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Cross-regulation between Egr-1 and APE/Ref-1 during early response to oxidative stress in the human osteoblastic HOBIT cell line: Evidence for an autoregulatory loop

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Abstract

The Early Growth Response protein (Egr-1) is a C_2H_2–zinc finger-containing transcriptional regulator involved in the control of cell proliferation and apoptosis. Its DNA-binding activity is redox regulated *in vitro* through the oxidation–reduction of Cys residues within its DNA-binding domain. APE/Ref-1 is a DNA-repair enzyme with redox modulating activities on several transcription factors. In this study, by evaluating the effects of different stimuli, we found a similar timing of activation being suggestive for a common and co-linear regulation for the two proteins. Indeed, we show that APE/Ref-1 increases the Egr-1 DNA-binding activity in unstimulated osteoblastic HOBIT cells. H_2O_2 stimulation induces a strong interaction between Egr-1 and APE/Ref-1 at early times upon activation, as assayed by immunoprecipitation experiments. By using a cell transfection approach, we demonstrated the functional role of this interaction showing that two specific Egr-1 target genes, the PTEN phosphatase and the thymidine kinase (TK) genes promoters, are activated by contransfection of APE/Ref-1. Interestingly, by using a cell transfection approach and Chromatin immunoprecipitation assays, we were able to demonstrate that Egr-1 stimulates the transcriptional activity of APE/Ref-1 gene promoter by a direct interaction with specific DNA-binding site on its promoter. Taken together, our data delineate a new molecular mechanism of Egr-1 activation occurring soon after H_2O_2 stimulation in osteoblastic cells and suggest a model for a positive loop between APE/Ref-1 and Egr-1 that could explain the early transcriptional activation of APE/Ref-1 gene expression.

Keywords: *APE/Ref-1, Egr-1, transcriptional regulation, PKC, redox regulation, DNA-repair*

Abbreviations: *Egr-1, Early Growth Response Protein 1, PMA, phorbol 12-myristate 13-acetate, APE/Ref-1, Apurinic Apyrimidinic Endonuclease/Redox effector factor 1, DTT, Dithiothreitol, PAGE, polyacrylamide gel electrophoresis, EMSA, Electrophoretic Mobility Shift Assay, TF, transcription factor*

Introduction

Osteoblasts are largely exposed to toxic and non-toxic doses of Reactive Oxygen Species such as H_2O_2, $O_2^{\cdot-}$, $^{\cdot}OH$ (collectively named as ROS) due, for instance, to bone resorption processes by osteoclasts [1–3]. Moreover, it has been reported that high doses of ROS play an important role in controlling osteoblastic differentiation [4], by acting at the transcriptional level through NF-κB transcription factor [5] and Egr-1 [6–8]. However, molecular mechanisms responsible

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for ROS-mediated osteoblastic functions and signalling pathways involved in oxidative stress-induced inhibition of osteoblast differentiation are far from being understood, yet. Several lines of evidence have found a tight association between oxidative stress and pathogenesis of osteoporosis: a link between increased oxidative stress and reduced bone mineral densities (BMD) in aged men and women has been described [9]; a general decrease in plasma antioxidants was described in aged osteoporotic women [10] and that oxidative stress increases differentiation and function of osteoclasts [3].

The Early Growth Response Protein 1[1] (Egr-1, also named NGFIA, TIS8, Krox-24, and Zif-268) is a member of the immediate-early transcription factors that are rapidly and transiently induced by reactive oxygen species, growth factors and UV radiation and is also implicated in cell proliferation and/or differentiative events in several cell types including osteoblasts [11–13]. Egr-1 contains a zinc-finger DNA-binding domain and activates regulatory elements containing the sequence GCGGGGGCG, which is present in a large number of genes involved in the control of cell growth and/or differentiation such as the thymidine kinase gene [14], collagen α2(I) [15], PTEN [16], TNF-α gene [17], PDGF-A [18], EGF and IGF receptors [19,20] and Egr-1 TF itself [13]. We have recently demonstrated an important involvement of Egr-1 activation following mechanical cell stimulation through release of cellular ATP [21]. Moreover, a role for ROS production during mechanical stimulation of different cell types has been well ascertained [22,23]. Therefore, comprehension of Egr-1 inducible activation in response to redox signaling is of utmost importance particularly for the osteoblastic cell model. Interestingly, it has been recently demonstrated the existence of an inducible activation of Egr-1 by estrogen in osteoblasts reinforcing its biological importance for this cell system [24].

Transcription factors can be regulated by post-translational events, such as phosphorylation, glycosylation, subcellular localization or redox state. Redox regulation of Egr-1 occurs as a consequence of oxidative stress or due to the production of intracellular oxygen species generated after stimulation of membrane receptors and thus acting as second messengers [25,26]. It has been previously demonstrated that the DNA binding activity of Egr-1 is redox regulated *in vitro* [27]. A reducing environment is required, *in vitro*, for a proper DNA-binding activity whereas oxidation promotes the complete loss of the DNA-binding, which is rescued by the addition of the reducing agent DTT or the recombinant protein APE/Ref-1 [27]. This protein has been identified as capable of both apurinic–apyrimidinic endonuclease DNA repair and nuclear redox activity. Due to this latter activity, it induces the DNA binding of several transcriptional regulators [28–30]. APE/Ref-1 gene expression is selectively induced by non-toxic levels of a ROS variety. This is thought to be due to a translational induction, being inhibited by treating cells with cycloheximide [31]. Moreover, we have recently demonstrated that ROS induce APE/Ref-1 nuclear translocation in several cell types [32,33]. From the biological point of view, APE/Ref-1 plays a pivotal role in controlling cell responses to redox-based stimuli and its expression is always inversely correlated with the onset of apoptosis, suggesting a role as an antiapoptotic molecule [34]. APE/Ref-1 is an ubiquitous protein, however neither the expression nor its functional role in osteoblastic cells has been addressed before.

By using the well-differentiated human osteoblastic HOBIT cell line, here we show that the redox control of DNA binding activity demonstrated *in vitro* for Egr-1 [27] is exerted, *in vivo*, by APE/Ref-1. Moreover, we demonstrate that Egr-1 directly binds and transactivates the APE/Ref-1 promoter, thus suggesting a positive regulatory loop between APE/Ref-1 and Egr-1. These evidences could explain the molecular events occurring in osteoblastic cells at very early times after H_2O_2 exposure and responsible for the increase in the expression levels of APE/Ref-1 itself providing a molecular mechanism of early response to oxidative conditions in osteoblastic cells.

Experimental procedures

Reagents

All chemicals were purchased from Sigma (St. Louis, MO), whereas otherwise specified.

DNA constructs

The construct containing the PTEN minimal promoter (called *min PTEN-luc*) has been previously described [16]. In this construct, the fragments −1031 to −779, relative to the translation start site, of the PTEN gene promoter was cloned in the plasmid pGL3B-P10, 5′ to the luciferase (LUC) gene, which is used as reporter gene. The Egr-1 expression vector has been previously described [16]. CMV-βGAL plasmid contains the Cytomegalovirus promoter linked to the βgalactosidase (bGAL) gene.

