Ets regulates peroxiredoxin1 and 5 expressions through their interaction with the high-mobility group protein B1

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Peroxiredoxins (Prdxs) are thiol-specific antioxidant proteins that are highly expressed in human cancer cells. Prdxs have been shown to be involved in tumor cell proliferation under conditions of microenvironmental stress such as hypoxia. We hypothesized that Prdxs could be categorized into two groups, stress-inducible and non-inducible ones. In this study, we analyzed the promoter activity and expression levels of five Prdx family members in human cancer cells. We found that both Prdx1 and Prdx5 are inducible after treatment with hydrogen peroxide or hypoxia, but that Prdx2, Prdx3, and Prdx4 are not or are only marginally inducible. We also found that Ets transcription factors are the key activators for stress-inducible expression. High-mobility group protein HMGB1 was shown to function as a coactivator through direct interactions with Ets transcription factors. The DNA binding of Ets transcription factors was significantly enhanced by HMGB1. Silencing of Ets1, Ets2, Prdx1, and Prdx5 expression sensitized cells to oxidative stress. These data indicate that transcription of *Prdx* genes mediated by Ets/HMG proteins might protect cells from oxidative stress. (*Cancer Sci* 2008; 99: 1950–1959)

Redox regulation is important for various metabolic functions of cells.[1] Glutathione and thioredoxin are major reducing systems that regulate redox balance and communicate with cellular molecules to execute specific responses. Peroxiredoxins (Prdxs) are small antioxidant proteins that scavenge reactive oxygen species (ROS). Prdxs are also involved in the cellular response to ROS, and function as a cellular defense system against ROS. Like thioredoxin, expression of Prdxs increases during a variety of oxidative stress conditions. There are five members of the 2-Cys Prdx family, including three cytoplasmic forms, one mitochondrial form, and one secretary form.

Mice lacking Prdx1 have a shortened lifespan owing to the development of severe hemolytic anemia and several malignant cancers.[2] Prdx1-deficient cells showed increased sensitivity to oxidative stress and decreased cell proliferation. Thus, Prdx1 may function as a tumor suppressor. Elevated expression of Prdxs has been reported in human cancers, including lung cancer, breast cancer, and bladder cancer.[3–5] Knockdown of Prdx1 expression resulted in significant growth inhibition, radiosensitization, and reduced metastasis of lung cancer cells.[6] Elucidating the molecular mechanisms underlying the expression of Prdx family members will be critical to understanding Prdx function under physiological or stress conditions in tumor cells.

In this study, we demonstrate that Prdx1 and Prdx5 are significantly induced after H_2O_2 and hypoxia treatment, through the activation of Ets transcription factors. Further, the high-mobility group (HMG) protein HMGB1 was shown to interact directly with Ets transcription factors, enhancing their DNA binding ability and the promoter activity of target genes. This novel molecular interaction may contribute to our understanding of the functions of Prdxs and tumor biology from the aspect of redox regulation in cancer cells.

Materials and Methods

Cell culture. Human prostate cancer PC3 cells and human epidermoid cancer KB cells were cultured in Eagle's minimal essential medium. This medium was purchased from Nissui Seiyaku (Tokyo, Japan) and contained 10% fetal bovine serum. Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies. Antibodies against Sp1 (sc-59), GST (sc-138), Ets1 (sc-111), Ets2 (sc-351), Prdx2 (sc-23967), and Prdx4 (sc-23974) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Flag (M2) antibody and anti-Flag (M2) affinity gel were purchased from Sigma (St Louis, MO, USA). Anti-HA-peroxidase (3F10) and anti-Thio antibody were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and Invitrogen (San Diego, CA, USA), respectively. To generate anti-Prdx1, anti-Prdx5, and anti-BAF57 antibodies, the synthetic peptides PDVQKSKEYFSKQK, GLTCSLAPNIISQL, and EPPTDPIPEDEKKE were used as immunogens, respectively, and the antisera obtained from immunized rabbits were affinity purified using the synthetic peptide antigens. Anti-YB-1 and anti-HMGB1 antibodies were prepared as described previously.[7,8] The anti-Prdx3 antibody was kindly gifted by Dr H. Nanri (Seinan Jogakuin University).[9]

Plasmid construction. The plasmid construction of pGEX-HMGB1 expressing GST-HMGB1 and its deletion mutants (ΔA, ΔB) has been described previously.[8,10] For construction of pcDNA3-HA-HMGB1, the HA-tagged HMGB1 cDNA was ligated into the pcDNA3 vector (Invitrogen). To obtain full-length cDNAs for Ets1 and Ets2, polymerase chain reaction (PCR) was carried out on a SuperScript cDNA library (Invitrogen) using the following primer pairs: 5′-ATGAAGGCGGCCGTCGATCTCAAGC-3′ and 5′-TCACTCGTCGGCATCTGGCTTGACG-3′ for Ets1; and 5′-ATGAATGATTTCGGAATCAAGAATATGG-3′ and 5′-TCAGTCCTCCGTGTCGGGC-3′ for Ets2. PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). To construct pGEX-Ets1 and pGEX-Ets2, expressing GST-Ets1 and GST-Ets2, respectively,

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the *Not*I fragment of Ets1 cDNA and *Eco*RI fragment of Ets2 cDNA were ligated into the pGEX-4T vector (Pharmacia Biotech, Tokyo, Japan). For construction of TH-HA-Ets1 and TH-HA-Ets2, expressing N-terminal tagged HA-Ets1 and HA-Ets2, respectively, in bacteria, full-length Ets1 and Ets2 cDNAs were ligated into the TH-HA vector.[11] Their deletion mutants (TH-HA-Ets1 Δ1, TH-HA-Ets1 Δ2, TH-HA-Ets2 Δ1, TH-HA-Ets2 Δ2) were constructed from TH-HA-Ets1/2 full-length plasmids by deletion of the *Xag*I-*Mph1103*I, *Pae*I, *Pdi*I-*Xba*I, and *Bpu1102*I-*Xba*I fragments, respectively. To generate ThioHis-Ets1 Δ3, ThioHis-Ets1 Δ4, and ThioHis-Ets2 Δ3, *Psc*I-*Pst*I fragment of TH-HA-Ets1 Δ1, *Xag*I-*Pst*I fragment of TH-HA-Ets1, and *Xap*I-*Pst*I fragment of TH-HA-Ets2 Δ1 were ligated into the pThioHis vector (Invitrogen), respectively. For construction of pcDNA3-Flag-Ets1 and pcDNA3-Flag-Ets2, N-terminal Flag-tagged Ets1 and Ets2 cDNAs were ligated into the pcDNA3 vector (Invitrogen). The constructs Prdx1-Luc (−1065 to +83), Prdx2-Luc (−338 to +136), Prdx3-Luc (−357 to +42), Prdx4-Luc (−306 to +36), and Prdx5-Luc (−314 to +113) were made using the following primer pairs: 5′-CTCGAGGCCAAGGCGGGCGGATCACCTG-3′ and 5′-AAGCTTAACCACCGACACCAGGCAAGAAC-3′ for Prdx1-Luc; 5′-AGATCTTAGATGCTGCAGCCTCAGC-3′ and 5′-AAGCTTGGCAAAGGCTAGACGCACGG-3′ for Prdx2-Luc; 5′-AGATCTTAGCTTATTAACGGACTAAAAC-3′ and 5′-AAGCTTCAGTGCACTCGGGCGCCACGG-3′ for Prdx3-Luc; 5′-AGATCTGTGAGGGGCTTGTGTGCAG-3′ and 5′-AAGCTTCACGCGAGCGCAGAAACACG-3′ for Prdx4-Luc; and 5′-AGATCTAAGATGCAAATCATATGC-3′ and 5′-AAGCTTCCCACGGCCACTTCCACTCC-3′ for Prdx5-Luc. These PCR products were cloned and ligated into the *Bgl*II-*Hind*III sites or the *Xho*I-*Hind*III sites of the pGL3-basic vector (Promega). PCR was also performed to introduce the mutations of Ets binding sites into the Prdx1 and Prdx5 promoter, using the following primer pairs: 5′-CTCGAGTGGT*CCTGGAAGTA*AAGAGAATCC-3′ and 5′-AAGCTTAACCACCGACACCAGGCAAGAAC-3′ for Prdx1-Luc WT; 5-CTCGAGTGGT*CCTAAAAGTA*AAGAGAATCC-3′ and 5′-AAGCTTAACCACCGACACCAGGCAAGAAC-3′ for Prdx1-Luc MT; 5′-AGATCTGGGT*CCCGGAAGCT*CTGTTCTGC-3′ and 5′-AAGCTTCCCACGGCCACTTCCACTCC-3′ for Prdx5-Luc WT; and 5′-AGATCTGGGT*CCCAAAAGCT*CTGTTCTGC-3′ and 5′-AAGCTTCCCACGGCCACTTCCACTCC-3′ for Prdx5-Luc MT. Italicized nucleotides indicate wild-type Ets binding sites and mutated ones. These PCR products were cloned and ligated into the *Bgl*II-*Hind*III sites or the *Xho*I-*Hind*III sites of the pGL3-basic vector (Promega).

Western blotting analysis. Whole-cell lysates were prepared as described previously.[12,13] The indicated amounts of whole-cell lysates were separated by SDS-PAGE and transferred to polyvinylidine difluoride microporous membranes (Millipore, Bedford, MA, USA) using a semidry blotter. The blotted membranes were treated with 5% (w/v) skimmed milk in 10 mmol/L Tris, 150 mmol/L NaCl, and 0.2% (v/v) Tween 20, and incubated for 1 h at room temperature with primary antibody. The following antibodies and dilutions were used: a 1:5000 dilution of anti-Sp1, a 1:5000 dilution of anti-GST, a 1:500 dilution of anti-Ets1, a 1:2000 dilution of anti-Ets2, a 1:5000 dilution of anti-Flag (M2), a 1:5000 dilution of anti-Thio, a 1:10 000 dilution of anti-Prdx1, a 1:5000 dilution of anti-Prdx2, a 1:5000 dilution of anti-Prdx3, a 1:1000 dilution of anti-Prdx4, a 1:1000 dilution of anti-Prdx5, a 1:10 000 dilution of anti-YB-1, a 1:2000 dilution of anti-HMGB1, and a 1:10 000 dilution of anti-BAF57. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody or a 1:5000 dilution of anti-HA-peroxidase. Bound antibody was visualized using an enhanced chemiluminescence kit (GE Healthcare Biosciences, Piscataway, NJ, USA) and membranes were exposed to Kodak X-OMAT film (Kodak, Paris, France). For the correlation assay, the intensity of each signal was quantified by the NIH imaging program, version 1.62 (NIH, Bethesda, MD, USA).

Northern blotting analysis. Northern blotting analysis was performed as described previously.[12,13] To obtain cDNAs for Prdx1, Prdx2, Prdx3, Prdx4, and Prdx5, PCR was carried out on a SuperScript cDNA library (Invitrogen) using the following primer pairs: 5′-ATGTCTTCAGGAAATGCTAAAATTG-3′ and 5′-TCACTTCTGCTTGGAGAAATATTC-3′ for Prdx1; 5′-ATGGCCTCCGGTAACGCGCGC-3′ and 5′-CTAATTGTGTTTGGAGAAATATTCC-3′ for Prdx2; 5′-ATGGCGGCTGCTGTAGGACGG-3′ and 5′-CTACTGATTTACCTTCTGAAAGTAC-3′ for Prdx3; 5′-ATGGAGGCGCTGCCGCTGCTAGC-3′ and 5′-TCAATTCAGTTTATCGAAATACTTCAGC-3′ for Prdx4; 5′-ATGGGACTAGCTGGCGTGTGCG-3′ and 5′-TCAGAGCTGTGAGATGATATTGGG-3′ for Prdx5. Ets1 and Ets2 cDNAs were obtained as described in "Plasmid construction". RNA samples (20 μg/lane) were separated on a 1% formaldehyde–agarose gel and transferred to a Hybond N$^+$ membrane (GE Healthcare Biosciences) with 10 × SSC. After prehybridization and hybridization with radiolabeled cDNA fragment of Ets1, Ets2, Prdx1, Prdx2, Prdx3, Prdx4, and Prdx5, signal intensities were analyzed using a bio-imaging analyzer (FLA2000; Fujifilm, Tokyo, Japan).

