The Balance between Cu,Zn-Superoxide Dismutase and Catalase Affects the Sensitivity of Mouse Epidermal Cells to Oxidative Stress†

Paul Amstad, Alexander Peskin,‡ Girish Shah,§ Marc-Edouard Mirault,‖ Rêmy Moret, Irène Zbinden, and Peter Cerutti*

Department of Carcinogenesis, Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Lausanne, Switzerland

Received March 8, 1991; Revised Manuscript Received June 6, 1991

ABSTRACT: Oxidants are toxic, but at low doses they can stimulate rather than inhibit the growth of mammalian cells and play a role in the etiology of cancer and fibrosis. The effect of oxidants on cells is modulated by multiple interacting antioxidant defense systems. We have studied the individual roles and the interaction of Cu,Zn-superoxide dismutase (SOD) and catalase (CAT) in transfectants with human cDNAs of mouse epidermal cells JB6 clone 41. Since only moderate increases in these enzymes are physiologically meaningful, we chose the following five clones for in-depth characterization: CAT 4 and CAT 12 with 2.6-fold and 4.2-fold increased catalase activities, respectively, SOD 15 and SOD 3 with 2.3-fold and 3.6-fold increased Cu,Zn-SOD activities, respectively, and SOCAT 3 with a 3-fold higher catalase activity and 1.7-fold higher Cu,Zn-SOD activity than the parent JB6 clone 41. While the increases in enzyme activities were moderate, the human cDNAs were highly expressed in the transfectants. As demonstrated for the clone SOD 15, this discordance between message concentrations and enzyme activities may be due to the low stability of the human Cu,Zn-SOD mRNA in the mouse recipient cells. According to immunoblots the content of Mn-SOD was unaltered in the transfectants. While the activities of glutathione peroxidase were comparable in all strains, the concentrations of reduced glutathione (GSH) were significantly lower in SOD 3 and SOD 15. This decrease in GSH may reflect a chronic prooxidant state in these Cu,Zn-SOD overproducers. The Cu,Zn-SOD overproducers SOD 15 and SOD 3 were hypersensitive to the formation of DNA single-strand breaks, growth retardation, and killing by an extracellular burst of superoxide plus H_2O_2 while the CAT overproducers were protected relative to the parent clone JB6 clone 41. The double transfectant SOCAT 3 was well protected from oxidant damage because of its increased content in CAT, which counterbalances the increase in Cu,Zn-SOD. The inducibility of the growth-competence-related protooncogene c-*fos* was decreased in all transfectants, albeit probably for different reasons. We conclude that H_2O_2 represents the major intracellular oxidant on the pathways to DNA single-strand breakage and cytotoxicity and that the hypersensitivity of Cu,Zn-SOD transfectants is mostly due to the overproduction of H_2O_2. The balance of SOD and CAT plus glutathione peroxidase is more important for overall sensitivity than the level of Cu,Zn-SOD alone. Growth stimulation may occur when cells are protected from excessive oxidant toxicity but only when a sufficient oxidant signal remains to activate the necessary growth pathways.

Oxidants are ubiquitous in our aerobic environment and are formed in situ in tissues and cells by normal metabolism and the metabolism of certain xenobiotics. They are always toxic and produce macromolecular damage. At the same time oxidants can serve as (patho)physiological signals in growth and differentiation (Cerutti, 1985; Crawford et al., 1988; Shibanuma et al., 1988; Murrel et al., 1990; Cerutti & Trump, 1991). The sensitivity of cells to oxidants is attenuated by low molecular weight antioxidants and antioxidant enzymes. The biochemistry of the most important enzymes [i.e., superoxide dismutases (SOD),[1] catalase (CAT), GSH peroxidases (GPx), GSH reductase, and GSH *S*-transferases] has been studied in detail [see Cerutti et al. (1988)]. However, the physiological role of a single antioxidant enzyme in situ in the cell is only poorly understood because of complex interactions and interrelationships between the individual components. Complete deficiency in SOD sensitizes *Escherichia coli* (Natvig et al., 1987) and *Drosophila* (Phillips et al., 1989) to oxidative stress, and in several instances an increase in intracellular SOD was protective (Krall et al., 1988; Kyle et al., 1988; Elroy-Stein et al., 1986). However, there are remarkable exceptions. For example, a large increase in Fe- or Mn-SOD sensitized *E. coli* to paraquat toxicity (Scott et al., 1987; Bloch & Ausubel, 1986), and inactivation of Cu,Zn-SOD in human fibroblasts increased their growth rate (Michiels et al., 1988). There was no proportionality between the degree of resistance to paraquat and the complement of transfected Cu,Zn-SOD in HeLa cells. Cells with high complements were less protected and contained increased levels of peroxidized lipid (Elroy-Stein et al., 1986). Cytotoxic effects prevented the preparation of stable transfectants of bovine adrenocortical cells with human Cu,Zn-SOD (Norris & Hornsby, 1990). These results suggest that a fine