Plasmids CMV-CAT, RSV-CAT containing the CMV and the RSV promoters linked to the Chloramphenicol acetyl transferase reporter gene (CAT) [35–37] together with the plasmid pGL-2 (Promega), containing the promoter and enhancer sequences of SV-40 linked to the luciferase gene (LUC), were used in cotransfection studies with APE/Ref-1 expressing plasmid. Plasmid TK-CAT, containing the Egr-1 responsive promoter of the Thymidine Kinase gene [14] linked to the CAT gene was kindly provided by Dr A. Pardee.

Recombinant APE/Ref-1 His-tagged expressing plasmid pDS56Ref-1 was kindly provided by

Dr T. Curran (St. Jude Children's Research Hospital, Memphis, TN, USA) together with the eukaryotic expression vector CMV-APE/Ref-1.

A 2 kb promoter region of the APE/Ref-1 gene (obtained from Dr Sankar Mitra, University of Texas at Galveston, USA) was inserted into the SmaI and HindIII sites of the pGL2-Basic vector (Promega). This fragment contains the region of the APE/Ref-1 promoter from +99 to 1800.

The Egr-1 expressing plasmid pRSV-Egr-1, together with the mutant pRSV-Egr-1-AB in which the DNA binding domain has been deleted, were described in [38].

Cell lines and transfection

The HOBIT cell line, derived from normal adult human osteoblastic cells, retain most of the osteoblastic differentiation markers, including the expression of osteocalcin, alkaline phosphatase, type I collagen, osteopontin 1α and the sensitivity to steroid hormones [39]. HOBIT cells were kindly provided by Prof. B. Lawrence Riggs, Mayo Foundation, Rochester, Minnesota. Cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics and cultured at 37°C in a humidified atmosphere containing 5% CO_2. As the cultured cells approached confluence, they were subcultured at 1:8 splits. For this study, cells were used in passage number 4–9.

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum (FCS), glutamine and antibiotics. For transient transfection assays, cells were plated, 12 h before transfection, at 0.3×10^6 cells/100 mm culture dish. Transfections were carried out using a calcium phosphate method [40]. CAT was measured by an ELISA method (Boehringer Mannheim). A CMV-β-galactosidase expression plasmid was used as an internal control for transfection efficiency according the protocol provided by the manufacturer (Boehringer Mannheim). LUC activities were measured by a chemiluminescence procedure [41].

Protein expression and purification

Recombinant APE/Ref-1 protein (rAPE/Ref-1) was obtained as a hexahistidine-tag fusion protein from over-expression in *E. coli* and then purified by nickel-chelate chromatography from bacterial extracts and treated as previously described [42]. Sample concentrations were determined by Bradford colorimetric assay [43]. The purified proteins gave a single band on an overloaded SDS-PAGE. Fractions containing purified proteins were stored at −80°C.

Nuclear extracts preparation

Cell nuclear extracts were prepared as previously described [29]. Briefly, 10^7 cells were washed once with PBS and resuspended in 100 μl of hypotonic lysis buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF, pH 7.9). After 10 min, cells were homogenized by ten strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 500*g*, at 4°C, for 5 min in a microcentrifuge. The supernatant obtained after this centrifugation was considered as the cytoplasmic fraction. Nuclei were then washed three times with the same volumes of buffer A in order to minimize cytoplasmic contamination of the nuclear fraction. Nuclear proteins were extracted with 100 μl of buffer B (10 mM Hepes, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM PMSF, pH 7.9). After incubating for 30 min at 4°C, samples were centrifuged at 12,000*g* for 20 min, at 4°C. Nuclear extracts were then analyzed for protein content [43] and stored at −80°C in aliquots.

Western blot analysis

The indicated amounts of nuclear extracts, obtained from HOBIT cells incubated under different conditions, were electrophoresed onto a 10% SDS-PAGE. Then, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After transfer, membranes were saturated by incubation, at 4°C overnight, with 10% non-fat dry milk in PBS/0.1% Tween-20 and then incubated with the affinity purified polyclonal anti-APE/Ref-1 antibody [44] for 60 min at room temperature or with the polyclonal anti-Egr-1 antibody from Santa Cruz Biotechnology (sc-110) for 3 h. After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled to peroxidase (Sigma, St Louis, Missouri). After 60 min of incubation at room temperature, the membranes were washed several times with PBS/0.1% Tween-20 and the blots were developed using ECL chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy). Normalizations were performed with the polyclonal anti-Actin antibody (Sigma).

Electromobility shift assay (EMSA) analysis

Double-stranded oligodeoxynucleotides, labeled at the 5′ end with ^{32}P, were used as probes in gel retardation assays. The specific Egr-1 consensus binding site, here named *egr-1BS* oligonucleotide, is a 30-mer whose upper strand is 5′-GGATCCAGCG-GGGGCGAGCGGGGGCGAACG-3′. The mutant

Egr-1 binding site that was used for competitions, here named *egr-1MS* oligonucleotide, is a 30-mer whose upper strand is 5′-GGATCCAGCGGG*TA*CGAG-CGGG*TA*CGAACG-3′. Both the wild-type and mutant oligonucleotides were from Geneka Biotechnology Inc. (Montreal, Canada). The gel retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM *Tris*-HCl pH7.6, 75 mM KCl, 0.25 μg/ml bovine serum albumin (BSA) with calf thymus DNA (25 μg/ml) as reported in figure legends, 10% glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native 5% polyacrylamide gel run in 0.5 × TBE for 1.5 h at 4°C. The gel was dried and then exposed to an X-ray film at −80°C.

Preparation of cell lysates and immunoprecipitation

Cells were resuspended in 200 μl of lysis buffer containing 1% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM NaVanadate, 100 U/ml Aprotinin, 10 μg/ml leupeptin and 0.5 mM PMSF and incubated on ice for 20 min. Particulates were removed by centrifugation at 15,000 rpm for 30 min at 4°C. Supernatant were collected and protein amounts determined colorimetrically [43]. 500 μg of cell lysates were diluted to a final volume of 100 μl with TETN250 buffer (25 mM *Tris*-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl and 1% Triton X-100) and precleared by Protein-G Sepharose (Amersham Pharmacia Biotech, Milan, Italy) overnight at 4°C with agitation. A monoclonal antibody against APE/Ref-1 was then added to the precleared lysate and incubated for 1 h at 4°C with agitation to form an immunocomplex. 20 μl of protein-G Sepharose resin was then added and left to incubate for 20 min in agitation at 4°C. The immunoprecipitated material was then collected by centrifugation at 12,000 rpm for 30 s. The pellet was washed twice with TETN250 and once with TE buffer (10 mM *Tris*-HCl, pH 7.5, 5 mM EDTA). The APE/Ref-1 protein complex was then eluted by addition of 50 μl of a solution of 0.2 M glycine, pH 2.2 that was immediately buffered with a solution of 1.5 M Tris, pH 8.8. 5 μl of the eluted material were then used in EMSA analysis to test for the presence of Egr-1 transcription factor.