Hypoxia conditions. Hypoxic conditions were generated as described previously.[14] Briefly, plates seeded with PC3 cells or KB cells at 70% to 80% confluency, at a pH between 7.2 and 7.4, were incubated at 37°C in Anaerocult A mini (Merck, Darmstadt, Germany), creating oxygen-free circumstances. After 4 h, plates were removed from the Anaerocult A mini and used in assays immediately or after further incubation under normal oxygen.

Knockdown analysis using siRNAs. The following double-stranded RNA 25-base-pair oligonucleotides were commercially generated (Invitrogen): 5′-GCAAAGAAAUGAUGUCUCAAGCAUU-3′ for Ets1; 5′-GCAGCAACUUGAAUUUGCUCACCAA-3′ for Ets2; 5′-UGACCAUCUGGCAUAACAGCUGUGG-3′ for Prdx1; 5′-AGAACCUCUUGAGACGUCGAUUCCC-3′ for Prdx5. siRNA transfections were performed as described previously.[12,13] Ten microliters of Lipofectamine 2000 (Invitrogen) was diluted in 250 μL of Opti-MEM I medium (Invitrogen) and incubated for 5 min at room temperature. Next, 250 pmol of indicated siRNA or inverted control duplex Stealth RNAs (Invitrogen), diluted in 250 μL of Opti-MEM I, was added gently and incubated for 20 min at room temperature. Oligomer-lipofectamine complexes and aliquots of 1×10^6 cells in 500 μL of culture medium were combined and incubated for 10 min at room temperature. Aliquots of 1.5×10^5 PC3 cells were used in luciferase assays and aliquots of 2.5×10^3 PC3 cells were used in the WST-8 assay, as described below. The remaining cells were seeded into 35-mm dishes with 2 mL of culture medium and harvested after 72 h of culture for Western blotting analysis as described above.

Luciferase assay. Transient transfection and a luciferase assay were performed as described previously.[12,13] PC3 cells (1×10^5) were seeded into 12-well plates. The following day, cells were cotransfected with the indicated amounts of Prdx1, Prdx2, Prdx3, Prdx4, or Prdx5 reporter plasmid and expression plasmids using Superfect reagent (Qiagen, Hilden, Germany); cells were then transfected with siRNA as described above, followed by transfection with the indicated amounts of reporter plasmid at intervals of 12 h; finally cells were incubated under normal conditions or in the presence of 1 mmol/L H_2O_2, for 30 min, with or without recovery for 12 h, or incubated in hypoxic conditions for 4 h, with or without reoxygenation for 12 h. Forty-eight hours post-transfection, cells were lyzed with reporter lysis buffer (Promega) and luciferase activity was detected using a Picagene kit (Toyoinki, Tokyo, Japan); light intensity was measured using a luminometer (Luminescencer

JNII RAB-2300; ATTO, Tokyo, Japan). The results shown are normalized to protein concentrations measured using the Bradford method, and are representative of at least three independent experiments.

Chromatin immunoprecipitation assay (ChIP). The ChIP assay was performed as described previously.[12,13] Briefly, PC3 cells were transiently transfected with Flag, Flag-Ets1, or Flag-Ets2 plasmid and cultured for 48 h as described above. Soluble chromatin from 1×10^6 cells was incubated with 2 μg of anti-Flag (M2) affinity gel. Purified DNA was dissolved in 20 μL of dH_2O and 2 μL of DNA was used for PCR analysis with the following primer pairs for Prdx1: 5′-CTCGAGTCC-TGATATTTTATTTTCCTTTAC-3′ as a forward primer and 5′-AAGCTTAACCACCGACACCAGGCAAGAAC-3′ as a reverse primer; for Prdx2: 5′-AGATCTTAGATGCTGCAGCCTCAGC-3′ as a forward primer and 5′-AAGCTTGGCAAAGGCTAGAC-GCACGG-3′ as a reverse primer; for Prdx3: 5′-AGATC-TTAGCTTATTAACGGACTAAAAC-3′ as a forward primer and 5′-AAGCTTCAGTGCACTCGGGCGCCACGG-3′ as a reverse primer; for Prdx4: 5′-AGATCTGTGAGGGGCTTGT-GTGCAG-3′ as a forward primer and 5′-AAGCTTCACGCG-AGCGCAGAAACACG-3′ as a reverse primer; and for Prdx5: 5′-AGATCTGCAAAATAAACACATTTTACTCC-3′ as a forward primer and 5′-AAGCTTCCCACGGCCACTTCCACTCC-3′ as a reverse primer. PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide.

Coimmunoprecipitation assay. Transient transfection and immunoprecipitation assays were performed as described previously.[12,13] Briefly, 1×10^5 PC3 cells were seeded into 6-well plates. The following day, cells were transfected with 1 μg of each of HA- and Flag-fused expression plasmids using Superfect reagent (Qiagen) according to the manufacturer's instructions. Three hours post-transfection, the cells were washed with PBS, cultured at 37°C for 48 h in fresh medium, and then lyzed in buffer X containing 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylendiaminetetraacetic acid (EDTA), 120 mmol/L NaCl, 0.5% (v/v) Nonidet P-40 (NP-40), 10% (v/v) glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L dithiothreitol (DTT). The lysates were centrifuged at 21 000 *g* for 10 min at 4°C and the supernatants (300 μg) were incubated for 2 h at 4°C with anti-Flag (M2) affinity gel. Immunoprecipitated samples washed three times with buffer X and preimmunoprecipitated samples (30 μg) were subjected to Western blotting analysis with anti-Flag antibody and anti-HA antibodies, as described above. For immunoprecipitation assays without transient transfection, 70–80% confluent PC3 cells in 100-mm tissue-culture plates were harvested with PBS. After centrifugation at 500 *g* for 5 min, cells were resuspended in ice-cold 10 mmol/L Hepes/KOH (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, and 0.5 mmol/L PMSF and lyzed by adding NP40 to a final concentration of 0.5% (v/v), and lysates were centrifuged at 500 *g* for 5 min. The resulting nuclear pellet was resuspended in ice-cold 20 mmol/L Hepes/KOH (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L PMSF, and incubated for 15 min on ice. The lysates were centrifuged at 21 000 *g* for 10 min at 4°C and the supernatants (500 μg) were incubated for 2 h at 4°C with 2 μg of rabbit immunoglobulin G (IgG) or anti-Ets1 antibody. Immunoprecipitated samples washed three times with buffer X and preimmunoprecipitated samples (50 μg) were subjected to Western blotting analysis with indicated antibodies.

Expression of GST-, ThioHis-, and HA-fusion proteins in *E. coli*. For expression of GST-, ThioHis-, and HA-fusion proteins, bacteria transformed with expression plasmids were incubated with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (Boehringer Mannheim, Mannheim, Germany) for 2 h at room temperature, and collected by centrifugation. After sonication (TAITEC sonicator, Tokyo, Japan) in buffer X, the cell lysates were cleared by centrifugation at 21 000 *g* for 10 min at 4°C.

Purification of GST-fusion proteins. Purification of GST-fusion proteins has been described previously.[12,13] GST-fusion proteins prepared as described above were bound to glutathione-sepharose 4B (GE Healthcare Biosciences) in a 50% slurry in buffer X for 4 h at 4°C, washed three times with buffer X, and eluted with 50 mmol/L Tris-HCl (pH 8.0) and 20 mmol/L reduced glutathione, according to the manufacturer's protocol (GE Healthcare Biosciences). Purified GST-fusion proteins were used in electrophoretic mobility shift assays (EMSAs).

GST pulldown assay. GST-HMGB1 or its deletion mutants immobilized on glutathione-sepharose 4B were incubated with soluble bacterial extracts containing the indicated fusion proteins for 2 h at 4°C in buffer X. Bound samples were washed three times with buffer X and subjected to Western blotting analysis with anti-HA or anti-Thio antibody.

EMSA. The sequences of the oligonucleotides used as probes in EMSAs were 5′-GGATCTCGA*GCAGGAAG*TTCGA-3′ and 5′-GGTCGAA*CTTCCTGC*TCGAGAT-3′. Human Ets binding sites are italicized. Preparation of ^{32}P-labeled oligonucleotide probes and EMSAs with purified GST-fusion proteins were performed as described previously.[12,13] Briefly, oligonucleotides were annealed with their complementary strands. The double-stranded products were end-labeled with [α-^{32}P] dCTP (GE Healthcare Biosciences) using the Klenow fragment (Fermentas, Vilnius, Lithuania), and purified from gels. Then, GST-fusion proteins were incubated for 5 min at room temperature in a final volume of 20 μL containing 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L $MgCl_2$, 10 μmol/L $ZnCl_2$, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 mg/mL bovine serum albumin (BSA), 10% glycerol, 0.05% NP-40, and 4 ng of ^{32}P-oligonucleotide probe. The reaction mixtures were resolved by electrophoresis on 4% polyacrylamide gels (polyacrylamide/bisacrylamide, 80:1) at 10 V/cm for 90–120 min at room temperature in 0.5 × tris-borate-EDTA buffer (45 mmol/L Tris base, 45 mmol/L boric acid, and 1 mmol/L EDTA). Gels were dried and analyzed using a bio-imaging analyzer (FLA2000).

Cytotoxicity analysis. The water-soluble tetrazolium salt (WST-8) assay was performed as described previously.[12,13] Briefly, 2.5×10^3 PC3 cells transfected with the indicated amounts of siRNA were seeded into 96-well plates. The following day, the indicated concentrations of H_2O_2 were applied. After 72 h, surviving cells were stained with TetraColor ONE (Seikagaku Corporation, Tokyo, Japan) for 90 min at 37°C. The absorbance was then measured at 450 nm.

Results

Prdx family gene expression under oxidative stress conditions. To examine whether oxidative stress can activate Prdx family gene expression, human prostate cancer PC3 cells were exposed either to 30 min of 1 mmol/L H_2O_2 or 4 h of hypoxia, followed by recovery/reoxygenation in a CO_2 incubator. Western blot analysis showed a significant increase in the levels of Prdx1 and Prdx5 expression during recovery/reoxygenation (Fig. 1a). Similar results were also obtained by using human cancer KB cells (Suppl. Fig. 1a). Analysis of the nucleotide sequences of the promoters of Prdx family genes revealed multiple Ets transcription factor binding sites in the promoter regions of Prdx1 and Prdx5, suggesting that Ets transcription factors might mediate the stress induction of both Prdx1 and Prdx5.

Ets transcription factor expression under oxidative stress conditions. As expected, the expression levels of both Ets1 and Ets2 were also increased during recovery/reoxygenation after treatment with H_2O_2 and hypoxia in PC3 cells. More than two-fold elevated expression of both Ets1 and Ets2 was

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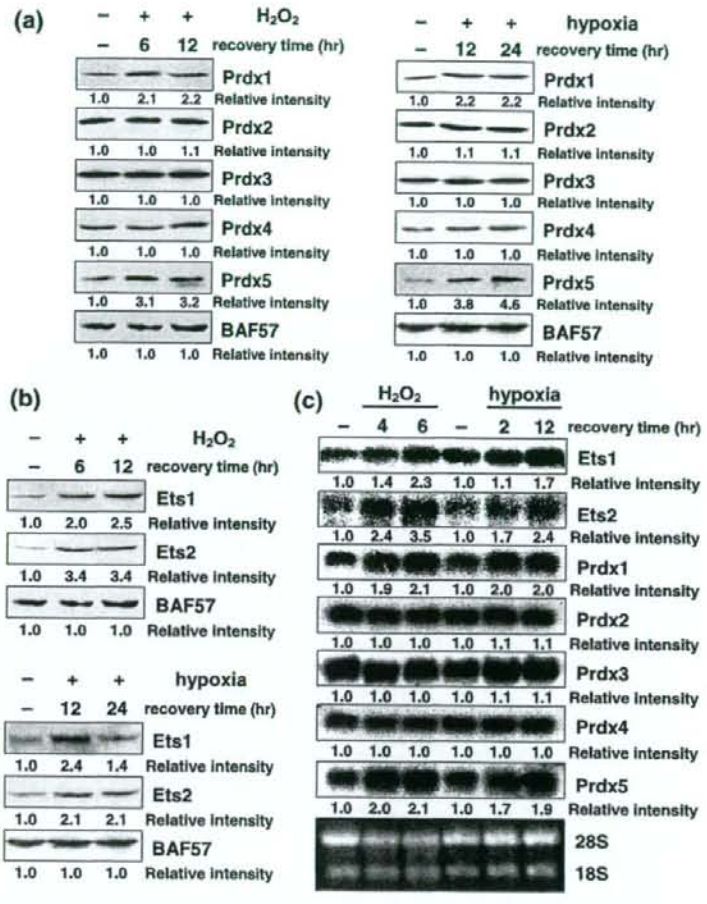

Fig. 1. H_2O_2 or hypoxia followed by recovery/reoxygenation increases Ets1, Ets2, peroxiredoxin1 (Prdx1), and Prdx5 expression. (a,b) PC3 cells were treated with or without 1 mmol/L H_2O_2 for 30 min after which time the media were replaced with fresh media and cells were recovered for the indicated time periods, or hypoxia for 4 h followed by reoxygenation for the indicated time periods. Whole-cell extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibodies. Immunoblotting of BAF57 is shown as a loading control. Relative intensity is shown at the bottom of the panel. (c) PC3 cells were treated as described in (a,b). Total RNA (20 μg) was separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N+ membrane. Northern blotting analysis was performed with the indicated cDNA probes. Gel staining with ethidium bromide is also shown.

observed after 12 h of H_2O_2 treatment (Fig. 1b), suggesting that stress induction of *Prdx* genes might be regulated by the transcription factors Ets1 and Ets2. In KB cells, similar results were also obtained (Suppl. Fig. 1b).

mRNA levels of Ets transcription factors and Prdx family genes under oxidative stress. To analyze the molecular mechanisms that up-regulate both Ets and Prdx in response to oxidative stress, we examined the mRNA levels of these genes. Northern blotting analysis confirmed that the amounts of Ets1, Ets2, Prdx1, and Prdx5 mRNA were significantly increased in PC3 cells as a result of oxidative stress (Fig. 1c).