† This work was supported by the Swiss National Science Foundation and the Swiss Association of Cigarette Manufacturers and the Association for International Cancer Research.

* Address correspondence to this author.

‡ Present address: Institute of Developmental Biology, Academy of Science of USSR, Moscow 117808, USSR.

§ On leave from the Biochemistry Division, Bhabha Atomic Research Centre, Bombay 400 085, India.

‖ Present address: Ontogênèse et Génétique Moléculaire, Le Centre Hospitalier de l'Université Laval, 2705 Boulevard Laurier, Québec, G1V 4G2 Canada.

[1] Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, selenium glutathione peroxidase; GSH, reduced glutathione; AO, active oxygen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; X/XO, xanthine/xanthine oxidase; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; $O_2^{\cdot-}$, superoxide anion radical.

0006-2960/91/0430-9305$02.50/0

balance between several antioxidant enzymes determines the physiological and pathophysiological effects of oxidants. It is interesting to note that mouse L-cells, neuroblastoma cells, and NIH 3T3 fibroblasts transfected with human Cu,Zn-SOD possessed a compensatory increase in glutathione peroxidase (GPx) (Ceballos et al., 1988; Kelner & Bagnell, 1990) and that promotable mouse epidermal cells JB6 clone 41) possess a superior antioxidant defense due to a coordinate increase in both Cu,Zn-SOD and CAT (Crawford et al., 1989).

Our present results with stable transfectants of mouse epidermal cells JB6 clone 41 indicate that at comparable levels of GPx the balance between Cu,Zn-SOD and CAT is a determining factor for their sensitivity to a burst of extracellular active oxygen (AO) produced by xanthine/xanthine oxidase (X/XO). Cu,Zn-SOD overproducers were hypersensitive to retardation of growth, cell killing, and DNA strand breakage by AO while CAT overproducers were protected. An increase in Cu,Zn-SOD was tolerated without an increase in oxidant sensitivity when it was counterbalanced by a corresponding increase in CAT in a double transfectant. Both increases in CAT and Cu,Zn-SOD diminished the inducibility of the growth-related protooncogene c-*fos*, albeit probably for different reasons. We propose that cells are stimulated to grow when they are protected from excessive AO toxicity as long as a sufficient AO signal remains to activate the necessary growth pathways.

Materials and Methods

Construction of Expression Vectors Containing Cu,Zn-SOD or CAT and Preparation of Stable Transfectants. The pD_5-*neo* vector containing the adeno 5 major late promoter, the SV40 promoter and enhancer sequences, and the neomycin resistance gene casette was used for the construction of a human Cu,Zn-SOD expression vector. A 600-bp cDNA fragment was removed by *Pst*I digestion from clone pS61-10 (Sherman et al., 1983), and the G- and C-tails were removed with the double-strand exonuclease *Bal*31. *Bam*HI linkers were added to the blunt-ended SOD cDNA fragment before ligation into the unique *Bam*HI site of the pD_5-*neo* vector. Transfection into mouse epidermal JB6 clone 41 cells was according to Chen and Okayama (1987), and resistant clones were selected in medium containing 400 μg/mL geneticin for 9 days. Control cell clones were also prepared which contained only the pD_5-*neo* vector without the SOD cDNA insert.