Chromatin Immunoprecipitation (ChIP) assay

Hobit cells (1×10^8) grown to 90% confluence in 100 mm plates were washed once with PBS at room temperature and fixed with 1% (v/v) formaldehyde in PBS at 37°C for 10 min. Cells were quickly rinsed twice with ice-cold PBS and scraped into 1 ml of ice-cold PBS. After spinning at 700*g* at 4°C, cell pellets were resuspended in 1 ml of lysis buffer (1% SDS, 5 mM EDTA, 50 mM *Tris*-HCl, pH 8.1, plus protease inhibitor cocktail) and incubated on ice for 10 min. After lysis, the sample was sonicated three times for 15 s each (Sonifer Power Supply, Branson, 2.8 mA, microtip) and then centrifuged for 10 min at 14,000*g* at 4°C. A 100 μl aliquot of the supernatant was set aside as the input fraction. NaCl and Triton X-100 were added to the remaining chromatin preparation to final concentrations of 150 mM and 1% (v/v), respectively, followed by incubation for immunoclearing with Protein A–Sepharose (300 μl of 50% slurry in 10 mM *Tris*-HCl, pH 8.1, 1 mM EDTA) for o/n at 4°C. After incubation, the sample was quickly centrifuged at 9000*g* and the supernatant was collected.

Immunoprecipitation was performed by adding 20 μl (0.2 μg/μl) of polyclonal anti-Egr-1 antibody (C-19, Santa Cruz Biotechnology) and, as a control, the same amount of total Rabbit IgG (from Sigma, St. Louis, MO) and by incubating the mixture overnight at 4°C; 300 μl of Protein A–Sepharose were then added and the slurries were incubated for a further o/n at 4°C. The Sepharose beads were harvested by centrifugation at 8000*g* and washed sequentially for 10 min in 1 ml each of TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 μM *Tris*-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM *Tris*-HCl, pH 8.1, 500 mM NaCl), TSE III (0.25 M LiCl, 1% Nonidet P40, 1% deoxycholate, 1 mM EDTA, 10 mM *Tris*-HCl, pH 8.1) and TE buffer (10 mM *Tris*-HCl, pH 8.1, 1 mM EDTA). DNA was eluted from beads with 100 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) at room temperature for 10 min and then heated at 56°C for 6 h to reverse the formaldehyde cross-link. DNA was extracted with Phenol–chloroform and then it was precipated with Ethanol and Sodium Acetate. The PCR was performed using the primers 5′-CGTTCAGACTGCCAGCGAAG-3′ (lower strand) and 5′ TGACCAGGTCCGCTAC-CCAC 3′ (upper strand) to Egr-1 binding site which amplifies a region of 143 bp comprised from nucleotide −81 to nucleotide +62, and the primers 5′-CATGAGGTCCACCACCCTGTTGCT-3′ (lower strand) and 5′-CAGCGACACCCACTCCTCCA-CCTT-3′ (upper strand) to GAPDH which amplifies a region of 124 bp and was conducted as follows: 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 30 cycles, using a Progene DNA Thermal Cycler. The PCR products were separated on a 1.5% (w/v) agarose gel and visualized by ethidium bromide staining.

Results

Egr-1 and APE/Ref-1 are co-regulated during short-times stimulation of HOBIT cells by PMA and H_2O_2 treatment

It has been previously demonstrated, by an *in vitro* experimental approach, that the DNA-binding

activity of Egr-1 is redox regulated [27]. In order to better understand the molecular mechanisms responsible for such activation, we performed a series of experiments in the osteoblastic HOBIT cell line, which retains most of the differentiative markers of human osteoblasts [39], by exposing cells to different stimuli, i.e. H_2O_2 and PMA, known to activate Egr-1 and APE/Ref-1 in other cell systems [13,28]. The osteoblast model has been successfully employed to demonstrate the functional activation of Egr-1 by H_2O_2 [8] and the production of H_2O_2 by Transforming Growth Factor-b1 in the same cells [6] points to oxidative stress as a physiological relevant phenomenon in osteoblast physiology. PMA, a phorbol ester mimicking the structure of 1,2-diacylglycerol, is known to activate PKC in the short terms and to down-regulate it after long exposures [45] and it is a very well known activator of Egr-1 in different cell models [13]. Both stimuli, i.e. H_2O_2 and PMA, are also known activators of APE/Ref-1 protein in different cell systems [31,46].

HOBIT cells were exposed to PMA and to sublethal doses of H_2O_2 and the expression levels of APE/Ref-1 and Egr-1, together with this latter's activation status, were assayed by Western blot and EMSA analysis. Treatment of HOBIT cells with 50 ng/ml PMA induced a time-dependent increase in Egr-1 DNA-binding activity, assayed by EMSA analysis (Figure 1, Panel A) and increased both Egr-1 and APE/Ref-1 protein expression levels (Panel B). Similarly, treatment of cells with 50 μM H_2O_2 induced a time-dependent activation of Egr-1 DNA-binding activity (Panel C) and an increase of both Egr-1 and APE/Ref-1 protein levels (Panel D).

APE/Ref-1 induces Egr-1 DNA-binding activity through direct protein–protein interaction

Our data not shown demonstrate that early functional activation of Egr-1 DNA-binding activity occurs in a protein-neosynthesis independent manner, since pretreatment of the cells with cycloheximide is not able to prevent H_2O_2 and PMA-induced Egr-1 activation (data not shown). This evidence suggests that a post-translational mechanism resposible for Egr-1 functional activation would occur. In order to test whether APE/Ref-1 directly acts as a "molecular switch" in the control of Egr-1 DNA-binding activity, nuclear extracts from unstimulated HOBIT cells were incubated with increasing amounts of recombinant purified APE/Ref-1 protein and then assayed by EMSA. Figure 2 (Panel A) shows that the APE/Ref-1 protein is capable, 'per se', to induce the DNA-binding activity of the endogenous Egr-1 protein, suggesting a direct role in the control of Egr-1 functional status.

Redox regulation of Egr-1 activity predicts the occurrence of a direct interaction between APE/Ref-1 and Egr-1 itself. In order to test this possibility, an immunoprecipitation approach was used and is shown in Figure 2 (Panel B). HOBIT cells were treated for the indicated times with 50 μM H_2O_2. Then, nuclear extracts were prepared and immunoprecipitated with an anti-APE/Ref-1 monoclonal antibody [47]. Proteins eluted from the immunocomplex were then analyzed for the presence of the Egr-1 DNA-binding activity by EMSA and the Egr-1 protein by Western blot analysis (Panel B). The time-dependent increase of Egr-1/DNA binding activity and Egr-1 protein levels in the anti-APE/Ref-1 immunoprecipitates demonstrates that H_2O_2 promotes the occurrence of a physical interaction between the two proteins (Panel B), that is already evident at early times after stimulation. Interestingly, the Egr-1-APE/Ref-1 interaction is already evident at 20 min, under conditions of basal APE/Ref-1 expression (see Figure 1D). This suggests that the Egr-1-APE/Ref-1 interaction occurs independently of APE/Ref-1 protein neosynthesis.