Ets proteins regulate *Prdx1* and *Prdx5* genes. To confirm these results, we first cloned the core promoter regions of all *Prdx* genes, including CpG islands, for reporter assays (Fig. 2a). Silencing of Ets expression significantly reduced the expression of Prdx1 and Prdx5, but not that of other *Prdx* genes (Fig. 2b). Reporter assays also showed that silencing of Ets expression significantly suppressed the promoter activities of both *Prdx1* and *Prdx5* genes to ~10–25% of the promoter activities of the other *Prdx* genes (Fig. 2c). Next, we performed cotransfection experiments using Ets1 and Ets2 expression plasmids. Both Ets1 and Ets2 could transactivate the Prdx1 and Prdx5 promoters, with ~three–four-fold activation (Fig. 2d). Furthermore, Prdx1 and Prdx5 promoter activities were significantly induced during recovery/reoxygenation after oxidative stress (Fig. 2e).

To prove that Ets transcription factors were involved in the expression of *Prdx1* and *Prdx5* genes, we introduced the mutation in the Ets binding sites of both Prdx1 and Prdx5 promoters and examined the transactivation of reporter genes. The transfection of either Ets1 or Ets2 transactivated the wild-type promoters of both *Prdx1* and *Prdx5* genes again. However, this transactivation was completely abolished by the introduction of mutations into Ets binding sites (Fig. 3a,b).

To determine whether the promoters of both Prdx1 and Prdx5 are direct target of Ets transcription factors, we performed ChIP assays covering the proximal promoter segments of five *Prdx* genes. We initially tried these experiments using commercial antibodies against Ets1 and Ets2, but they did not work well. Then, we employed transient transfection into PC3 cells with either Flag-tagged Ets1 or Ets2 expression plasmid. After 48 h, ChIP assays were performed with anti-Flag antibody. As shown in Fig. 3c, we found that Ets1 and Ets2 were observed in the promoters of both *Prdx1* and *Prdx5* genes. However, no binding of Ets was observed in Prdx2, Prdx3, and Prdx4 gene promoters. These results suggest that Ets binding sites in the proximal region of both Prdx1 and Prdx5 are the target of Ets binding *in vivo*.

Ets proteins interact with HMG protein. To investigate the mechanism of Ets-mediated transcriptional regulation, we previously used a membrane pulldown assay to identify proteins that interact with Ets.[15] One of the candidate proteins we

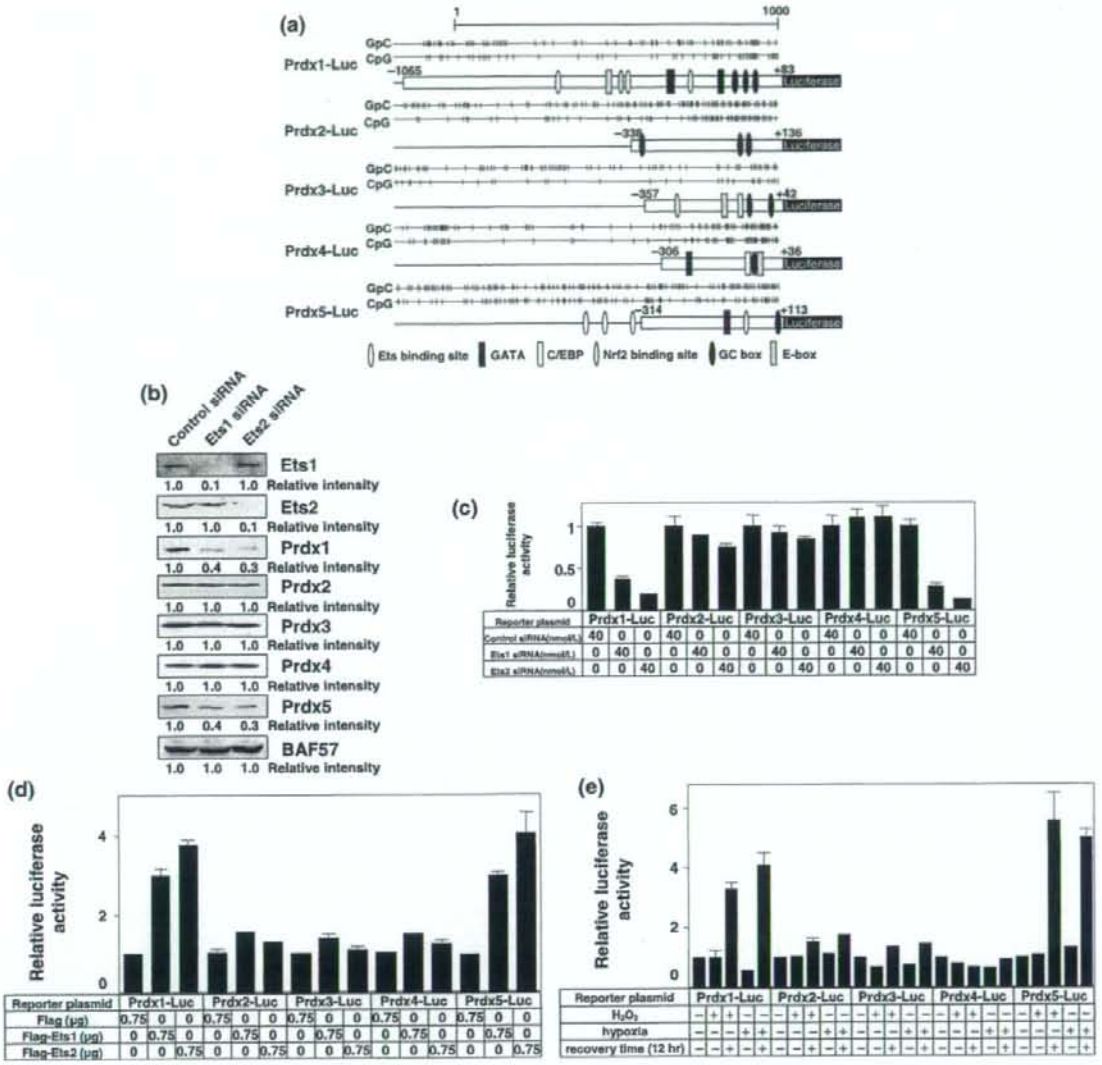

Fig. 2. Ets transcription factors and oxidative stress regulate peroxiredoxin1 (*Prdx1*) and *Prdx5* gene expression. (a) Schematic representations of Prdx1-Luc, Prdx2-Luc, Prdx3-Luc, Prdx4-Luc, and Prdx5-Luc are shown with their CpG islands. (b) PC3 cells were transiently transfected with the indicated amounts of control, Ets1, or Ets2 siRNA. After 48 h, whole-cell extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibodies. Immunoblotting of BAF57 is shown as a loading control. Relative intensity is shown at the bottom of the panel. (c) PC3 cells were transiently transfected with the indicated amounts of control, Ets1, or Ets2 siRNA followed by transfection with 1 μg of the indicated reporter plasmid at intervals of 12 h. The results were normalized to protein concentration measured using the Bradford method. All values are representations of at least three independent experiments. The luciferase activity of each Prdx-Luc alone corresponds to 1. Bars indicate ± SD (d) PC3 cells were transiently cotransfected with 0.75 μg of Flag, Flag-Ets1, or Flag-Ets2 expression plasmid in addition to 0.75 μg of the indicated reporter plasmid. A luciferase assay was performed as described in (c). The luciferase activity of each Prdx-Luc alone corresponds to 1. Bars indicate ± SD. (e) PC3 cells were transiently transfected with 1 μg of the indicated reporter plasmid. Thirty-six hours post-transfection, cells were incubated under normal conditions, treated with 1 mmol/L H_2O_2 for 30 min or incubated in hypoxic conditions for 4 h; cells were either lyzed immediately or after recovery/reoxygenation for 12 h. A luciferase assay was performed as described in (c). The luciferase activity of each Prdx-Luc construct without oxidative stress corresponds to 1. Bars indicate ± SD.

found to preferentially interact with Ets was HMGB1. Coimmunoprecipitation experiments using expression plasmids clearly showed that both Ets1 and Ets2 interact with HMGB1 (Fig. 4a). Moreover, endogenous Ets1 protein was shown to interact with HMGB1 in human prostate cancer PC3 cells (Fig. 4b). We also observed that Ets1 interacts with Sp1 as a positive control.[16] On the other hand, Ets1 could not interact with YB-1 (Fig. 4b). Next, we mapped the domain in Ets

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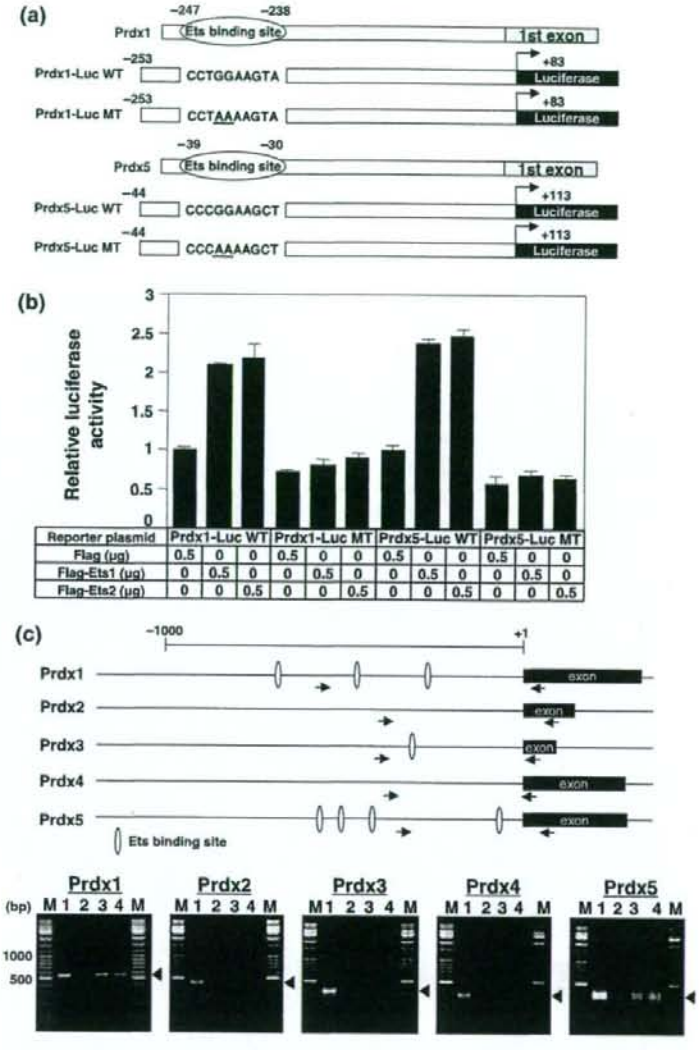

