RESEARCH COMMUNICATION

Functional activation of the *egr-1* (early growth response-1) gene by hydrogen peroxide

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The redox-based regulation of gene expression is one of the fundamental mechanisms of cellular functions, and hydrogen peroxide seems to act as an intracellular second messenger of signal transduction of cytokines. Hydrogen peroxide at non-toxic doses induced the accumulation of mRNA for the early growth response-1 (*egr-1*) gene in mouse osteoblastic cells. The Egr-1 protein is a transcription factor that binds the GCGG-GGGCG sequence and contains a zinc-finger structure that is essential for DNA binding. Egr-1 protein is sensitive to oxidative stress and loses specific DNA-binding activity when exposed to high levels of oxidative stress. Incubating cells with hydrogen

peroxide at about 50 μ M, however, increased the accumulation of Egr-1 protein, and the Egr-1 product seemed to be functional, judging by its binding activity to the GCGGGGGCG sequence and its ability to activate the chloramphenicol acetyltransferase reporter gene under the control of the human thymidine kinase enhancer containing the Egr-1 binding sequence. It was reported that the activity of Egr-1 protein as a transcription factor was negatively regulated by active oxygens. However, with appropriate concentrations of active oxygen, its capacity to bind a specific DNA sequence and to enhance the transcriptional activity of target genes is thought to be elevated.

INTRODUCTION

Oxidative stress and active oxygen species induce various alterations in gene expression, including those of stress-response genes and of growth factor-regulated genes such as *c-fos*, *c-jun*, *c-myc* and early growth response-1 (*egr-1*) [1–3]. As the production of active oxygen species is modulated by several growth factors, such as tumour necrosis factor α [4], tumour growth factor β 1 [5] and vascular endothelial growth factor [6], these active oxygens may participate in signal transduction pathways. In fact, oxidative stress affects the activities of many signal-transduction pathways, such as protein kinase [7], protein phosphatase [8] and several transcription factors. Serum response factor is one target of active oxygen species in the induction of the early response genes such as *c-fos* and *egr-1* [3,5]. The DNA-binding activity of nuclear factor κ B [9–11] or FOS [12] and the binding protein for the iron-responsive element of mRNA [13] are regulated by redox. As the thiol group is very sensitive to oxidation, proteins containing cysteine are good targets for redox-based modification, and it seems that such modifications constitute one basis of regulation following various stimuli. However, whether the induction of these genes at the mRNA level really reflects the functional activation of the gene products remains unknown. With regard to *c-fos* expression, activator protein-1 activity is activated by antioxidants but not by oxidants [14], even though the induction of mRNA is elevated by hydrogen peroxide and other active oxygen species [1,2].

The *egr-1* gene encodes a zinc-finger protein that binds and activates an enhancer element containing GCGGGGGCG (referred to as the GC sequence), like the tumour suppressor WT-1 gene product, thereby competing for binding [15]. The expression of the *egr-1* gene is induced by a variety of cellular stimuli, including growth factors and stress. The trans-acting

function of Egr-1 protein seems important for growth control, and human thymidine kinase gene expression is activated by the Egr-1 product [16]. The Egr-1 protein also participates in macrophage differentiation [17], although the real biological roles remain unclear. We have previously shown that hydrogen peroxide is involved in the transcriptional induction of *egr-1* by tumour growth factor β 1 [5], but several others have indicated that the activity of the Egr-1 protein deteriorates under oxidation *in vitro* [18,19]. Here, we assess the DNA-binding activity of the Egr-1 product induced in cells incubated with hydrogen peroxide to understand the biological relevance of *egr-1* induction caused by oxidative stress.

MATERIALS AND METHODS

Cell culture

The mouse osteoblastic cells, MC3T3-E1 [20], were cultured in Dulbecco's modified minimal essential medium containing 10% (v/v) fetal bovine serum in a CO₂-incubator.

Gel shift assay

Nuclear extracts were prepared from confluent cells by the method of Dignam et al. [21] as described previously [11]. Cells were collected with a rubber policeman, suspended in hypotonic buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM PMSF and 1 mM dithiothreitol (DTT)], centrifuged and disrupted with a Dounce homogenizer. The nuclear pellet was extracted with buffer C [10 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF and 1 mM DTT] at 4 °C for 30 min. The extract was dialysed against 5 vol. of buffer D [20 mM Hepes, pH 7.9, 0.1 M

Abbreviations used: *egr-1*, early growth response-1; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; CRE, cAMP response element; GC sequence, GCGGGGGCG.