APE/Ref-1 increases the Egr-1 transcriptional activity in vivo

In order to test whether the stimulatory effect of APE/Ref-1 on the Egr-1 activity could have an *in vivo* relevance, a cell transfection approach was used (Figure 2, Panel C). Due to the poor transfection efficiency of HOBIT cells, we used the HeLa cell system. The PTEN gene is a known target gene for the activity of Egr-1 [16]. The effects of APE/Ref-1 on PTEN promoter activity mediated by Egr-1 was assayed. HeLa cells were co-transfected with a construct in which the PTEN minimal promoter was linked to the LUC gene [16] and with an Egr-1 expression vector. Since the Egr-1 gene is ubiquitously expressed, the Egr-1 expression vector was used to increase Egr-1 protein levels. Figure 2 (Panel C) shows that the presence of the Egr-1 expression vector significantly enhanced the LUC gene expression of two fold, indicating that overexpression of Egr-1 in HeLa cells induces an increase of PTEN promoter activity, as expected. The co-transfection of increasing amounts of APE/Ref-1 encoding plasmid was able to markedly enhance the Egr-1-mediated PTEN activation. The amount of this increase is suggestive for a synergistic effect of APE/Ref-1 and Egr-1 on PTEN promoter activity. The stimulatory effect of APE/Ref-1 is also evident, although to a lesser extent, when using only the PTEN promoter suggesting a role of APE/Ref-1 on the endogenous Egr-1 protein. In order to test whether the stimulatory role of APE/Ref-1 is exerted by means of its "redox activity", cell transfections were also performed with the redox-defective mutant APE/Ref-1C65A [48]. As it is evident, this mutant was completely unable to affect PTEN promoter activity. In order to test for a general activatory role of APE/Ref-1 on Egr-1 transcriptional

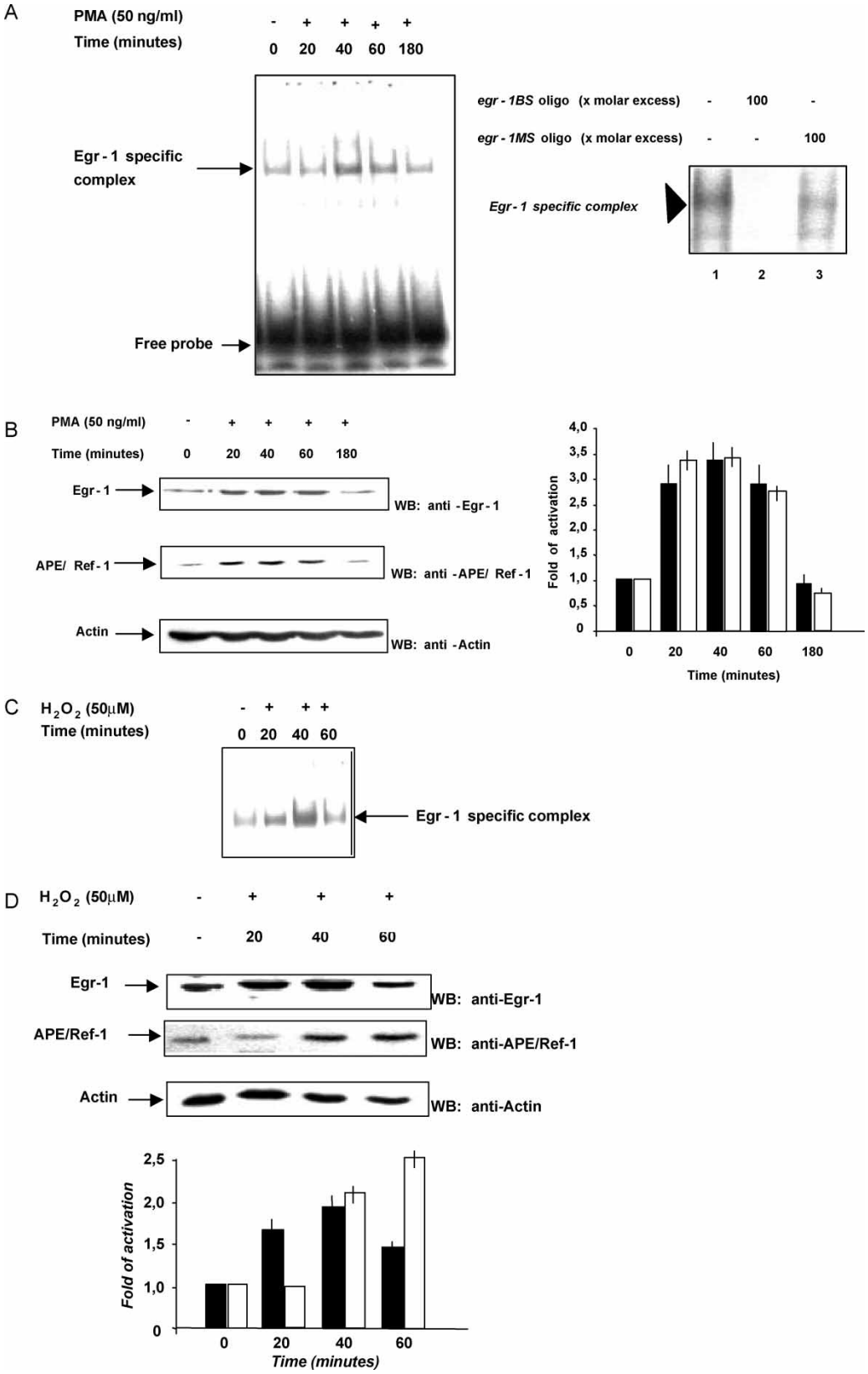

Figure 1. *PMA treatment of HOBIT cells stimulates Egr-1 and APE/Ref-1. Panel (A)*: *PMA induces a rapid induction of Egr-1 DNA-binding activity. Left*. After treatment of HOBIT cells with 50 ng/ml PMA for the indicated times, cells were lysed and nuclear extracts were prepared for the EMSA analysis with the ^{32}P-labeled specific oligo called egr-1BS (see Section 2). 5 μg of each nuclear extracts were incubated with 200 fmol of labeled probe in the presence of 500 ng of calf thymus DNA as aspecific competitor for 30 min at RT and then analyzed by native 5% polyacrylamide gel electrophoresis. After drying, the gel was exposed o/n at −80°C for autoradiography. *Right*. To test for the specific protein–DNA complex a competition assay was performed with a molar excess of the cold specific egr-1BS (lane 2) and with a mutant binding

activity, another Egr-1- target gene promoter was used. The Thymidine kinase (TK) gene is a known target gene for the activity of Egr-1. The CAT reporter under the control of the TK gene enhancer [14], was transfected into cells without (empty box) or with increasing amounts of the APE/Ref-1 expression vector (black boxes). The co-transfection of the APE/Ref-1 expression vector increases the Egr-1-induced activation of the TK construct by two-three fold (Figure 2, Panel C). The activity of APE/Ref-1 is specific, since the activities of the CMV and RSV promoters, that are not controlled by Egr-1, are not modified by the co-transfection of the APE/Ref-1 expression vector (Panel C).