Fig. 3. Ets transcription factors regulate peroxiredoxin1 (*Prdx1*) and *Prdx5* gene expression through Ets binding sites located in the promoter region. (a) Schematic representation of the promoter regions of the *Prdx1* and *Prdx5* genes. Prdx1-Luc WT, Prdx1-Luc MT, Prdx5-Luc WT, and Prdx5-Luc MT used in (b) are also shown. (b) PC3 cells were transiently cotransfected with the indicated amounts of Ets1 or Ets2 expression plasmids and 0.5 μg of the indicated reporter plasmids shown in (a). The results were normalized to protein concentration measured using the Bradford method. All values are representations of at least three independent experiments. The luciferase activity of Prdx1-Luc WT or Prdx5-Luc WT alone corresponds to 1. Bars indicate ± SD (c) Schematic representation of the promoter region and 5′ end of *Prdxs* genes. White circles and arrows indicate Ets binding sites and polymerase chain reaction primer regions, respectively. Chromatin immunoprecipitation assays using PC3 cells transiently transfected with Flag vector (lane 2), Flag-Ets1 plasmid (lane 3), or Flag-Ets2 plasmid (lane 4) was performed using an anti-Flag (M2) antibody. Soluble chromatin (lane 1) and immunoprecipitated DNAs (lane 2–4) were amplified by polymerase chain reaction using specific primer pairs for the Prdxs promoters (Prdx1, −491 to +83 bp; Prdx2, −338 to +136 bp; Prdx3, −357 to +42 bp; Prdx4, −306 to +36 bp; Prdx5; −264 to +113 bp). Lane M shows a DNA size marker and the arrowhead indicates PCR products (574 bp for Prdx1, 474 bp for Prdx2, 399 bp for Prdx3, 342 bp for Prdx4, 377 bp for Prdx5).

proteins required for interactions with HMGB1 by a pulldown assay, using a GST-fusion protein containing HMGB1 and HA- or ThioHis-fusion proteins containing deletion mutants of Ets1 and Ets2 (Fig. 4c). A fusion protein lacking the D domain and part of the transcription-activating domain was unable to bind HMGB1 (Fig. 4d). We next mapped the domain of HMGB1 responsible for the interaction with Ets1 and Ets2. A GST-fusion protein containing HMGB1 with a deletion of the A box was unable to interact with Ets1 and Ets2 (Fig. 4e).

HMGB1 protein increases the DNA binding affinity of Ets proteins and enhances *Prdx* genes. It has been previously shown that HMGB1 can stimulate the DNA binding of p53.[17] We examined the influence of HMGB1 on the sequence-specific binding of Ets to DNA. GST-fusion proteins for EMSAs were purified and stained with Coomassie Brilliant Blue (Fig. 5a). Fig. 5b shows the binding of Ets1 and Ets2 in the presence and absence of HMGB1. HMGB1 protein markedly enhanced the binding of Ets to DNA. HMGB1 protein increased the DNA binding affinity of Ets in a dose-dependent manner (Fig. 5b). We next examined whether HMGB1 could transactivate the Ets-dependent promoters of *Prdx* genes. As shown in Fig. 5c, the promoter activities of Prdx1 and Prdx5 were greatly enhanced by ~five–10-fold by HMGB1 overexpression. Next, we tested whether HMGB1 as well as Ets transcription factors can increase expression of endogeneous *Prdx* genes. As shown in Fig. 5d, expression of HMGB1 could increase expression of both endogeneous *Prdx1* and *Prdx5* genes. Transfection of both Ets1 and Ets2 expression plasmids could also increase expression of these genes. These data also support the results shown in Fig. 2c.

Down-regulation of Ets1, Ets2, Prdx1, or Prdx5 sensitizes cells to oxidative stress. Initially, we analyzed whether the expression of *Prdx* genes correlates with sensitivity to H_2O_2. Knockdown of each *Prdx* gene by siRNA rendered cells more sensitive to

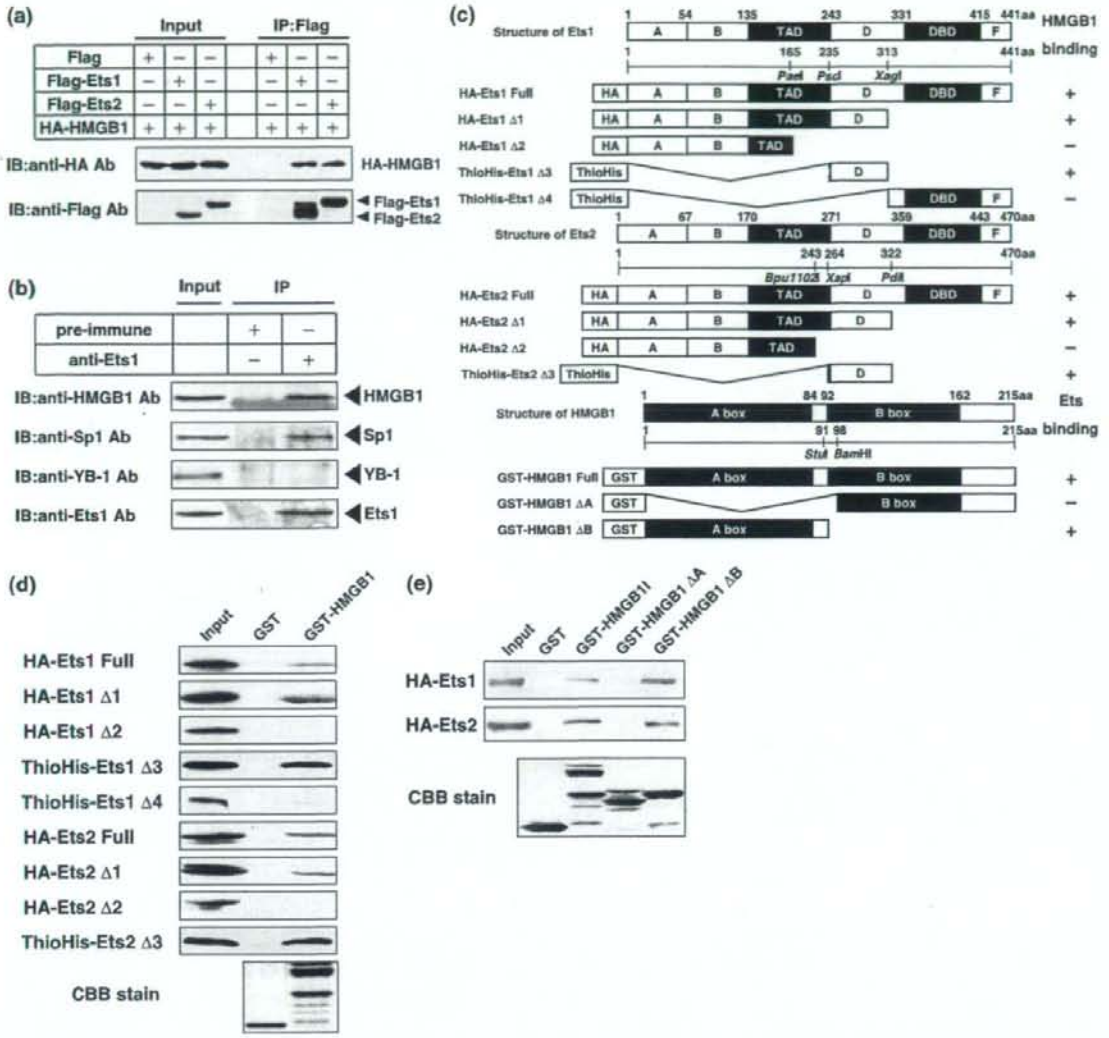

Fig. 4. Ets transcription factors interact with HMGB1. (a) Whole-cell extracts (300 μg) prepared from PC3 cells cotransfected with 1 μg of each of the indicated expression plasmids were immunoprecipitated with anti-Flag (M2) antibody. The resulting immunocomplexes and whole-cell extracts (30 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using anti-HA and anti-Flag (M2) antibodies. (b) Nuclear extracts (500 μg) prepared from PC3 cells were immunoprecipitated with 2 μg of rabbit IgG or anti-Ets1 antibody. The resulting immunocomplexes and nuclear extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using indicated antibodies. (c) Schematic representations of GST-, HA-, or ThioHis-fusion proteins containing deletion mutants of Ets1, Ets2, and HMGB1. TAD, transcription activating domain; DBD, DNA binding domain. (d) Equal amounts of GST and GST-HMGB1 fusion proteins immobilized on glutathione-sepharose 4B were incubated with HA- or ThioHis-fusion proteins containing deletion mutants of Ets1 and Ets2 shown in (c). Bound protein samples and 10% of input were subjected to SDS-PAGE, and Western blotting analysis was performed using anti-HA or anti-Thio antibody. Gel staining with Coomassie Brilliant Blue is also shown. (e) Equal amounts of GST, GST-HMGB1, GST-HMGB1-ΔA, and GST-HMGB1-ΔB fusion proteins immobilized on glutathione-sepharose 4B were incubated with HA-Ets1 or HA-Ets2 fusion proteins. Bound protein samples and 10% of input were subjected to SDS-PAGE, and Western blotting analysis was performed using an anti-HA antibody. Gel staining with Coomassie Brilliant Blue is also shown.

H_2O_2 (Fig. 6a). We next examined whether Ets1 and Ets2 could protect cells against oxidative stress. As shown in Fig. 6b, silencing of either Ets1 or Ets2 sensitized cells to oxidative stress, suggesting that Ets activity influences the sensitivity to ROS through the expression of Prdx1 and Prdx5.

Discussion

ROS are highly reactive oxygen metabolites that include the superoxide radical, hydrogen peroxide (H_2O_2) and the hydroxyl radical.[1,18] ROS can be produced under aerobic conditions or when an organism is exposed to a variety of stress conditions.[1,18]

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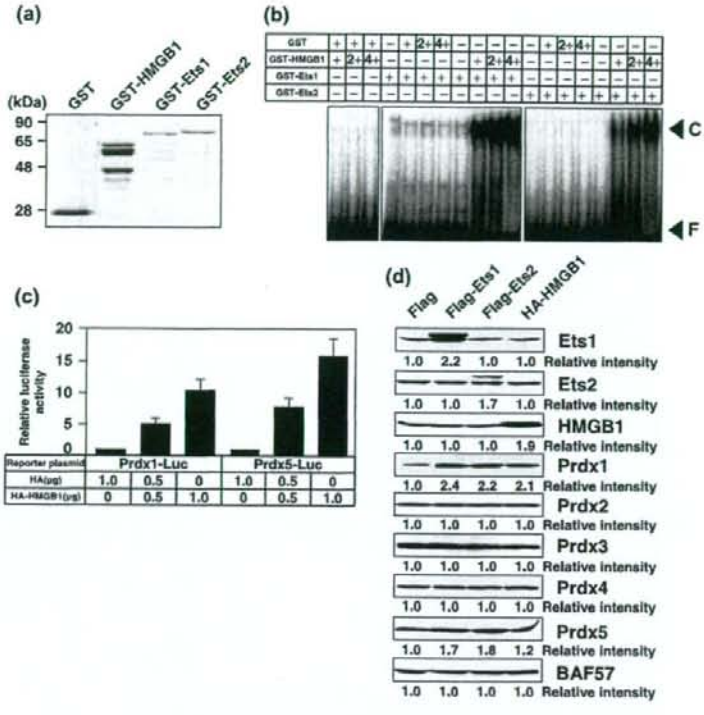

Fig. 5. HMGB1 increases the DNA binding affinity of Ets and enhances the promoter activity of peroxiredoxin (*Prdx*) genes. (a) Purified GST, GST-HMGB1, GST-Ets1, and GST-Ets2 fusion proteins used for EMSAs were stained with Coomassie Brilliant Blue. (b) GST or GST-HMGB1 fusion proteins (+, 50 ng; 2+, 100 ng; 4+, 200 ng) were mixed with GST-Ets1 or GST-Ets2 fusion proteins (+, 50 ng), and incubated with 4 ng of ^{32}P-labeled oligonucleotides containing the Ets binding site. Reaction mixtures were resolved by electrophoresis on 4% polyacrylamide gels in 0.5 × tris-borate-EDTA buffer. Gels were dried and analyzed using a bio-imaging analyzer (FLA2000). F and C indicate free probe and DNA-protein complex, respectively. (c) PC3 cells were transiently cotransfected with the indicated amounts of HA or HA-HMGB1 expression plasmid in addition to 0.5 μg of Prdx1-Luc or Prdx5-Luc. The results were normalized to protein concentration, which was measured using the Bradford method. All values are representations of at least three independent experiments. The luciferase activity of Prdx1-Luc alone or Prdx5-Luc alone corresponds to 1. Bars indicate ± SD (d) PC3 cells were transiently transfected with 2 μg of the indicated expression plasmid. After 48 h, whole-cell extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibodies. Immunoblotting of BAF57 is shown as a loading control. Relative intensity is shown at the bottom of the panel.