An expression vector containing complete CAT cDNA was constructed by using the pCAT1 clone of Korneluk et al. (1984), which lacks the 5′ portion coding for 77 amino acids. In order to complement the 5′ portion, the 5′ 450-bp *Pst*I to *Pvu*II fragment of the rat catalase cDNA clone pMJ1010 (Furuta et al., 1986) was inserted into the *Pst*I and *Pvu*II sites of pCAT1. Immediate early promoter sequences of cytomegalovirus (CMV), i.e., 1.2-kb *Pst*I to *Ava*II, were then inserted between the *Pst*I and *Nco*I sites of the vector containing the fused rat–human CAT cDNA. Finally the vector was completed by the insertion of a hygromycin gene resistance casette. For this purpose pSP65–SV40–hygromycin was restricted with *Sal*I and *Hin*dIII, and the ends of the 2.5-kb SV40–hygromycin expression casette were filled in with Klenow enzyme. This blunt-ended fragment was then ligated into the unique *Hpa*I site of the modified pCAT1 plasmid containing fused rat–human CAT cDNA under direction of the CMV promoter.

Growth Conditions and Antioxidant Enzyme Activities for the Parent Strain and Transfectants of JB6 Clone 41 Cells. Mouse epidermal cells JB6 clone 41 originally had been received from Dr. N. Colburn and were cultured in monolayers with 8% fetal calf serum (GIBCO, Grand Island, NY) in MEM supplemented with 50 ng/mL Na_2SeO_3. To the medium of stable CAT transfectants was added 10 μg/mL hygromycin; for stable SOD transfectants 100 μg/mL geneticin was added, and for CAT plus SOD double transfectants both antibiotics were added for routine culturing in order to guard against the loss of the transfected DNAs. These antibiotics were omitted immediately before experiments measuring biological properties. For the determination of CAT and GPx activities, the monolayers were rinsed twice with ice-cold phosphate-buffered saline and the cells collected by scraping with a sterile rubber policeman. The cells were sedimented for 4 min at 1600*g* and processed either for enzyme/protein or for mRNA analyses. For enzyme/protein lysates, cells were resuspended in 50 mM potassium phosphate buffer containing 0.5% Triton X-100 and sonicated (in an ice–water bath) for two 30-s bursts on a Branson sonicator B15 (position 2, continuous setting; Branson Ultrasonics Corp., Danbury, CT) with a 30-s cooling interval. Total protein concentration was determined according to the procedure of Peterson (1977). For CAT and GPx activities, sonicates were first spun 5 min at 800*g* (4 °C). The supernatants were assayed according to the procedures of Clairborne (1985) for CAT activity and Günzler and Flohé (1985) for GPx activity.

For SOD measurements cells were suspended in 100 mM triethanolamine–diethanolamine buffer and homogenized with a Teflon glass Dounce homogenizer. The homogenate was centrifuged at 105000*g* for 1 h (4 °C), and the supernatant was passed through a small Sephadex G25 (coarse) column to remove low molecular weight substances which interfere with the enzyme assay according to the procedure of Paoletti et al. (1986). An aliquot of the eluate was applied onto a 5.5% polyacrylamide gel in order to localize SOD activity according to the procedure of Beauchamp and Fridovich (1971) with the exception that no tetramethylethylenediamine was used for staining. Mn-SOD activity was determined in mitochondrial fractions prepared by differential centrifugation. Mitochondria were disrupted by freezing–thawing in a high ionic strength buffer (0.25 mM sucrose, 0.12 M KCl, 10 mM Tris-HCl, pH 7.4). Mitochondrial membranes were removed by sedimentation at 105000*g* for 1 h, and enzyme activity was measured in the supernatant. Reduced glutathione (GSH) concentration was determined by the monobromobimane method of Cotgreave and Moldeus (1986).

