band (filled arrow) and two 42–46-kDa AOE372-related bands (open arrow) were detected in T cell lines (C81 and MT2). Lane 6 is a 55-kDa purified GST-AOE372 fusion protein. Blot was also probed for α-tubulin (bottom). B, inhibition of HIV-1 replication by overexpression of AOE372. AOE372-expressing plasmid (pSV372; 5 μg) was co-transfected with 7 μg of an infectious HIV-1 molecular clone, pNL4-3 (NL43) into HeLa cells. The production of supernatant p24 and reverse transcriptase (RT) was assayed 40 h after transfection. Similar results were obtained from three independent experiments.
three subfamilies (I, II, and III). AOE372 represents the prototype for a fourth subfamily (IV). Why there are more than one thioredoxin peroxidase can be explained by the fact that different forms of enzyme have varying tissue distributions (Fig. 3). Furthermore, in contrast to MER5, which resides predominantly in the mitochondria (23), we find that AOE372 and Pag are localized to the cytoplasm (Fig. 5). Taken with data from others (19, 20, 23, 24), our findings support a model in which different peroxiredoxins serve restricted functions in a subcellular and tissue-specific manner. The absence or low level of expression of AOE372 in primary blood leukocytes and lymphoid organs (thymus and spleen) suggests the interesting possibility that in some cells in which antioxidants may need to be less tightly controlled to allow their primary functions, this class of thioredoxin peroxidase is down-regulated. That AOE372 specifically interacts with Pag (Table I and Fig. 6) and that a heterodimer of two different peroxiredoxins is more active than counterpart homodimers (Fig. 10) further suggest a mechanism of regulation of antioxidant activity based on combinatorial assortment of different forms of enzymes. The generality of this mechanism is supported by findings that proteins from other subfamilies (I, II, and III) of peroxiredoxins have also been observed to heterodimerize in vitro with members from a different subfamily. The fact that AOE372 and MER5 do not form protein-protein complexes inside cells (Table I) suggests specificity to dimerization.

**Peroxiredoxins Are Redox Regulators of Signal Transduction**—Cells have multiple pathways to transduce extracellular signals to the nuclear compartment. These pathways are complex networks that ultimately modulate gene expression. Intermediating proteins in the transmission of signals from cell surface to nucleus are numerous and incompletely understood. Kinases and phosphatases represent signal transducers that regulate activity by phosphorylation and dephosphorylation. Oxidants and antioxidants represent a different set of signaling molecules that modify function through redox. Similar to phosphorylation, redox can serve as the critical switch in many processes. Biologically relevant oxidants (e.g. hydrogen peroxide and nitric oxide) that serve as pleiotropic signaling molecules have been well documented (47–49). Balancing these oxidants are antioxidants such as glutathione, thioredoxin, and glutaredoxin (1). The delicate interplay inside cells between oxidants and antioxidants ultimately determines the activity profile for many transcription factors.

NF-κB is redox-regulated (2, 50). Oxidants such as H₂O₂ rapidly activate NF-κB (2, 51). This effect is genetically separable from another downstream redox-sensitive step, which primarily affects NF-κB’s DNA binding activity (8). Since neither H₂O₂ nor antioxidant has an effect on NF-κB activation in cell-free systems, more than one intracellular redox-sensitive molecule must be involved directly or indirectly in H₂O₂-triggered redox regulation (8). Our results here show for the first time that an early H₂O₂-triggered regulation of NF-κB involves the human thioredoxin peroxidase, AOE372. The fact that AOE372 has a similar effect on many different activators of NF-κB (Figs. 7 and 8) suggests that it targets a common signaling step.

**An Effect on IκB-α Phosphorylation**—The mechanism through which human thioredoxin peroxidase affects the nuclear NF-κB activity is poorly understood. We suggest that AOE372 modulates IκB-α phosphorylation in the cytoplasm and thus affects a peroxiredoxin-dependent redox step (Fig. 7D). Our results are consistent with a recent report ascribing an activity to glutathione peroxidase on IκB-α phosphorylation (52). However, it is noteworthy that among several antioxidant pathways the thioredoxin pathway seems to have the greatest selectivity for NF-κB (14–16).

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3 S. W. Kang and S. G. Rhee, unpublished data.
IxB-α can be phosphorylated either on serine 32/36 (9–11) or on tyrosine 42 (12). Recently, an IxB kinase has been described (53, 54). Our findings here suggest that oxidants and/or antioxidants might influence IxB kinase activity. The identification of a specific peroxiredoxin, AOE372, that influences IxB phosphorylation provides a reagent to test this hypothesis.

Acknowledgments—We thank Elizabeth Rich, Ileana Quinto, Hua Xiao, Sang Won Kang, and Vadim Gladyshev for critical readings of this manuscript.

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