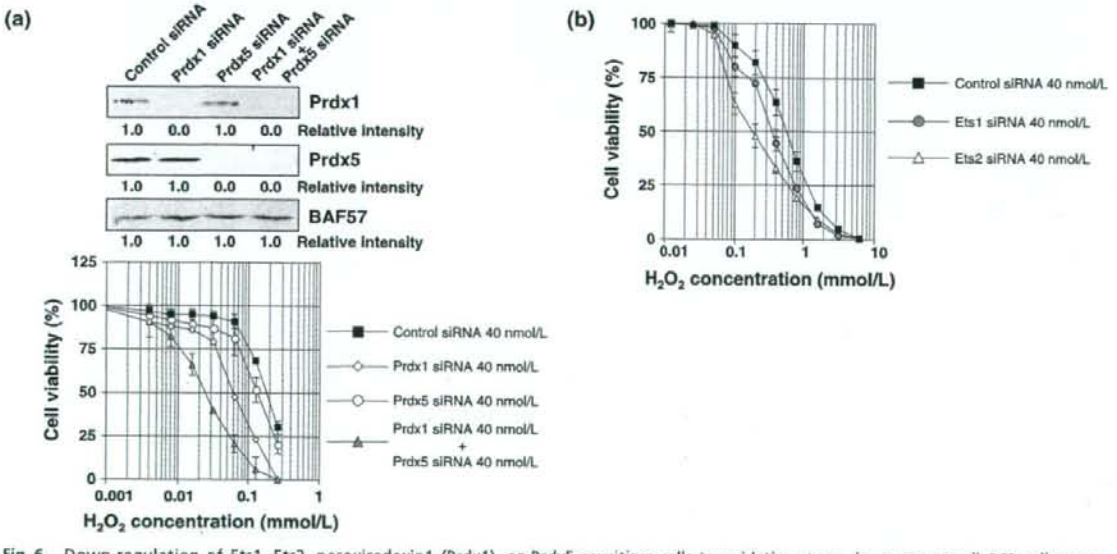

Fig. 6. Down-regulation of Ets1, Ets2, peroxiredoxin1 (Prdx1), or Prdx5 sensitizes cells to oxidative stress. (a, upper panel) PC3 cells were transiently transfected with 40 nmol/L of control, Prdx1, and/or Prdx5 siRNAs. After 48 h, whole-cell extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibodies. Immunoblotting of BAF57 is shown as a loading control. Relative intensity is shown at the bottom of the panel. (lower panel) 2.5 × 10^3 PC cells were transfected with 40 nmol/L of Control siRNA (black square), Prdx1 siRNA (white triangle), Prdx5 siRNA (white circle), or Prdx1 siRNA and Prdx5 siRNA (gray triangle). The following day, various concentrations of H_2O_2 were applied. After 72 h, cell survival was analyzed by a WST-8 assay. Cell survival in the absence of H_2O_2 corresponds to 100%. All values are the means of at least three independent experiments. Bars indicate ± SD (b) 2.5 × 10^3 PC cells were transfected with 40 nmol/L of control siRNA (black square), Ets1 siRNA (gray circle), or Ets2 siRNA (white triangle). WST-8 assay was performed as described in (a).

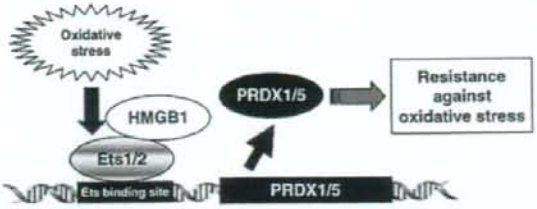

Fig. 7. A model that shows a novel relation between peroxiredoxin (*Prdx*) gene expression and Ets/HMGB1 transcription system. Oxidative stress induces the expression of Ets transcription factors. Then, Ets transcription factors induce both Prdx1 and Prdx5 expression via binding to Ets binding site. HMG proteins enhance the DNA binding ability of Ets transcription factors and function as a coactivator. Increased Prdxs lead to resistance against oxidative stress.

Human tumor cells produce significant amounts of ROS. To protect cells from oxidative stress, all organisms are equipped with antioxidant proteins, including superoxide dismutase/catalase, glutathione, and many kinds of peroxidase.[1,18] Peroxiredoxins (Prdxs) are a new type of antioxidant protein that reduces ROS. Prdx1 and Prdx2 are mainly found in the cytoplasm, Prdx3 is present in mitochondria, and Prdx4 is secreted into the extracellular space. Prdx5 is an atypical, cytosolic type, which possesses more effective antioxidant activity against ROS than other Prdxs. It has been shown that ROS are involved in signaling in biological processes, including cell proliferation and apoptosis.[1] The expression of Prdxs is closely associated with human cancers, such as thyroid cancer,[19] breast cancer,[4] and lung cancer.[3] However, little is known about the regulation of *Prdx* gene expression in cancer cells. We speculated that Prdxs may be categorized into stress-inducible and non-inducible types.

Identification of the target genes regulated by specific transcription factors and cofactors interacting with these transcription factors is critical for understanding gene expression. Our observations allow us to construct a model that shows a novel relation between Ets transcription factors, HMG proteins, and *Prdx* gene expression (Fig. 7). The experimental data clearly demonstrate that Prdxs can be categorized into two groups from the viewpoint of transcriptional regulation. Prdx1 and Prdx5 are stress inducible, whereas the other Prdxs are constitutively expressed (Fig. 1a and Suppl. Fig. 1a). In addition, we showed that Ets1 and Ets2 are key transcription factors for the stress induction of Prdx1 and Prdx5 expression (Figs 1–3), and that HMGB1 functions as a coactivator through direct interactions with Ets1 and Ets2 (Figs 4 and 5). Although we found one Ets binding site in the promoter region of Prdx3 (Fig. 2a), the expression of Prdx3 was not induced by oxidative stress or by the transfection of Ets expression plasmids (Figs 1 and 2). This site is also a high-affinity binding site of the other transcription factor NRF2, indicating that this site of the Prdx3 promoter may not function as an Ets binding site. Furthermore, it has been shown that Prdx3 expression is regulated by c-Myc, suggesting that E-box located in the core promoter region may function mainly through c-Myc dependent transcription.[20]

The human Ets transcription factor family comprises more than 25 members that retain a conserved DNA binding domain called the Ets domain.[21,22] The Ets transcription factor family is involved in various important biological processes.[23–25] Protein–protein interactions often regulate DNA binding, subcellular localization, and transcriptional activity. So far, many partners that interact with Ets family members have been identified. There has been only one report on the interaction between an Ets family member and an HMG protein.[26] In the present study, we showed that Ets1 and Ets2 interact with HMGB1, and that this molecular interaction is involved not only in the DNA binding ability of these transcription factors, but also in the transactivation of their target genes (Fig. 5). The region in Ets1 and Ets2 that is required for the interaction with HMGB1 is located between amino acid residues 250–330, and includes a phosphorylation site.[27] Since the Ets domain is not necessary for this interaction, HMGB1 may not participate in target gene selection, but exclusively inhibit the Ets domain–dependent interaction with other molecules. As *in vitro* phosphorylation of Ets proteins has been shown to inhibit sequence-specific DNA binding,[28] the molecular interaction with HMGB1 might inhibit phosphorylation of Ets proteins and thereby stimulate sequence-specific DNA binding.

Both Ets proteins and HMGB1 have been shown to be highly expressed in tumor cells.[29–31] Thus, we speculate that mutual cooperation of these proteins is involved in various aspects of tumor biology, including transformation, antiapoptotic ability, and malignant progression.[30,32,33] Ets transcription factors have been shown to be involved in tumor angiogenesis and metastasis.[30,34,35] The expression levels of Prdx3, Prdx4, and Prdx5 were significantly higher in tumors that were poorly differentiated. A significant positive association between prognosis and Prdx expression has previously been shown in breast cancers.[4] Taken together, these data do not contradict our results that *Prdx1* and *Prdx5* are Ets target genes. As shown in Fig. 6, our data clearly indicate that Ets activity influences sensitivity to ROS through the expression of both *Prdx1* and *Prdx5* genes. These results supports previous reports that overexpression of either Prdx1 or Prdx5 contributes to protect cells against apoptosis caused by oxidative stress.[36–39] Thus, both Prdx1 and Prdx5 may be required for cancer cells to remain viable under conditions of oxidative stress. There are also data to show that the level of ROS is increased by the knockdown of either Prdx1 or Prdx5 expression.[40,41] Therefore, the expression of Ets transcription factors might play a critical role in the early development of tumors in a hypoxic environment. Further investigations are required to show the correlation between Ets and Prdx expression in clinical tumors.

In summary, although Prdxs function as antioxidant proteins, Prdx1 and Prdx5 expression are stress inducible. The current work suggests the presence of functional differences among members of the Prdx family and reveals important information contributing to our understanding of tumor biology.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. H_2O_2 or hypoxia followed by recovery/reoxygenation increases Ets1, Ets2, peroxiredoxin1 (Prdx1), and Prdx5 expression in KB cells. (a,b) (left panel) KB cells were treated with or without 1 mmol/L H_2O_2 for 30 min, after which time the media were replaced with fresh media and cells were recovered for the indicated time periods. (right panel) KB cells were treated with or without hypoxia for 4 h followed by reoxygenation for the indicated time periods. Whole-cell extracts (100 μg) were subjected to SDS-PAGE, and Western blotting analysis was performed using the indicated antibodies. Immunoblotting of BAF57 is shown as a loading control. Relative intensity is shown at the bottom of the panel.

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CONFERENCE PAPER

Symposium: Molecular target therapy

Preclinical studies of molecular-targeting diagnostic and therapeutic strategies against breast cancer

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Abstract We have investigated protein kinase C (PKC) signaling, a putative differentiation-related and metastasis suppressor gene Cap43/NDRG1/Drg-1, and Y-box binding protein-1 (YB-1) to identify new molecular targeting for breast cancer. PKC is a family of serine/threonine kinases that is involved in the regulation of cell growth. We have demonstrated that PKC caused G_1 arrest in a breast cancer cell line through a mechanism involving a PKC–ERK MAPK–JNK–Rb protein signaling pathway. We have also characterized a novel mechanism through which all-*trans* retinoic acid (ATRA) and antineoplaston, anticancer drug, caused cell growth inhibition in breast cancer cells through effects on intracellular pathways. ATRA decreased the expression of PKCα, as well as reduced ERK MAPK phosphorylation, and consequently caused G_1 arrest. Antineoplaston caused the down-regulation of PKCα protein expression, resulting in inhibition of ERK MAPK phosphorylation, with resultant inhibition of Rb phosphorylation leading to G_1 arrest. PKC signaling represents a promising target for development of novel therapeutic agents. Cap43 is known as *N*-myc downstream-regulated gene 1 (NDRG1). Treatment with estradiol (E_2) significantly decreased the expression of Cap43 in ER-α-positive breast cancer cell lines. Co-administration of tamoxifen abrogated the E_2-induced downregulation of Cap43 in ER-α-positive cell lines. These results suggested that Cap43 may hold the potential of being a molecular marker to determine the therapeutic efficacy of anti-estrogenic anticancer agents in breast cancer. YB-1 is a member of the cold shock domain protein family. The expression of nuclear YB-1 was correlated with HER2 positively in clinical specimens of human breast cancer. Immunostaining studies showed that nuclear YB-1 expression was an independent prognostic factor of overall survival. Expression of nuclear YB-1 played an essential role in acquirement of malignant characteristics through HER2-dependent pathways in breast cancer patients. PKC, Cap43 and YB-1 may be useful in new molecular-targeting diagnosis and therapeutics in breast cancer.

Keywords Breast cancer · Molecular targeting · Protein kinase C · Cap43 · YB-1

Abbreviations

PKC	Protein kinase C
NDRG1	*N*-myc downstream-regulated gene 1

This article is based on a presentation delivered at Symposium 3, "Molecular target therapy: basics and clinical application," held on 30 June 2007 at the 15th Annual Meeting of the Japanese Breast Cancer Society in Yokohama.