Egr-1 activates APE/Ref-1 gene promoter

Molecular mechanisms responsible for the early activation of APE/Ref-1 gene expression have not been well elucidated, yet. Interestingly, the APE/Ref-1 promoter contains putative Egr-1 binding sites [49]. In order to test whether Egr-1 is able to control the APE/Ref-1 promoter activity, we used a cell transfection approach in HeLa cells. The APE/Ref-1 gene promoter, linked to the luciferase gene, was transfected either without (empty boxes) or with increasing amounts of wild-type Egr-1 expression vector (black boxes) or with a truncated form of Egr-1, in which the DNA-binding domain has been previously deleted (hatched boxes). The co-transfection of wild-type Egr-1 increases the APE/Ref-1 promoter activity by three-four fold (Figure 3) demonstrating that Egr-1 is able to control the APE/Ref-1 transcriptional activity.

Egr-1 binds to APE/Ref-1 gene promoter in vivo

The transfection experiments shown above indicated that Egr-1 should be a potential regulatory transcriptional factor involved in the control of the inducible APE/Ref-1 gene expression. Indeed, the APE/Ref-1 basal promoter region (about 300 bp) contains one Egr-1 putative binding site (see Figure 4, Panel A), in a region rich of consensus sequences for other important transcription factors, such as Sp1 and USF.

To demonstrate whether Egr-1 directly binds the *Ape/Ref-1* promoter *in vivo*, a Chromatin Immunoprecipitation assay was carried out using extracts from HOBIT cells (Figure 4, Panel B). For this purpose, DNA-binding proteins of H_2O_2-treated (50 μM, for 40 min) and untreated HOBIT cells were covalently linked to genomic DNA by treatment of the cells with formaldehyde. The DNA-protein complexes were then sheared by sonication, and specific DNA-protein complexes were immunoprecipitated with the specific polyclonal antibody against Egr-1. Covalent linkage was reversed and the purified double-stranded DNA was used as a template to amplify, by PCR, a 143 bp region internal to the APE/Ref-1 gene promoter. In a 1.5% (w/v) agarose gel, a band of the expected amplified product could be observed both in the input fraction and, with a more intense signal, in the chromatin fraction obtained after immunoprecipitated material with the anti-Egr-1 antibody (lane 5). As a positive control we amplified the 143 bp region using, as template, the 2 kb promoter of APE/Ref-1 gene used for transfection studies. As a negative control, we amplified a 124 bp region internal to the GAPDH gene (lanes 2 and 3) using the same immunoprecipitated sample. The expected GAPDH amplified product could be observed only in input fractions. The lack of its amplification in the eluted fractions demonstrated the immunoprecipitation specificity devoid of genomic-contamination.

Altogether these data are suggestive for the existence of an autoregulatory loop between APE/Ref-1 and Egr-1.

PKC stimulation plays a role in controlling the autoregulatory loop between APE/Ref-1 and Egr-1

It has been demonstrated that the redox activity of APE/Ref-1 over the transcription factor AP-1, in response to oxidative stimuli, is a PKC-dependent phosphorylation event [46]. We wondered whether this could be also the case for Egr-1. To test this

site called egr-1MS (lane 3). *Panel (B)*: *PMA induces an increase in Egr-1 protein levels*. *Left*. Representative Western blot analysis of Egr-1 and APE/Ref-1 expression levels of samples used in EMSA assays. 15 μg of nuclear extracts from HOBIT cells were separated onto a 10% SDS-PAGE, blotted onto nitrocellulose membranes and assayed for the presence of Egr-1 protein by using the polyclonal anti-Egr-1 antibody from Santa Cruz and by the anti-APE/Ref-1 specific polyclonal antibody [44]. Actin was always measured, as loading control, by using the anti-actin polyclonal antibody from Sigma. *Right*. Values obtained from densitometric analysis of Western blot experiments, normalized vs. Actin, were reported as histograms: black boxes represent Egr-1 and empty boxes represent APE/Ref-1. Bars indicate the mean value ± SD of at least three independent experiments. *Panel (C)*: *H_2O_2 stimulates the DNA-binding activity of Egr-1 in the HOBIT cells*. Representative EMSA analysis of Egr-1 DNA-binding activity performed by nuclear extracts from HOBIT cells after stimulation, for the indicated times, with H_2O_2. 5 μg of each nuclear extract was incubated with 200 fmol of labeled probe in the presence of 500 ng of calf thymus DNA as aspecific competitor for 30 min at RT and then analyzed by native 5% polyacrylamide gel electrophoresis. *Panel (D)*: *Left*. Representative Western blot analysis of Egr-1 and APE/Ref-1 expression levels of samples used in previous EMSA assays. 15 μg of nuclear extracts from HOBIT cells were separated onto a 10% SDS-PAGE, blotted onto nitrocellulose membranes and assayed for the presence of Egr-1 protein, by using the polyclonal anti-Egr-1 and the anti-APE/Ref-1 antibodies [44]. Actin was always measured, as loading control. *Right*. Values obtained from densitometric analysis of Western blot experiments, normalized vs. Actin, were reported as histograms: black boxes represent Egr-1 and empty boxes represent APE/Ref-1. Bars indicate the mean value ± SD of at least three independent experiments.