Western Blot Analysis. Total cellular sonicates were prepared in 50 mM potassium phosphate, pH 7.0–0.5% Triton X-100. The sonicates were then mixed with an equal volume of 2× SDS buffer, boiled for 3 min, frozen, boiled again for 3 min, and applied to a 12.5% SDS–polyacrylamide gel. Forty micrograms of protein was applied to each lane and, after electrophoresis, transferred to an Immobilon PVDF membrane (Millipore Corp., Bedford, MA) by electrotransfer. CAT protein concentration was determined after 2 h of prehybridization by overnight incubation with a mixture of rabbit anti-human CAT antibody and rabbit anti-human Mn-SOD antibody at 4 °C. The filter was washed five times followed by hybridization with 100000 cpm/mL ^{125}I-labeled goat anti-rabbit immunoglobulin.

Northern Blot Analysis. Total RNA was prepared according to the procedure of Chirgwin et al. (1979). A total of 10 μg of total RNA was electrophoresed on a 1.4% agarose–formaldehyde gel and then transferred to gene screen membranes. The filters were prehybridized in 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% poly(vinylpyrrolidone), 0.2% Ficoll, 5 mM EDTA, 50% for-

mamide, 0.2% BSA, 1× SSC, and 150 μg/mL denatured salmon sperm DNA at 65 °C for 6 h. For hybridization, 1 × 10^6 cpm/mL of ^{32}P-labeled probe solution was used. The filteres were washed at 65 °C twice for 15 min with 2× standard sodium citrate (SSC)–0.1% SDS and twice for 15 min with 0.1× SSC–0.1% SDS. The probe was a transcript of an SP65 recombinant containing in the antisense direction a cDNA fragment of human Cu,Zn-SOD (450-bp *Alu* to *Taq*) (Sherman et al., 1983).

The stability of Cu,Zn-SOD mRNAs transcribed from the endogenous and transfected genes, respectively, was estimated by using actinomycin D in the parent strain JB6 clone 41 and the SOD transfectant SOD 15. Actinomycin D (5 μg/mL) in DMSO was added to cultures which were approximately 80% confluent and growth continued for 2, 4, 6, 8, and 10 h before the preparation of total RNA according to Chirgwin et al. (1979) and Northern blotting using a ^{32}P-labeled riboprobe as described above.

Induction of c-*fos* by AO treatment in JB6 clone 41 and in the antioxidant gene transfected clones was determined as described previously (Crawford et al., 1988) in monolayer cultures grown for 24 h in only 0.25% fetal calf serum. The cultures were exposed to AO generated by 20 μg/mL X and 2.0 μg/mL XO (obtained from Boehringer Mannheim) for 30 min. The production of $O_2^{\bullet-}$ was determined spectrophotometrically by the reduction of cytochrome. The rate of $O_2^{\bullet-}$ release was close to linear during the first 15 min at 0.45 nmol of $O_2^{\bullet-}$/min and had decreased to less than 0.2 nmol/min of this value after 30 min. Cells were harvested and total RNA was prepared for Nothern blotting as described above. The probe was prepared by the transcription of an SP6 recombinant containing a 685-bp *Sal*I to *Pst*I fragment of v-*fos* (Van Beveren et al., 1986).

RNase Protection Analysis of CAT Expression. RNase protection analysis was used to demonstrate the presence of human CAT message in the transfectants CAT 4 and SOCAT 3. For this purpose the SP65 recombinant described above containing in the antisense direction the 1250-bp *Hin*dIII to *Pvu*II human CAT cDNA insert was linearized with *Xho*I, and a ^{32}P-labeled riboprobe of 468 nucleotides was prepared according to the procedure of Melton et al. (1984). The probe was passed through a spin column and precipitated with 2 M ammonium acetate and 2.5 volumes of ethanol. The pellet was washed with 75% ethanol–25% 0.1 M sodium acetate, pH 5.7, dried under vacuum, and dissolved in 100 μL of hybridization buffer (80% formamide–40 mM Pipes, pH 6.4–400 mM NaCl–1 mM EDTA).