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KCl, 0.2 mM EDTA, 20 % (v/v) glycerol, 1 mM PMSF and 1 mM DTT] at 4 °C for 5 h, and aliquots were stored at -80 °C. The synthetic probes for the Egr-1-binding sequence (the GC sequence), 5'-GATCCGCGCGGGGGCGAGGGCA and 5'-AGCTTGCCCTCGCCCCGCGCCG, were annealed and subcloned into pUC18 (*Hind*III/*Bam*HI site). The plasmid was cut with the restriction nucleases and end-labelled with [³²P]dCTP and Klenow fragment. The labelled insert was isolated by PAGE and purified with Elutip D (Schleicher & Shuell). The cAMP response element (CRE) was as described previously [12].

For the binding assay, nuclear extracts (10 µg of protein) were incubated in 24 µl of binding buffer [20 mM Hepes, pH 7.9, 50 mM KCl, 4 mM MgCl₂, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM DTT, 0.05 % (v/v) Nonidet P-40, 20 % (v/v) glycerol, and 1 mg/ml BSA containing 1 µg of poly(dI-dC)]. The ³²P-labelled probe (0.2 ng, 5000 c.p.m.) was added and the mixture was incubated for 30 min at room temperature. Samples were analysed on a native 4%-polyacrylamide gel containing 45 mM Tris, 45 mM boric acid and 1.25 mM EDTA.

Western blots

Nuclear extracts (10 µg of protein per lane) were resolved by electrophoresis on 10%-polyacrylamide gels, containing SDS, electro-transferred to nitrocellulose membrane (Hybond C; Amersham). The membrane was blocked with BSA, then immersed in polyclonal anti-Egr-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) that was diluted 1:500. Bound antibody was detected with horseradish peroxidase-conjugated anti-rabbit antiserum and 3,3'-diaminobenzidine using a kit (Vector Laboratory, Inc.).

Transient expression assay

The chloramphenicol acetyltransferase (CAT) expression vector under the control of the mouse thymidine kinase upstream region (pmTKCAT [16]) containing the Egr-1-binding site was provided by Dr. A. B. Pardee (Dana-Farber Cancer Institute, Boston, MA, U.S.A.). For transfection, cells were plated at 3 × 10⁶ cells/100 mm dish. They were transfected 24 h later with plasmid DNA by calcium phosphate precipitation [22]. The precipitates were removed 6 h later and the cells were treated with 15 % (v/v) glycerol. After rinsing with PBS, the cells were cultured for 24 h after transfection, then incubated with H₂O₂ for another 24 h. Cell lysates were prepared and CAT activity was measured as described [23].

RESULTS AND DISCUSSION

The Egr-1 product is a zinc-finger protein that binds to a specific sequence (the GC sequence [24]), like WT-1, a protein identified in Wilm's tumour [25]. Nuclear extracts were prepared from cells incubated with phorbol 12-myristate 13-acetate (PMA) or with various concentrations of hydrogen peroxide, and binding activity was compared by means of electrophoretic mobility-shift assays. The results in Figure 1(a) show that incubating MC3T3 cells with PMA for 2 h increased the GC sequence-binding activity of the nuclear extracts, as found with other cell lines [15], and that this binding was extinguished in the presence of an antibody against Egr-1 protein. Pre-immune serum did not affect the pattern of gel shift (results not shown). These results indicate that the major factor involved in the gel shift is the Egr-1 protein.

Mild oxidative stress applied to the cells induced *egr-1* gene transcription that led to the accumulation of *egr-1* mRNA [3,5].

Figure 1 GC sequence-binding activity of nuclear extracts

(a) Nuclear extracts were prepared from MC3T3 cells of control (lane 1), or treated with 50 ng/ml PMA for 2 h (lane 2). GC sequence-binding activity was measured by an electrophoretic mobility shift assay using a ³²P-labelled probe. Lane 3 contained 2 ml of anti-Egr-1 serum in the reaction mixture. (b) Cells were either untreated (lane 1) or treated with 50 ng/ml PMA (lane 2) or 20 (lane 3), 50 (lane 4), 100 (lane 5) or 200 (lane 6) µM H₂O₂ for 2 h. Nuclear extracts were prepared and GC sequence-binding activity was measured. (c) GC sequence-binding activity was measured using the extracts shown in lane 2 of (a), that were treated for 5 min on ice in the absence (lane 1), or presence of 0.2 mM H₂O₂ (lane 2) or 0.05 mM diamide (lane 3) before addition of the probe.