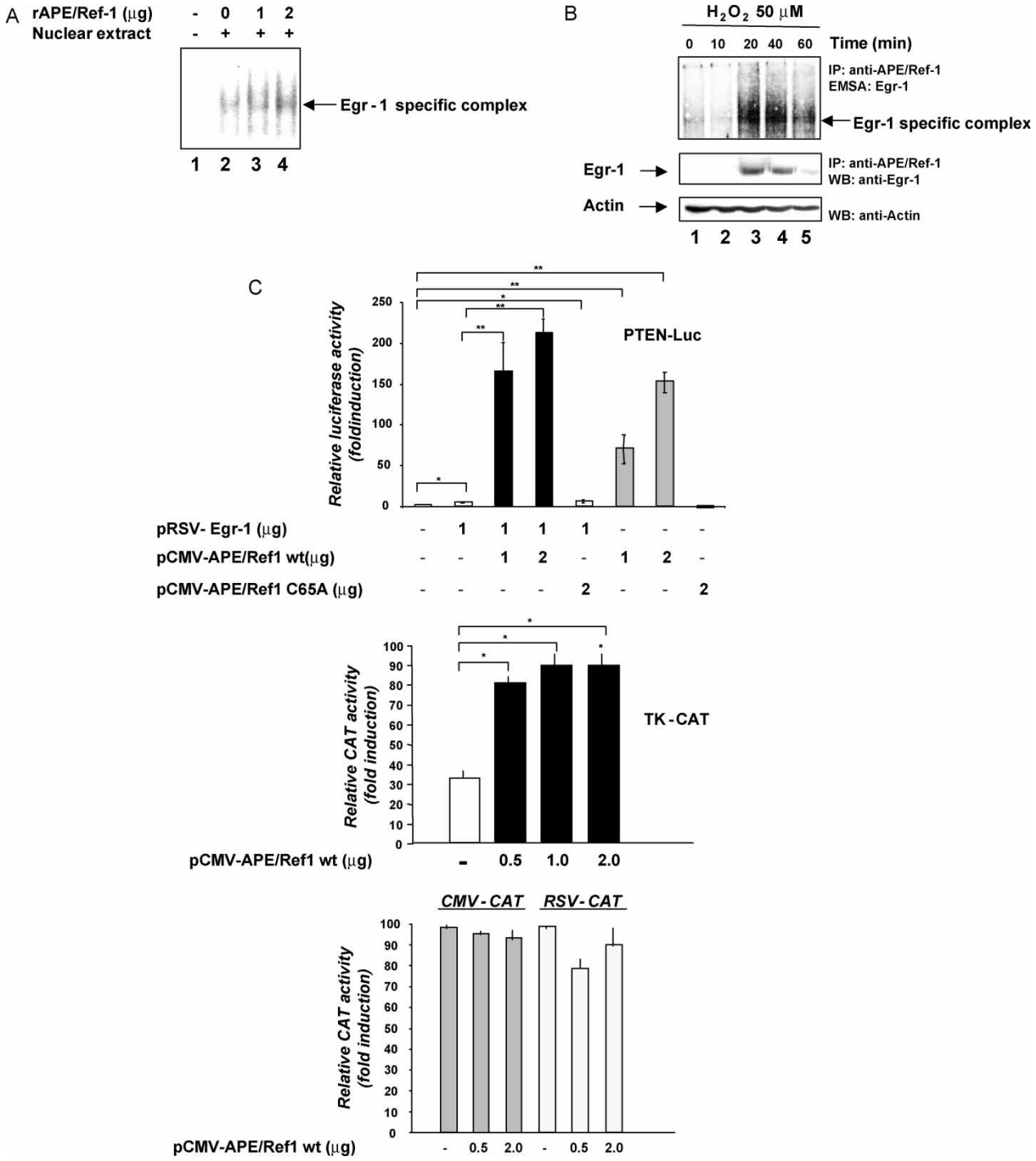

Figure 2. *APE/Ref-1 activates Egr-1 DNA-binding activity through direct protein–protein interaction and increases its transcriptional activity "in vivo". Panel (A): Recombinant APE/Ref-1 (rAPE/Ref-1) is a stimulator of endogenous Egr-1 DNA-binding activity.* EMSA analysis of Egr-1 DNA binding by 5 μg of nuclear extract from unstimulated HOBIT cells, in the absence (lane 2) or presence of increasing amounts of purified rAPE/Ref-1 (lanes 3 and 4). After incubating nuclear extracts with rAPE/Ref-1, each sample was incubated with 200 fmol of labeled specific probe in the presence of 500 ng of calf thymus DNA as aspecific competitor for 30 min at RT and then analyzed by native 5% polyacrylamide gel electrophoresis. Lane 1 contains probe alone. *Panel (B): H_2O_2 treatment of HOBIT cells induces a time-dependent increase in Egr-1-APE/Ref-1 interaction*. HOBIT cells were treated, for the indicated times, with 50 μM H_2O_2 and total extracts were prepared and subjected to immunoprecipitation procedure with the anti-APE/Ref-1 monoclonal antibody [47], as described in the methodological section. Eluted samples were analyzed for the presence of Egr-1 protein by EMSA analysis (Upper Panel) and by Western blot analysis (Lower Panel), as described above. Actin was measured, as loading control. *Panel (C): APE/Ref-1 increases the Egr-1 transcriptional activity in vivo*. Plasmids were transfected in HeLa cells at indicated amounts. 48 h after transfections, cells were harvested and LUC, CAT and β-Gal activities were measured. *Top*: Effect of APE/Ref-1 (wild type and redox mutant C65A) on Egr-1-induced activity of PTEN minimal promoter. [16]. *Middle*: Effect of APE/Ref-1 on Egr-1-induced activity of TK promoter. *Bottom*: Effect of APE/Ref-1 on the CMV and RSV promoters. In all panels, bars indicate the mean value ± SD of three independent experiments. (*, $p < 0.05$; **, $p < 0.001$ by ANOVA).

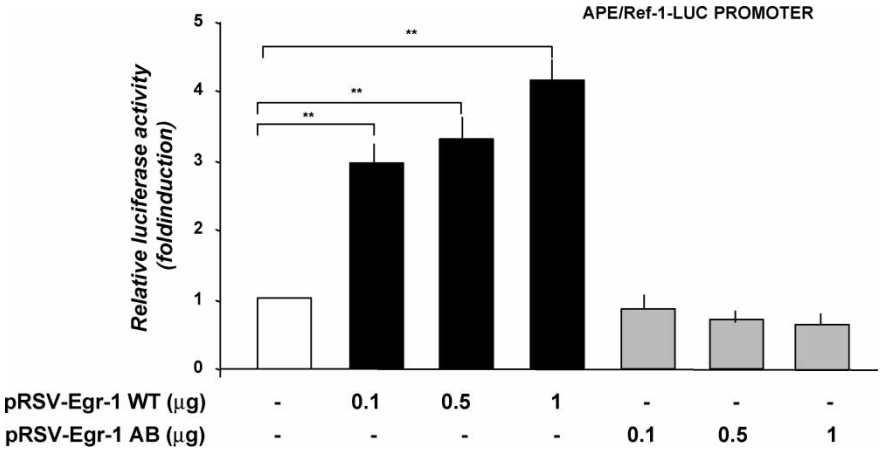

Figure 3. *Egr-1 transactivates the APE/Ref-1 gene promoter*. Egr-1 transactivates expression from the APE/Ref-1 5′ region. Plasmids encoding the wild-type Egr-1 protein (pRSV-Egr-1-WT) or a truncated form, in which the zinc-finger has been previously deleted (pRSV-Egr-1-AB), were transfected in HeLa cells at indicated amounts together with the 2-kb promoter of the human APE/Ref-1 protein linked to a luciferase gene reporter (Luc). 48 h after transfections, cells were harvested and Luc and β-Gal activities were measured.