Total cellular RNA (10 μg), prepared as described above, was taken up in 30 μL of hybridization buffer, and 5 × 10^5 cpm of the CAT riboprobe was added. After the mixture was heated for 15 min to 85 °C and incubated overnight at 45 °C, 300 μL of RNase buffer (10 mM Tris-HCl, pH 7.8–5 mM EDTA–300 mM NaCl) containing 40 μg/mL RNase A and 2 μg/mL RNase T1 was added, and the samples were incubated for 30 min at 30 °C. The RNase was then inactivated by the addition of 10 μL of 20% SDS and 50 μg of proteinase K for 15 min at 37 °C. The RNA was extracted with 400 μL of phenol–chloroform–isoamyl alcohol and the aqueous layer transferred to a centrifuge tube containing 10 μg of tRNA. The RNA was taken up in 2 M ammonium acetate and precipitated with an equal volume of 2-propanol and the pellet washed once with 75% ethanol before it was dried under vacuum. The RNA was then dissolved in 5 μL of gel loading buffer (80% formamide–40 mM Tris–borate, pH 7.5); the sample was heated to 85 °C for 10 min and loaded in a buffer consisting of 89 mM Tris–89 mM boric acid–2 mM EDTA onto a 5% denaturing polyacrylamide gel [19:1 acrylamide: bis(acrylamide)] which was run for 3 h at 50 W. The dried gel was then exposed to an X-ray film.

Southern Blot Analysis. DNA was extracted, restricted with *Bam*HI, and electrophoresed in 0.8% agarose gels. The DNA was transferred to nitrocellulose essentially as described by Southern. The filters were hybridized with a nick-translated 450-bp probe removed by *Hin*dIII/*Eco*RI from an SP65 vector containing a 450-bp *Alu*I to *Taq*I human Cu,Zn-SOD cDNA fragment (Sherman et al., 1983). As expected, only the SOD-transfected clones showed a band at approximately 0.5 kb for the transfected gene in addition to a double band at approximately 10 kb for the endogenous mouse gene, which is present in all cell clones. From a comparison of the intensities of the bands for the transfected and the endogenous gene, we estimate that the SOD transfectants contain no more than one to two copies of the tranfected human SOD gene (data not shown). For CAT a nick-translated probe was prepared by using a 1250-bp fragment removed by *Hin*dIII and *Eco*RI from an SP64 construct containing a 1250-bp *Hin*dIII to *Pvu*II fragment of human CAT cDNA (Korneluk et al., 1984). The blots contain two clearly separated bands at approximately 1.8 kb for the transfected human CAT gene in transfected clones and a band at approximately 10 kb for the mouse CAT gene in all cell clones. Ratios of densitometer readings for the bands attributed to the transfected over the endogenous CAT gene indicate that the transfectants contain two to three human CAT copies per cell (data not shown).

Growth Properties, DNA Synthesis Capacity, and Colony-Forming Ability of Antioxidant Gene Transfected JB6 Cells in the Presence and Absence of Oxidant Stress. For the determination of the effect of AO on the growth of the parent JB6 clone 41 cells and the antioxidant gene transfected clones, 10^5 cells were plated into 10-cm Petri dishes in MEM supplemented with 8% serum and 50 ng/mL Na_2SeO_3 and allowed to grow for 2 days before treatment with X/XO at the indicated doses. Without changing the culture medium the cells were harvested at the indicated times by trypsinization, and their number was counted in a hemocytometer (Muehlematter et al., 1988). For the determination of the survival of colony-forming ability, 500 cells for each strain were plated into 6-cm Petri dishes and allowed to grow for 14 h under the usual culture conditions. The cultures were then treated without medium change with increasing doses of X/XO (0.5–1.75 μg/mL XO and 10 μg/mL X). Control cultures received only 10 μg/mL X. Growth was then continued for 7 days without medium change when the colonies were fixed with methanol and stained with 2% crystal violet and counted.

DNA Single-Strand Breakage. DNA single-strand breakage was measured by the alkaline elution method of Kohn et al. (1976) as described previously (Muehlematter et al., 1988). JB6 cells were seeded at low density in Petri dishes and cultured as described above. After 12 h they were labeled in their DNA by the addition of [^{14}C]thymidine over a period of 48 h. Subsequently, the cultures were grown in fresh medium containing nonradioactive thymidine for 12 h. Before treatment with active oxygen generated by X/XO, fresh medium supplemented with 8% fetal calf serum was added. Following treatment for 30 min, the medium was removed and the cells were scraped and collected directly on the filters used for alkaline elution.