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Drg-1	Differentiation-related gene-1
YB-1	Y-box binding protein-1
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
JNK	c-Jun NH_2-terminal kinase
Rb	Retinoblastoma
ATRA	All-*trans* retinoic acid
E_2	Estradiol
VEGF	Vascular endothelial growth factor
EGFR	Epidermal growth factor receptor
DAG	Diacylglycerol
PMA	Phorbol 12-myristate 13 acetate

Introduction

The development of effective adjuvant therapy has improved survival of breast cancer patients. Furthermore, molecular cancer therapeutics is attracting attention as an area of fundamental importance, and molecular targeting therapy such as trastuzumab has contributed to the treatment of breast cancer patients. Recently, other molecular targeting, including EGFR, VEGF and cyclooxygenase-2 (COX-2), has been clinically evaluated in breast cancer, and the results of clinical trials using gefitinib, bevacizumab and celecoxib may be promising [1–3]. To identify this kind of unique molecular target, we are continuing our efforts to investigate molecular targets that may be useful for both diagnosis and treatment. In particular, we have focused on three projects, including PKC signaling, putative metastasis suppressor gene Cap43/NDRG1/Drg-1 and Y box binding protein (YB-1).

Protein kinase C (PKC) signaling

Role of PKC in the breast cancer cell

PKC is a family of phospholipid-dependent serine/threonine kinases that are involved in regulating basic cell functions, such as proliferation, differentiation and cell death. To date, at least 12 isozymes of PKC have been identified and have been divided into three subgroups according to their structural and functional properties [4, 5]. PKCα, -βI, -βII and -γ are considered to be classical or conventional PKC (cPKC) isozymes, which are activated by calcium, diacylglycerol (DAG) and phorbol esters. PKCδ, -ε, -η and -θ are classified as novel PKC (nPKC) isozymes, which are also activated by DAG and phorbol esters, but are not calcium dependent. Both cPKCs and nPKCs have two C1 domains that confer DAG and phorbol ester responsiveness. PKCζ, $-\lambda/\iota$ and μ are atypical PKC (aPKC) isozymes, which are neither calcium dependent nor activated by DAG or phorbol esters.

When a certain stimulus is applied to the cell membrane, phospholipase C, an effector, is activated via the G protein. As a result, phosphatidylinositol-4,5-bisphosphate (PIP_2), which is a trace component of the cell membrane, is broken down to DAG. The DAG plays an important role in the activation of PKC by causing an increase in the affinity of PKCs for cell membranes, which is accompanied by PKC activation and pseudo-substrate release [5]. The DAG thus formed activates cPKC and nPKC, leading to phosphorylation of substrates downstream such as the mitogen-activated protein kinase (MAPK) pathway [4, 6]. By contrast, phorbol esters, such as phorbol 12-myristate 13 acetate (PMA), are promoters of carcinogenesis that pass through the cell membrane and activate cPKC and nPKC isozymes by directly binding them.

To clarify the role of PKC in the breast cancer cell, we have investigated the effects of activating endogenous PKC on cell proliferation and the cell cycle by treating the breast cancer cell line SKBR-3 with PMA. This inhibited cell growth in a concentration-dependent manner, causing a marked arrest of cells in G_1. Pretreatment with GF109203X, PKC inhibitor, completely blocked the antiproliferative effect of PMA, and pretreatment with the PKCδ inhibitor rottlerin partially blocked it. Infecting SKBR-3 cells with an adenovirus vector containing wild-type PKCδ, WTPKCδAdV, had similar effects to PMA. Downstream of PKC, PMA treatment inhibited ERK MAPK phosphorylation, up-regulated JNK phosphorylation and inhibited Rb phosphorylation. These results strongly implicated PKC (mainly PKCδ) in the G_1 arrest induced by PMA. Thus, PKC might be an important cell-cycle regulator through the PKC-MAPK pathway, and suggested PKC as a target for breast cancer treatment [7].

Agents that affect PKC and/or MAPK in breast cancer

The intracellular signaling, including PKC and MAPK, represents a promising target for the development of novel therapeutic agents. We have investigated the effect of agents that would affect PKC and MAPK in breast cancer cells.

Retinoid

Retinoid is a generic term for natural and synthetic derivates of vitamin A-related compounds, of which all-*trans* retinoic acid (ATRA) is a biologically active prototype. Retinoids exert most of their cellular effects through the activation of specific nuclear receptors, the retinoic acid

receptors (RARs). Binding of retinoids to the nuclear receptor, which ligand-activated transcription factors, leads to regulation in cell growth, differentiation and apoptosis. The anticancer effects of retinoids are a subject of intense investigation. Indeed, retinoids have been reported to be effective in the treatment of breast cancer [8]. Furthermore, retinoids are being extensively evaluated in clinical trials of breast cancer prevention [9].

Although retinoids have been considered to exert their antitumor effects through the activation of intranuclear receptor RAR and retinoid X receptor (RXR), recent reports have suggested that these compounds also have significant effects on signal transduction kinases located in the cytoplasm, such as PKC and MAPK [10]. To elucidate the mechanism through which ATRA causes cell growth inhibition, we have examined changes in the cell cycle and intracellular signaling pathways. We have found that treatment with ATRA significantly decreased the expression of PKCα, as well as reduced ERK MAPK phosphorylation. ATRA treatment leads to dephosphorylation of Rb, and consequently to G_1 arrest. Marked changes in the expression of cyclins were observed in SKBR-3 cells treated by ATRA. Using a series of pharmacological and molecular approaches, we have found that ATRA-induced SKBR-3 cell growth inhibition involved deregulation of the PKCα-MAPK pathway (Fig 1) [11]. This experimental evidence suggested that retinoids were likely to involve multiple signal transduction pathways that are crucial for cell cycle progression and suggested the potential of retinoid in the treatment or prevention of breast cancer.

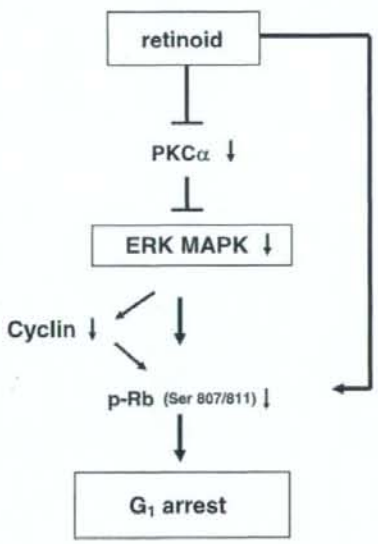

Fig. 1 Mechanism of inducing G_1 arrest by retinoid in SKBR-3 breast cancer cells

Antineoplaston

Antineoplastons are naturally occurring peptides, amino acid derivatives and organic acids found in blood, tissues and urine. The first synthetic antineoplaston was produced from extracted antineoplaston A2 and designated A10. Its chemical composition is 3-phenylacetylamino-2, 6-piperidinedione, and it is hydrolized in pancreatic juice to phenylacetylglutamine and phenylacetylisoglutamine. Clinical studies have described the effectiveness of antineoplastons on many different tumors [12]. The mechanism underlying the antitumor effect is considered to involve regulation of p53 and p21 gene expression through demethylation of their promoter sequences and acetylation of histones [12]. However, both the mechanism and effect on breast cancer cells have been poorly studied to date. We have investigated the mechanism underlying this antitumor effect using the breast cancer cell line SKRB-3. A10 markedly inhibited SKBR-3 proliferation due to arrest in the G_1 phase. A10 down-regulated the expression of PKCα protein, leading to inhibition of ERK MAPK phosphorylation. This increased the expression of p16 and p21 protein, with resultant inhibition of Rb phosphorylation, leading to G_1 arrest. This study has defined a pathway in which A10 arrested SKBR-3 cells in the G_1 phase via PKCα and MAPK (Fig. 2) [13]. Our results indicated that the antineoplaston A10 antitumor effect could be utilized as an effective therapy for breast cancer patients.

A putative differentiation-related and metastasis suppressor gene, Cap43/NDRG1/Drg-1

Cap43/NDRG1/Drg-1 (Cap43), a 43-kDa protein, is identical to the *N*-myc downstream-regulated gene 1 (NDRG1) and also to the differentiation-related gene-1 (Drg-1). Furthermore, the expression of Cap43 is markedly influenced by a number of stimuli including oxidative stress, metal ions, hypoxia, phorbol esters, vitamin A and D, steroids, as well as oncogenes (*N*-myc and C-myc) and tumor suppressor genes (p53 and VHL).

Although a number of studies have elucidated various characteristics of the Cap43 protein, its exact function remains unclear. In breast cancer, overexpression of the Cap43 gene inhibited cell growth as well as metastasis [14], suggesting that one of the Cap43 function was suppression of metastasis. Cap43 expression was up-regulated in normal cells and highly differentiated cancer cells, but down-regulated in poorly differentiated cancer cells in

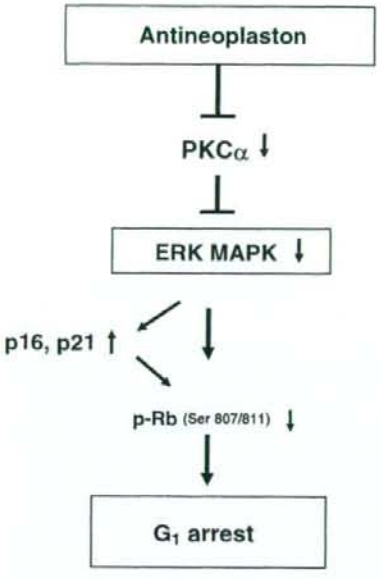

Fig. 2 Mechanism of inducing G_1 arrest by antineoplaston in SKBR-3 breast cancer cells

colon and prostate cancers [15, 16]. Low levels of Cap43 expression in breast cancer cells have been closely correlated with poor clinical outcomes [14]. Cap43 thus appears to be closely associated with the differentiation and/or malignant states of breast cancers.

Since previous studies have reported that Cap43 played a key role in breast cancer, we have examined how the expression of the Cap43 gene could be modulated during therapeutic treatment by antiestrogenic drugs, and we discuss the potential of Cap43 as a molecular target for the therapeutic efficacy of antiestrogenic anticancer agents in breast cancer. Treatment with estradiol (E_2) decreased the expression of Cap43 dose-dependently in ER-α-positive cell lines, but not in ER-α-negative lines. Administration of anti-estrogens, tamoxifen, abrogated the E_2-induced down-regulation of Cap43 (Fig. 3). Over-expression of ER-α in both ER-α-negative cell lines, SKBR-3 and MDA-MB-231, resulted in down-regulation of Cap43. Immunostaining studies showed that Cap43 expression was inversely correlated with the expression of ER-α. E_2-induced down-regulation of Cap43 appears to be mediated through ER-α-dependent pathways in breast cancer cells, both in culture and in patients [17]. The expression of Cap43 was modulated by E_2 and/or antiestrogen in ER-α-positive breast

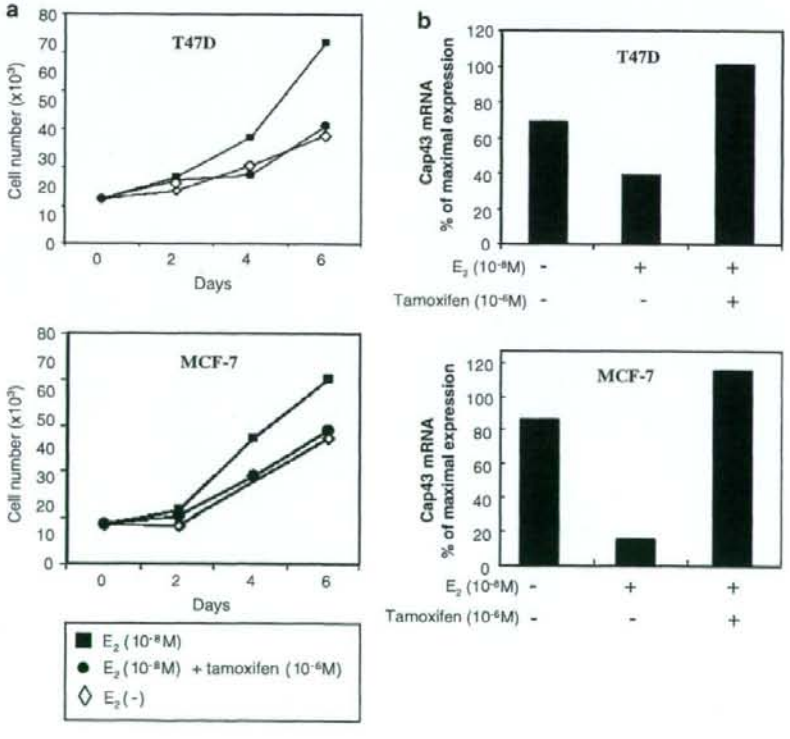

Fig. 3 **a** The addition of estradiol (E_2) enhanced growth in the estrogen receptor (*ER*)-α-positive cell lines T47D and MCF-7. Administration of a tamoxifen (antiestrogenic drugs) inhibited E_2-induced cell growth in T47D and MCF-7 cell lines. **b** Expression of Cap43 mRNA was down-regulated by addition of E_2 in T47D and MCF-7. Administration of tamoxifen abrogated the E_2-induced down-regulation of the Cap43 gene in T47D and MCF-7