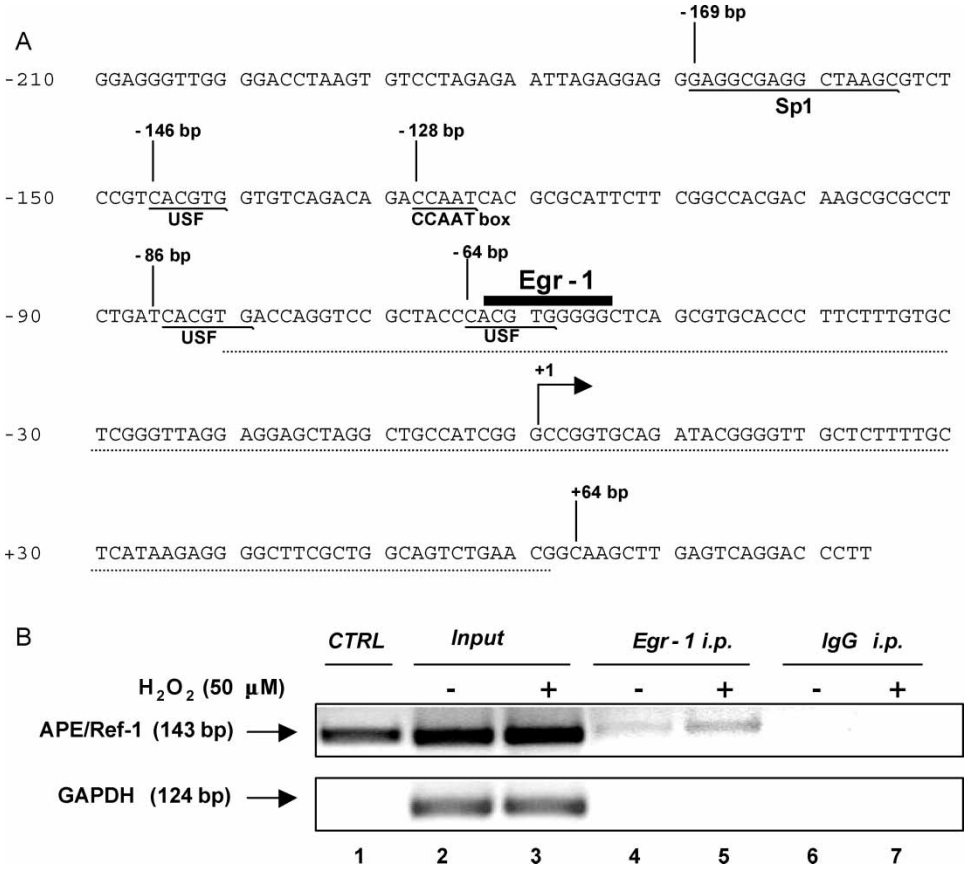

Figure 4. *Putative transcription factor-binding sites in the minimal promoter sequence of APE/Ref-1 gene. Panel (A)*: *Consensus binding sites for factors with a potential role in APE/Ref-1 gene expression*. The position of the transcription start site as described by Harrison *et al.* [55]. The region dotted underlined represents the amplified region used for CHIP experiments. *Panel (B)*: *Egr-1 binds to the 5′ region of APE/Ref-1 gene promoter*. PCR amplification of the APE/Ref-1 5′ region from cross-linked chromatin. Either total cross-linked chromatin (Input), Egr-1 immunoprecipitates (Egr-1 i.p.) or control rabbit IgG immunoprecipitates were screened directly for the presence of APE/Ref-1 5′ sequences by PCR using primers designed to amplify a 143-bp fragment (dotted underlined) within the basal promoter of APE/Ref-1 gene. The same samples were also used for amplifications using primers for GAPDH giving a band of 124 bp. The same primers were used to analyze Egr-1 immunoprecipitates from untreated or H_2O_2 treated HOBIT cells. As a positive control of amplification APE/Ref-1 promoter-containing plasmid was used (lane 1). Input and Egr-1-immunoprecipitated fractions were amplified by using specific primers for APE/Ref-1 and GAPDH genes (lanes 2–5), as indicated in the methodological section. The immunoprecipitated control was performed by using total rabbit IgG (lanes 6 and 7).

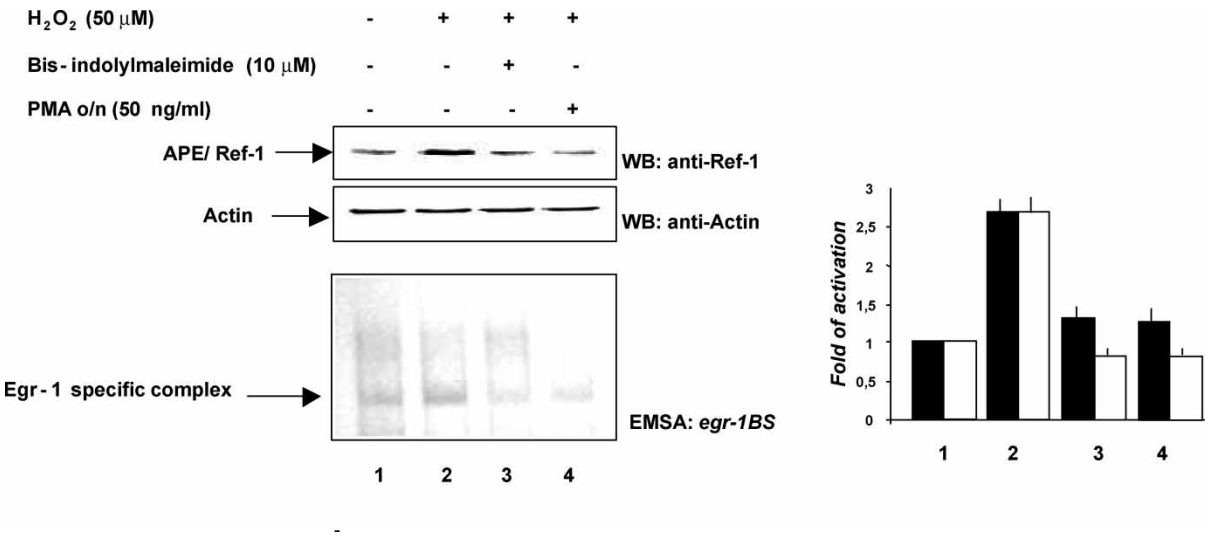

Figure 5. *Activation of Egr-1 and APE/Ref-1 expression is dependent on PKC. Left Panel*, in order to abolish the activity of PKC, HOBIT cells were pretreated, over night, with 50 ng/ml of PMA or with bis-indolylmaleimide for 1 h. Thereafter, cells were treated for 30 min with 50 μM H_2O_2. Then, cells were collected and lysed to obtain nuclear extracts that were subsequently used for Western blot (for evaluating the presence of APE/Ref-1 protein) and EMSA analysis (for evaluating the activation of Egr-1 DNA-binding activity). In Western blot analysis, 15 μg of nuclear extracts from HOBIT cells were separated onto a 10% SDS-PAGE, blotted onto nitrocellulose membranes and assayed for the presence of APE/Ref-1 protein by using the monoclonal anti-APE/Ref-1 antibody. Actin was always measured, as loading control. The same samples used in Western blot were assayed for the DNA-binding activity by Egr-1 through EMSA analysis with the ^{32}P-labeled specific *egr-1BS* oligonucleotide. 5 μg of each nuclear extract were incubated with 200 fmol of labeled probe in the presence of 500 ng of calf thymus DNA as aspecific competitor for 30 min at RT and then analyzed by native 5% polyacrilamide gel electrophoresis. *Right Panel*, values obtained from densitometric analysis of specific signals of APE/Ref-1 protein levels (from Western blot analysis) and Egr-1/DNA specific complex (from EMSA analysis) from three independent experiments, normalized vs. Actin, were reported as histograms to evaluate the activation of APE/Ref-1 expression levels (black boxes) and Egr-1 DNA-binding activity (empty boxes).