Results

Transfection of Mouse Epidermal Cells JB6 Clone 41 with Human Cu,Zn-SOD, CAT, or Both Enzymes. JB6 clone 41

Table I: Activities of Antioxidant Enzymes in Transfected JB6 Clone 41 Cells[a]

cell strain	CAT (units/mg of protein)	SOD (units/mg of protein)	SOD/CAT	GPx (units/mg of protein)	GSH (nmol/10^6 cells)
JB6 cl 41	5.6 ± 1.1 (5)	21 ± 6 (11)	3.8	33 ± 4 (4)	2.6 ± 0.3 (4)
SOD 15	6.7 ± 1.1 (3)	**48** ± 14 (11)	7.2	45 ± 5 (4)	**1.5** ± 0.1 (4)
SOD 3	4.8 ± 1.2 (2)	**76** ± 9 (5)	15.8	45 ± 5 (4)	**0.9** ± 0.1 (4)
CAT 4	**14.5** ± 1.7 (4)	27 ± 3 (5)	0.5	45 ± 8 (4)	2.7 ± 0.1 (4)
CAT 12	**23.6** ± 1.2 (4)	23 ± 4 (4)	1.0	35 ± 11 (4)	3.9 ± 0.2 (4)
SOCAT 3	**17.2** ± 4.3 (*n*)	**36** ± 6 (8)	2.1	**26** ± 5 (4)	3.9 ± 0.2 (4)

[a] Cells were grown in monolayer cultures and extracted when confluency was approximately 80%. Values for enzyme activities are given in units per milligram of protein cell extract; means ± SD are listed; values in parentheses represent the number of independent determinations used for these calculations. Units are defined as follows: catalase, micromoles of H_2O_2 consumed per minute; SOD, amount of SOD inhibiting the rate of control NADH oxidation by 50%; GPx, nanomoles of NADPH oxidized per minute at 1 nM GSH. The values for reduced glutathione are nanomoles per 10^6 cells.

cells were transfected with expression vectors containign cDNA of human Cu,Zn-SOD and a neomycin resistance casette or fusion rat–human cDNA of CAT and a hygromycin resistance casette, respectively. Several transfectants in each series were grown in mass culture, and SOD, CAT, and glutathione peroxidase (GPx) activites were determined in lysates. Representative transfectants with a moderate increase in the particular antioxidant enzyme were chosen for detailed characterization on the molecular and cellular level.

CAT 4 was transfected in a second round with the human Cu,Zn-SOD expression vector, and neomycin/hygromycin resistant clones were selected. A particular clone, SOCAT 3, with moderate levels of both human Cu,Zn-SOD and CAT, was chosen for in-depth investigation.

Expression of Human Cu,Zn-SOD mRNA and Rat–Human CAT mRNA in JB6 Transfectants. Stationary mRNA concentrations were determined by using transcripts from SP65 recombinants containing cDNA fragments of human Cu,Zn-SOD and human CAT cDNA, respectively. The Northern blots under high stringency conditions in Figure 1A show the messages for endogenous mouse Cu,Zn-SOD and transfected human Cu,Zn-SOD, which possess different lengths in the clones SOD 3, SOD 15, and SOCAT 3. CAT 4 and a pD5-*neo* transfected clone serve as controls. It is evident that the amount of stationary mRNA is severalfold higher for the transcripts of the transfected than for the endogenous Cu,Zn-SOD gene in SOD 3 and SOCAT 3. A rough measure of the level of expression of the transfected gene can be obtained by comparison with the amount of message transcribed from the corresponding endogenous gene. In order to distinguish the endogenous mouse CAT mRNA from the transfected rat–human CAT mRNA, we performed the RNase protection experiment shown in Figure 1B. A 468-nucleotide transcript was prepared from an SP6 recombinant which contained in the antisense direction a 1200-bp human CAT cDNA fragment reaching from the *Pvu*II site to the *Hin*dIII site and which was linearized with *Xho*I. Correspondingly, a 486-nucleotide RNA fragment was protected from RNase digestion in the transfectants CAT 4, CAT 12, and SOCAT 3 but not in the parent clone 41. In contrast, a short fragment of approximately 100 nucleotides was protected in all clones and originates from the endogenous mouse CAT message. As noted for the Cu,Zn-SOD transfectants, it is evident that the transcripts from the transfected CAT gene are more abundant than those originating from the endogenous gene.