At these concentrations, hydrogen peroxide was not toxic to the cells, as determined by dye exclusion or plating efficiency (results not shown). Nuclear extracts were prepared from cells incubated with various concentrations of hydrogen peroxide for 2 h, then binding activity to the GC sequence was examined. The binding activity increased with the dose of hydrogen peroxide, reaching a maximum at 0.05 mM (Figure 1b). This finding indicated that the increased level of *egr-1* mRNA in cells incubated with hydrogen peroxide, as reported previously [5], correlates with that in GC sequence-binding activity.

Western blots using antibody against Egr-1 protein showed that the amount of the protein was also increased in cells incubated with low concentrations of hydrogen peroxide (Figure 2). This finding suggests that the increase in *egr-1* mRNA is

Figure 2 Increase in Egr-1 protein induced by H₂O₂

Proteins of the nuclear extracts were resolved by electrophoresis on 10%-polyacrylamide gels containing SDS and transferred to a nitrocellulose membrane. The membrane was stained with anti-Egr-1 antibody as described in the text. Lanes 1–6, nuclear extracts from cells treated either with none, 50 ng/ml PMA, 0.02, 0.05, 0.1 and 0.2 mM H₂O₂ for 2 h respectively. Nuclear extracts from PMA-treated (lanes 7 and 9) or H₂O₂-treated (lanes 8 and 10) cells were run on the gel and stained with pre-immune (lanes 7 and 8) or anti-Egr-1 (lanes 9 and 10) antibody. The positions of molecular-mass markers are indicated on the left: 86.8 (BSA), 47.8 (ovalbumin) and 33.3 (carbonic anhydrase) kDa from top to bottom respectively.

Table 1 Activation of mTKCAT by H₂O₂

MC3T3 cells were transfected with pmTKCAT (10 mg/100 mm dish), and 12 h later H₂O₂ was added. Cells were collected 24 h after the addition, and CAT activity (expressed as arbitrary units) was measured. The results are means $\pm$ S.D. of three independent experiments.

Treatment	CAT activity
–	1332 $\pm$ 165
0.1 mM H ₂ O ₂	3245 $\pm$ 544
0.2 mM H ₂ O ₂	5417 $\pm$ 493

related to the accumulation of its protein, resulting in an increase in GC sequence-binding activity.

Several reports have shown that the DNA-binding activity of Egr-1 protein is impaired by treatment of isolated protein or nuclear extracts with oxidizing agents, such as diamide, which catalyse the oxidation of free thiol groups, or with *N*-ethylmaleimide, which irreversibly alkylates free thiol groups [17,18]. Incubating nuclear extracts prepared from cells exposed to PMA with 0.1 mM hydrogen peroxide or 0.05 mM diamide *in vitro* decreased the GC-binding activity (Figure 1c), whereas it did not significantly affect CRE-binding activity (results not shown). Since zinc fingers that contain cysteine are essential for DNA-binding activity [14], the direct oxidation of Egr-1 protein should cause a loss of binding ability. However, mild oxidative stress applied to the cells induced several early response genes, and the induction of mRNAs may reflect an increase in the amount and activity of their products.

Furthermore, we tested the possibility that the target gene of Egr-1 protein is activated. The CAT reporter under the control of the thymidine kinase gene enhancer that is activated by the Egr-1 product was transfected into cells, which were then exposed to hydrogen peroxide. CAT activity was increased, indicating that GC-binding activity correlates with the transcriptional activation of its target gene (Table 1). Thus, we conclude that *egr-1* is functionally activated in cells exposed to mild oxidative stress. The intracellular redox state is maintained under relatively reduced conditions by numerous factors, of which the major one is glutathione [26], but it is modulated following stimulation with

growth factors [5,6]. A subtle change in the redox state may constitute one of the physiological signalling pathways elicited by growth factors or extracellular stimuli, as discussed previously [5,7]

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