hypothesis, PKC was inhibited before treatment with H_2O_2, either by pretreating HOBIT cells with the specific PKC-inhibitor Bis-indolyl maleimide or by an overnight-treatment with 50 ng/ml PMA [45]. As reported in Figure 5, PKC inhibition totally prevented both the increase of APE/Ref-1 expression and the stimulation of the DNA-binding activity of Egr-1. These evidences suggest that PKC activity is required for triggering the autoregulatory loop between APE/Ref-1 and Egr-1.

Discussion

Early molecular events triggered by oxidative stress on osteoblastic cells are largely unknown. However, some molecular effectors involved in cell response to this injury are already known in different cell models. The Egr-1 transcription factor and the APE/Ref-1 redox coactivator have been largely described to play a role in these complex cellular mechanisms as single molecules. However, these studies did not appreciate the possible existence of an interlinked action of these two molecular factors, beside than a single work performed "*in vitro*" [27].

The central mechanism(s) responsible for the APE/Ref-1 inducible expression in response to different stimuli remain largely unknown. A role for CREB and AP-1 transcription factors in controlling APE/Ref-1 expression has been suggested by different authors [28]. However, the molecular mechanisms responsible for APE/Ref-1 upregulation, upon oxidative injury, have not been delineated. In this regard, the present data, demonstrating the existence of co-regulation between Egr-1 and APE/Ref-1, suggest the presence of an autosustaining loop composed of two chronologically distinct mechanisms. Oxidative stimuli or PKC stimulation induce, at first, an early activation exerted by APE/Ref-1 over Egr-1 by means of direct interaction. This early activation does not require protein neosynthesis and, possibly, accounts for a post-translational modification, i.e. redox, exerted by APE/Ref-1 upon Egr-1, which ultimately leads to an increase of Egr-1 DNA-binding activity. In the second phase, through a protein neosynthesis dependent-mechanism, Egr-1 could activate the expression of APE/Ref-1, as suggested by the stimulatory effects of Egr-1 over APE/Ref-1 promoter. Moreover, since the Egr-1 promoter contains several putative binding sites for Egr-1 itself [13], it seems likely that Egr-1 could up-regulate its own expression by a self-autosustaining loop, thus explaining the observed increase of Egr-1 protein levels. The autostimulatory role of Egr-1 on itself has been recently demonstrated by Yu *et al.* [50] In this context, the stimulatory role exerted by APE/Ref-1 over preconstituted inactive Egr-1 protein would explain the molecular mechanism at the basis of this loop. At longer times, the autostimulatory loop would be shut off by the negative effect that APE/Ref-1 plays

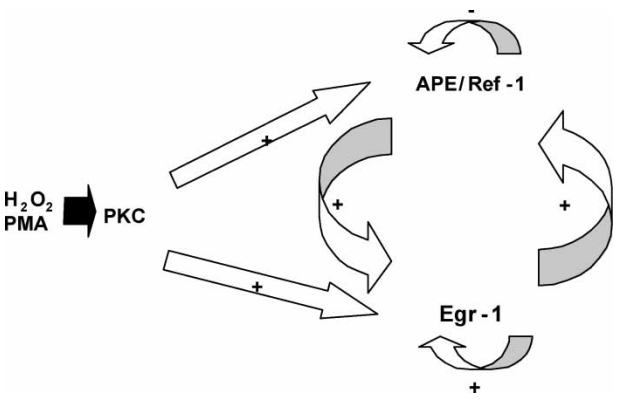

Figure 6. *Proposed model of the autoregulatory loop existing between APE/Ref-1 and Egr-1 proteins*. Data reported in the present paper, together with previously published data on the auto-inhibitory role of APE/Ref-1 on its own expression [56], allow to depict this regulatory model.

on its own expression by directly binding on its promoter [56]. The functional relevance of the proposed regulatory loop is reinforced by co-transfection experiments here presented. Interestingly, a number of evidences showed that PTEN gene expression is stimulated by oxidative stress conditions [57,58]. Our present data, shed new light in the molecular mechanisms responsible for the inducible expression of PTEN tumor suppressor upon oxidative stress.

While these data shed light into a new molecular mechanism to explain the rapid induction of APE/Ref-1 protein levels after oxidative stimuli, they do not explain the nature of the initial activation on APE/Ref-1 itself. In the light of data obtained by PMA treatment, known to stimulate PKC functionality, together with previously published data on the role of PKC in modulating the APE/Ref-1 stimulatory activity over AP-1 [46], it can be hypothesized that PKC could play a master role in this loop. It is well known that PKC activity is induced by H_2O_2 treatment of cells [51]. The inhibitory effects on APE/Ref-1 expression played by the two PKC inhibitors used in this study would suggest that PKC is central in controlling the proposed loop. Therefore, in light of our data, we could suggest the model depicted in Figure 6.

It has been recently demonstrated that, in a fibroblast cell line, H_2O_2 exposure can exert a stimulatory effect on Egr-1 by an unknown soluble factor, possibly released after cell exposure to the stimulus [52]. Future studies will elucidate if the H_2O_2-mediated induction is directed on an intracellular molecular target, such as PKC, or requires a soluble released factor able to trigger the stimulatory cascade.

APE/Ref-1 has been already demonstrated to control, in a redox-dependent manner, the DNA binding activity of different important TFs (such as p53, c-Myb, AP-1, NF-κB and members of the ATF/CREB family, (for a review see ref. [28]) by modulating the redox state of the DNA-binding domain. Our data demonstrate, for the first time, the presence and regulation of this protein in the osteoblastic cell type. Therefore, due to the central role this protein plays in several biologic processes and to the fact that the redox based-mechanisms controlling osteoblast transcriptional regulation in response to oxidative bursts are scarcely known at the molecular level, the study of APE/Ref-1 could represent an interesting aspect to osteoblast physiology.

Since APE/Ref-1 and Egr-1 play pivotal roles in the control of eukaryotic cell physiology [13,28], the study of these two molecules, particularly their coregulatory actions, in the context of osteoblastic physiology, would be important to explain, at the molecular level, some aspects of bone diseases. In fact, recent literature points to oxidative stress-based mechanisms as central etiopathogenetic event for osteoporosis [3,53,54].

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