The stabilities of the endogenous mouse SOD and transfected human SOD mRNAs were compared in clone SOD 15 in an experiment with the transcriptional inhibitor actinomycin D. The Northern blots are shown on Figure 2. Transcripts of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured as reference. Our results indicate a much lower stability for the human Cu,Zn-SOD message

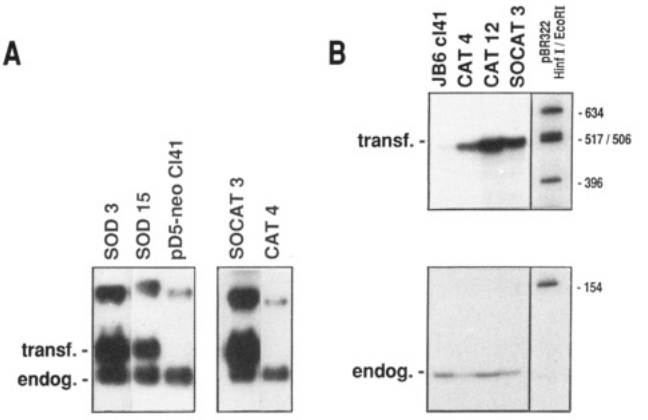

FIGURE 1: Expression of endogenous and transfected Cu,Zn-SOD and CAT genes in transfectants of JB6 clone 41. (A) The concentrations of endogenous mouse message and message transcribed from transfected human cDNA for Cu,Zn-SOD were measured by Northern blotting of total RNA extracted from monolayer cultures of the stable transfectants SOD 3, SOD 15, and SOCAT 3. For comparison, corresponding Northern blots were also performed for the parent JB6 clone 41, for CAT 4, and for cells which had been transfected with the pD_5-*neo* vector which did not contain a cDNA insert. A labeled transcript of an SP65 recombinant containing in the antisense direction a cDNA fragment of human Cu,Zn-SOD was used as the probe and is described under Materials and Methods. (B) The expression of transfected and endogenous CAT mRNA in parent clone 41, CAT 4, CAT 12, and SOCAT 3 was determined in RNase protection experiments. Total cellular RNA was hybridized with a 468-nucleotide ^{32}P-labeled riboprobe transcribed from an SP65 recombinant containing in antisense direction an insert of human CAT cDNA and digested with RNases A and T1. The protected RNA was isolated and separated by gel electrophoresis and the dried gel exposed to an X-ray film as outlined under Materials and Methods.

transcribed from the transfected gene than for the endogenous mouse message.

Levels of Antioxidant Enzymes in JB6 Clone 41 Transfectants. CAT, SOD, and GPx activities as well as concentrations of reduced glutathione were measured in lysates of the parent clone 41 and the antioxidant gene transfectants and are given in Table I. It is evident that CAT 4 possesses 2.6-fold and CAT 12 4.2-fold higher CAT activities than the parent clone 41. In SOD 15 the total SOD activity is 2.3-fold and in SOD 3 3.6-fold above the parent JB6 cells while CAT levels are essentially unaltered. In SOCAT 3 the total SOD activity is 1.7-fold higher than in the parent clone 41 and 1.3-fold higher than in CAT 4 from which it was derived. No significant differences were observed in the GPx activities between all six cell strains. In contrast, the concentrations of reduced GSH were lower in SOD 15 and SOD 3 (see Table I) while total glutathione levels were comparable (data not shown).