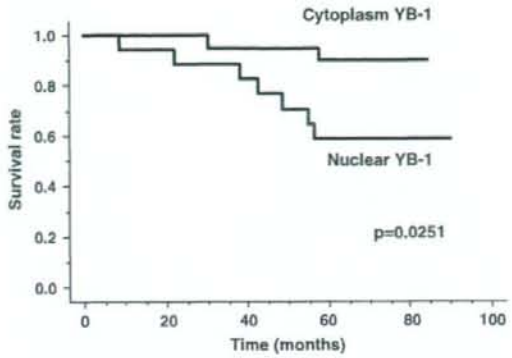

Fig. 4 In 39 breast cancer patients, univariate analysis for 5-year postoperative overall survival showed significant association between nuclear YB-1 expression and postoperative prognosis ($P = 0.0251$)

cancer cells. Therefore, Cap43 may hold some potential as a molecular marker to determine the therapeutic efficacy of anti-estrogenic anticancer agents in breast cancer.

Y-box binding protein-1 (YB-1)

YB-1 is a member of the cold shock domain protein family, which is found in the cytoplasm and nucleus of human cells. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance and cellular responses to environmental stimuli [18]. YB-1 is normally present in the cytoplasm, although it is translocated to the nucleus when cells are exposed to anticancer drugs, hyperthermia or UV light irradiation. Nuclear localization of YB-1 is required for its transcriptional control of multidrug resistance-related genes, but also for its repair control of DNA damages induced by anticancer agents or radiation in cancer cells, resulting in acquirement of global drug resistance to a wide range of anticancer agents [18]. On the other hand, nuclear expression of YB-1 is often associated with a poor prognosis in breast cancer [19]. These findings have suggested that the nuclear expression of YB-1 may be a predictive factor in breast cancer.

As YB-1 might play a key role not only in the expression of drug-resistance-related genes, but also in the expression of cell growth-related genes, we have investigated whether nuclear YB-1 localization was associated with expression of EGFR family proteins that might affect the poor prognosis in breast cancer patients. The expression of nuclear YB-1 was correlated with HER2 positively in clinical specimens of human breast cancer. Immunostaining studies showed that nuclear YB-1 expression was a prognostic factor of 5-year postoperative overall survival (Fig. 4). Nuclear YB-1 localization may thus be expected to be the molecular target of intrinsic importance not only for acquirement of multidrug resistance, but also for tumor growth dependent upon HER2 and other growth factor receptors in breast cancer.

Conclusion

We have investigated PKC signaling, Cap43 and YB-1 to identify new molecular targeting for breast cancer. PKC signaling represents a promising target for the development of novel therapeutic agents. As expression of Cap43 was modulated by estrogen and/or antiestrogen in ER-α-positive breast cancer cells, Cap43 may hold some potential as a molecular marker to determine the therapeutic efficacy of anti-estrogenic anticancer agents in breast cancer. Expression of nuclear YB-1 played an essential role in the acquirement of malignant characteristics through HER2-dependent pathways in breast cancer patients. The relevant efforts of basic research to identify the key and selective molecular alterations such as PKC, Cap43 and YB-1, which sustain breast cancer growth and progression, opens the possibility to develop a specific molecular target diagnosis and therapy.

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Preclinical and Clinical Studies of Novel Breast Cancer Drugs Targeting Molecules Involved in Protein Kinase C Signaling, the Putative Metastasis-Suppressor Gene Cap43 and the Y-box Binding Protein-1

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Abstract: Breast cancer is a common cause of tumors in women. The development of effective adjuvant therapies using drugs such as anthracyclines, taxanes, and aromatase inhibitors has improved the survival of breast cancer patients. Molecular cancer therapeutics are also attracting attention, and targeted molecular therapies, such as trastuzumab, have already contributed to effective new treatments for breast cancer. Other candidate targeted molecular therapies for breast cancer, including erlotinib, gefitinib, lapatinib, bevacizumab, and celecoxib, are currently undergoing clinical evaluation, and promising results are expected. The current review provides an up-to-date summary of the preclinical and clinical development of these drugs for breast cancer. In particular, we focus on therapies targeting protein kinase C (PKC) signaling, the putative metastasis-suppressor gene Cap43/N-myc downstream-regulated gene 1 (NDRG1)/differentiation-related gene-1 (Drg-1), and the Y-box binding protein-1 (YB-1). The PKC signaling pathway is widely considered to be a promising target for the development of novel therapeutics. Cap43 expression is significantly modulated by estrogen and/or anti-estrogens in breast cancer cells that are positive for estrogen receptor-α (ER-α). Cap43 is therefore of particular interest as a molecular indicator of the therapeutic efficacy of anti-estrogenic agents in breast cancer. The nuclear expression of YB-1 plays an essential role in the acquisition of malignant characteristics by breast cancer cells, through epidermal growth factor receptor 2 (HER2)–Akt-dependent pathways. Basic research investigating the key selective molecular changes that sustain breast cancer growth and progression, as demonstrated for PKC, Cap43, and YB-1, is allowing the development of specific targeted molecular diagnostics and therapeutics.

Keywords: Breast cancer, molecular targeting, protein kinase C, Cap43, YB-1.

1. INTRODUCTION

Emerging technologies are improving our understanding of basic biochemical processes and our ability to characterize tumors from individual patients. This progress is creating opportunities to improve the treatment of malignant disease, and to accelerate the development of new therapeutics. For example, members of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, including EGFR (HER1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4), are attractive targets for anticancer strategies. The roles of EGFR family members and their downstream signaling molecules as targets for cancer therapy are illustrated in Fig. (**1**). HER2 over-expression, in particular, has been implicated in the genesis and progression of a subset of breast cancers [1]. Targeting HER2 signaling has led to the development of therapies that reduce the activation of this receptor. One such novel therapeutic is the monoclonal antibody trastuzumab, which has clinical benefits not only against metastatic cancer [2–4], but also as part of adjuvant and neoadjuvant treatment [5–9]. Other targeted molecular agents that are currently undergoing clinical evaluation and are showing promising results are summarized below.

1.1. Erlotinib

Erlotinib is a highly potent and reversible inhibitor of EGFR tyrosine kinase. It acts by binding extracellularly to EGFR, and its effect is potentiated by the dimerization of activated receptors. In preclinical studies, erlotinib inhibited cell growth in several human tumors [10, 11]. In clinical trials, erlotinib had anti-tumor effects in patients with non-small-cell lung cancer, squamous cell carcinoma of the head and neck, and hepatocellular carcinoma [12–14]. Interestingly, Cho *et al.* [15] reported that erlotinib was effective in non-small-cell lung cancer patients who had previously failed to respond to gefitinib. Tan *et al.* [16] reported on 18 breast cancer patients who were treated orally with 150 mg/d erlotinib and expression of Ki67, EGFR, phosphorylated EGFR (pEGFR), phosphorylated mitogen-activated protein kinase (pMAPK), and phosphorylated Akt (pAkt), in total, 15 paired tumor, skin, and buccal mucosa biopsies from these patients were examined by immunohistochemistry. In the EGFR-positive tumors, the pEGFR, pMAPK, and pAkt levels were reduced after treatment. The authors concluded that erlotinib had inhibitory biological effects on EGFR-positive tumors.

1.2. Gefitinib

Gefitinib is a low-molecular weight anticancer agent, which acts as a selective EGFR tyrosine kinase inhibitor that blocks the signal-transduction pathway implicated in the proliferation and survival of cancer cells [17, 18]. We demonstrated that sensitivity to growth inhibition by gefitinib was associated with dependence on Akt and extracellular signal-regulated kinase (ERK)1/2, and that HER2 over-expression in non-small-cell lung cancer increased the sensitivity to gefitinib, through the inhibition of HER2/HER3 heterodimer formation [19, 20]. Preclinical studies established that gefitinib potently inhibited growth in a variety of human cancers [21–23]. Similar growth inhibition by gefitinib was reported in breast cancer models [24–26]. Combined treatment with drugs targeting EGFR (gefitinib) and HER2 (trastuzumab) resulted in the

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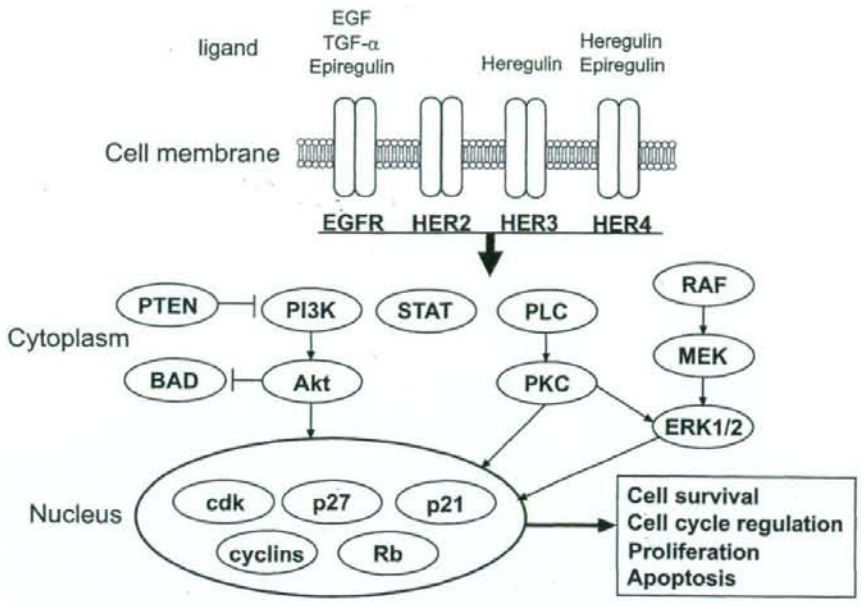

Fig. (1). EGFR family members and downstream signaling molecules as targets for cancer therapy.

efficient inhibition of tumor growth in breast cancer cells that co-expressed both receptors [27]. Clinically, a significant anti-tumor effect was observed in the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) clinical trial, and gefitinib is now approved for use in several countries to treat advanced non-small-cell lung cancer [28, 29]. Clinical trials have been carried out using gefitinib on its own to treat advanced breast cancer [30, 31], and gefitinib in combination with anastrozole, a non-steroidal aromatase inhibitor, and trastuzumab as a preoperative treatment for metastatic breast cancer [32, 33]. Two recent phase II clinical trials of gefitinib in combination with docetaxel in patients with metastatic breast cancer concluded that this regimen was effective [34, 35].

1.3. Lapatinib

Lapatinib is an orally-available low-molecular weight tyrosine kinase inhibitor that reversibly inhibits both EGFR and HER2. Preclinical studies have shown that lapatinib is active not only on its own but also in combination with trastuzumab [36–38]. Recently, Nahta *et al.* [39] reported that lapatinib induced apoptosis in trastuzumab-resistant breast cancer cells, and that its effects were mediated through insulin-like growth factor-1 (IGF-1) signaling. This result suggests that lapatinib is a potential candidate therapeutic for breast cancer patients who are resistant to trastuzumab. In clinical trials, lapatinib has been reported to be well tolerated, and to show early evidence of clinical efficacy, especially in EGFR-positive and/or HER2-positive breast cancer patients [40]. In a randomized phase III study, patients with HER2-positive metastatic breast cancer, which had progressed after anthracycline, taxane, and trastuzumab therapy, were treated with a combination of lapatinib and capecitabine; this combination was found to be superior to capecitabine alone, without causing an increase in serious side effects [41].

1.4. Bevacizumab

Bevacizumab is a recombinant humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF) [42]. The addition of bevacizumab to combined fluorouracil and irinotecan chemotherapy resulted in a statistically significant and clinically meaningful improvement in the survival of patients with metastatic colorectal cancer [43]. In breast cancer patients, phase I/II trials evaluated the safety and efficacy of bevacizumab in patients with previously treated metastatic breast cancer [44]. Ramaswamy *et al.* [45] reported the results of a phase II trial of bevacizumab combined with weekly docetaxel treatment in metastatic breast cancer patients; the authors concluded that this combination therapy was effective with acceptable side effects. Furthermore, a randomized phase III trial comparing capecitabine alone and capecitabine combined with bevacizumab, in patients with metastatic breast cancer who had failed to respond to standard therapies, showed that bevacizumab was well tolerated and that its co-administration with capecitabine significantly increased the response rate [46].

1.5. Celecoxib

Cyclooxygenase-2 (COX-2) is an inducible enzyme that catalyzes the synthesis of prostaglandins; it is over-expressed in various human cancers, and has been associated with a particularly poor prognosis in breast cancer [47, 48]. Celecoxib is a selective COX-2 inhibitor that inhibits tumor growth by inactivating Akt, which has been shown to reduce lymphangiogenesis in a xenograft model of breast cancer [49]. Clinical trials of celecoxib in combination with the steroidal aromatase inhibitor exemestane, in postmenopausal patients with advanced breast cancer, showed promising efficacy and the drugs were well tolerated [50]. However, the use of celecoxib combined with trastuzumab in breast cancer patients with tumors over-expressing HER2 was not effective [51].

Table 1 summarizes the novel agents targeting specific molecules that are currently being evaluated in clinical trials for breast cancer. Although promising results are expected, new molecular targeting treatments should currently be used only as third-line or fourth-line therapies for advanced or metastatic breast cancers (Fig. 2). Further clinical studies are needed before these molecular targeting drugs can be recommended as first-line therapies or for treating early breast cancer.

Table 1. Clinical Trials of Molecular Targeting Therapy for Breast Cancer

Agent	Target	Clinical trial (phase)	Results	Side effects	References
Gefitinib	EGFR	Monotherapy (II)	SD: 38.7%	Diarrhea, skin flash	[30]
		Monotherapy (II)	PR: 1.7%	Diarrhea, pruritus	[31]
		Combined with anastrozole (II)	PR: 50%	Skin disorder	[32]
		Combined with docetaxel (II)	CBR: 51.5%	Neutropenia	[34]
		Combined with docetaxel (II)	CR+PR: 54%	Neutropenia, diarrhea	[35]
Lapatinib	EGFR/HER2	Monotherapy (I)	SD: 36%*	Diarrhea, rash	[40]
		Combined with capecitabine (III)	TTP: 8.4M	Diarrhea, HFS	[41]
Bevacizumab	VEGF	Monotherapy (I/II)	ORR: 9.3%	Hypertension	[44]
		Combined with docetaxcel (II)	ORR: 52%	Neutropenia, fatigue	[45]
		Combined with capecitabine (III)	RR: 19.8%	Hypertension	[46]
Celecoxib	COX-2	Combined with exemestane (I)	CBR: 74%	Hot flushes, nausea	[50]
		Combined with trastuzumab (II)	No response	Abdominal pain	[51]

Abbreviations: EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; SD, stable disease; PR, partial response; CBR clinical benefit rate; CR, complete response; TTP, time to progression; ORR, overall response rate; RR, response rates; HFS, hand–foot syndrome; *included other solid tumor.

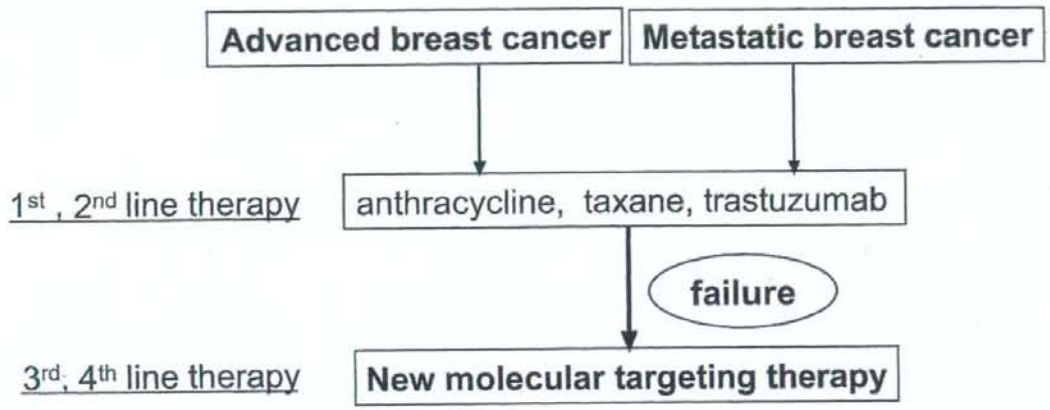

Fig. (2). The strategy of molecular targeting therapy for breast cancer.

We are continuing to investigate other molecules of this type that might be useful for both diagnosis and treatment. We aim to determine their potential to be translated into novel approaches to diagnosis and therapy in breast cancer patients, with the ultimate intention of moving promising lead compounds rapidly from the bench to the bedside. Below, we discuss the preclinical molecular-targeting strategies that we are using against breast cancer, focusing on protein kinase C (PKC) signaling, Cap43/N-myc downstream-regulated gene 1 (NDRG1)/differentiation-related gene-1 (Drg-1), and Y-box binding protein-1 (YB-1).

2. PRECLINICAL MOLECULAR-TARGETING STRATEGIES FOR NOVEL DIAGNOSTICS AND THERAPEUTICS AGAINST BREAST CANCER

2.1. PKC Signaling

2.1.1. PKC

Phorbol esters, such as phorbol 12-myristate 13 acetate (PMA) induce proliferation, differentiation, malignant transformation, and death in many cell types [52–55]. These effects are realized throug

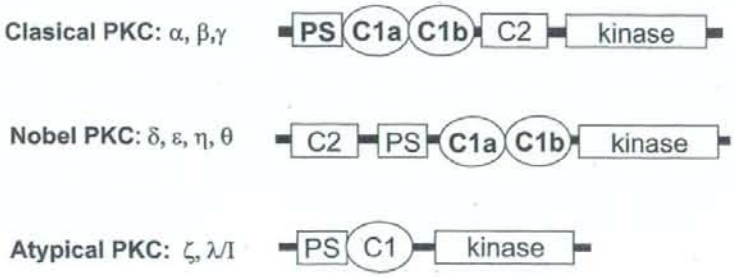

Fig. (3). Structure of PKC.

Abbreviations: PS, pseudo-substrate; C1, DAG and phorbol ester binding domain; C2, Ca^{++} binding domain; kinase, substrate binding domain.

various receptors, including several PKC isozymes [56] and the novel non-kinase receptors α-chimaerin, β-chimaerin, and RasGRP [57–59].

Members of the PKC family of phospholipid-dependent serine/threonine kinases [60] are involved in regulating basic cell functions, such as proliferation, differentiation, and cell death. This has led to PKC being considered as a target for anticancer therapy. To date, 12 isozymes of PKC have been identified, and they have been divided into three subgroups according to their structural and functional properties (Fig. **3**) [53, 61–64]. The members of the first group (PCK-α, PCK-βI, PCK-βII, and PCK-γ) are considered to be classical or conventional PKC (cPKC) isozymes, which are activated by calcium, diacylglycerol (DAG), and PMA. The members of the second group (PKC-δ, PKC-ε, PKC-η, and PKC-θ) are referred to as non-classic or novel PKC (nPKC) isozymes, which are also activated by DAG and PMA but are not calcium dependent. Both cPKCs and nPKCs have two C1 domains that confer DAG and phorbol ester responsiveness. The members of the third group (PKC-ζ, PKC-λ/ι and PKC-μ) are atypical PKC (aPKC) isozymes, which are neither calcium dependent nor activated by DAG or PMA (Fig. **3**).

As illustrated in Fig. (**4**), when certain stimuli are applied to the cell membrane, the effector molecule phospholipase C (PLC) is activated by G proteins. As a result, phosphatidylinositol-4,5-bisphosphate (PIP_2), which is a trace component of the cell membrane, is broken down to form DAG. This plays an important role in the activation of PKCs, increasing their affinity for cell membranes, and is accompanied by PKC activation and pseudo-substrate release [64]. DAG formed in this way activates cPKCs and nPKCs, leading to the phosphorylation of downstream substrates [63, 65, 66]. By contrast, aPKCs are activated by phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [57] (Fig. **4**).

2.1.2. Role of PKC in Breast Cancer

Several reports have described the biological effects of phorbol esters on cancer cells [67–73]. In the breast cancer cell line MDA-MB-231, PMA induced the translocation of PKC-α from the cytoplasm to the cell membrane, where it inhibited cell spreading and motility by negatively regulating the signaling pathway downstream of EGFR [74]. PMA has also been reported to inhibit the growth of MCF7 cells by up-regulating PKC-δ and $p21^{Waf1}$ [75]. Another phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), not only acts as a tumor promoter, but also induces apoptosis in breast cancer cell lines by a mechanism that is thought to involve the up-regulation of $p21^{Waf1}$ and BAX, but to be independent of p53 [76]. These results strongly implicate PKC in the cell proliferation and cell death induced by phorbol ester in breast cancer cells.

The distinct expression patterns and functions of different PKC isozymes are gradually being clarified [77–84]. For example, four breast cancer cell lines (MDA-MB-231, SKBR-3, MCF-7, and T47D) showed relatively high levels of PKC-α, PKC-δ, and PKC-ε expression (Fujii *et al.* unpublished data). Over-expression of PKC-α enhanced the proliferation rate of MCF-7 cells and led to increased tumorigenicity in nude mice [85]. Both PKC-α and PKC-θ have been shown to regulate transcription from the multidrug-resistance 1 (MDR1) promoter [86]. PKC-β enhanced the growth rate and cyclin D1 expression level of human breast cancer cells [87]. Inositol hexaphosphate blocked the proliferation of breast cancer cells through a PKC-δ-dependent increase in $p27^{Kip1}$ and a

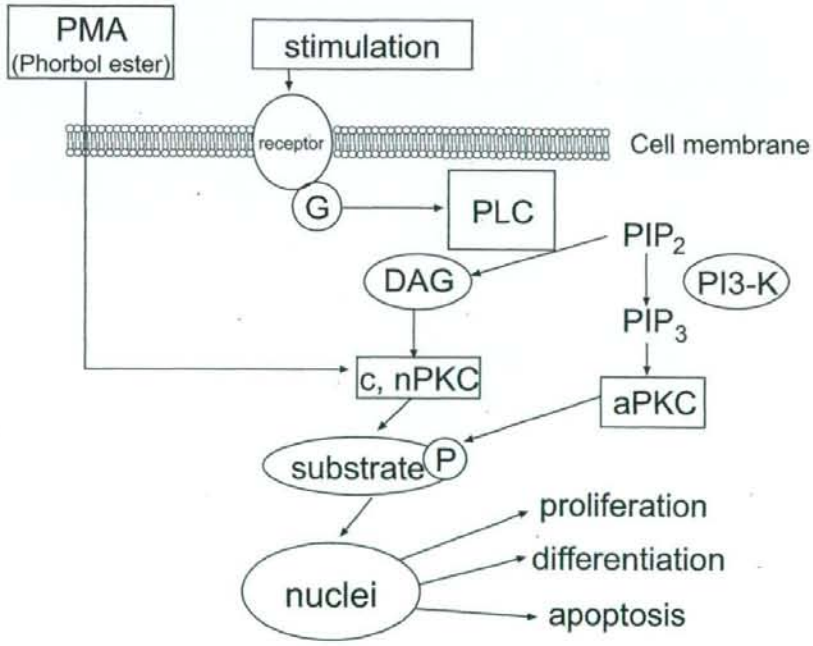

Fig. (4). Mechanism of PKC activation. When an appropriate stimulus is applied to the cell membrane, PLC is activated *via* a G protein. As a result, PIP_2, is broken down to DAG, which activates cPKCs and nPKCs, leading to the phosphorylation of substrates downstream. Phorbol esters pass through the cell membrane and activate cPKC and nPKC isozymes by directly binding to them. aPKCs are activated by PIP_3 *via* phosphatidylinositol-3-kinase (PI3-K).