Mn-SOD activity was determined in soluble mitochondrial preparations of the parent clone 41 and CAT 4 and found to

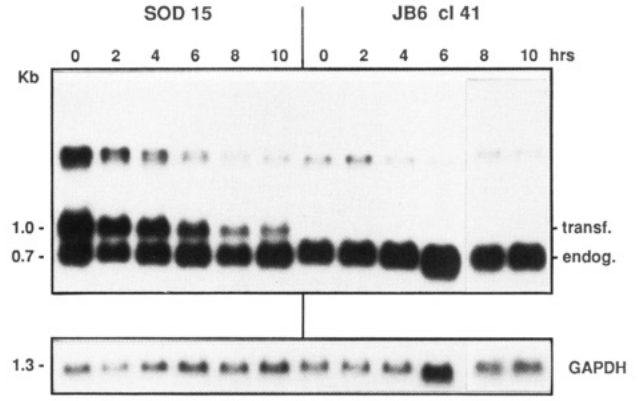

FIGURE 2: Stability of Cu,Zn-SOD mRNA transcribed from the transfected human Cu,Zn-SOD cDNA and from the endogenous Cu,Zn-SOD gene, respectively. Monolayer cultures were treated with 5 μg/mL actinomycin D and total RNA prepared after the indicated duration of growth for Nothern blotting with a human Cu,Zn-SOD riboprobe as described in the legend to Figure 1.

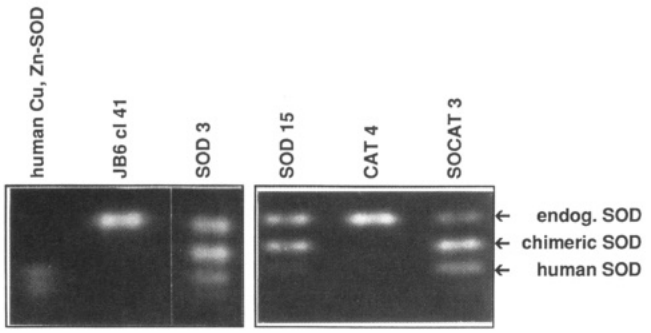

FIGURE 3: SOD activity gel of extracts of the parent JB6 clone 41 and its transfectants. Cellular homogenates were centrifuged at high speed, and the supernatant was passed through a Sephadex G25 column as described under Materials and Methods. Aliquots of the eluates were applied to a 5.5% PAGE, and the SOD activity was localized as described by Beauchamp & Fridovich (1971).

be 10–15-fold lower than Cu,Zn-SOD in both clones. Mn-SOD protein levels were estimated by immunoblotting (see below).

A SOD activity gel is shown in Figure 3. Because of its higher mobility human Cu,Zn-SOD can be readily distinguished from endogenous mouse Cu,Zn-SOD. Since Cu,-Zn-SOD is a dimer, the band between the two activities most probably represents the mouse–human heterodimer. It is evident that the SOD-transfected clones SOD 3, SOD 15, and SOCAT 3 contain active human Cu,Zn-SOD.

The presence of human CAT protein in CAT 4, CAT 12, and SOCAT 3 was demonstrated by immunoblotting with rabbit anti-human CAT antibody. The immunoblots in Figure 4 show double bands for the CAT transfectants which correspond to human and mouse CAT, which cross-reacts with the antibody. The parent clone 41, SOD 15, and SOD 3 show single bands. Indeed, the filters had been reacted simultaneously with a mixture of rabbit anti-CAT and rabbit anti-human Mn-SOD antibodies. The high-mobility band in all cell clones corresponds to endogenous mouse Mn-SOD, which cross-reacts with the latter antibody. According to these data all six cell clones contained comparable amounts of Mn-SOD.

Susceptibility to DNA Strand Breakage upon Exposure to Superoxide plus Hydrogen Peroxide. We measured the induction of DNA single-strand breaks (alkali-labile sites) in four representative cells strains, i.e., parent, SOD 15, CAT 4, and SOCAT 3, at two doses of $O_2^{\bullet-}$ plus H_2O_2 produced extracellularly by 40/3 and 40/4 μg/mL X/XO by the alkaline elution method. From Figure 5 we can derive the following order of decreasing susceptibilities: SOD 15 > parent > CAT 4 ≃ SOCAT 3. Our data indicate that H_2O_2 is the major intracellular oxidant species which ultimately leads to DNA breakage by an extracellular burst of $O_2^{\bullet-}$ plus H_2O_2 but give no information on the mechanism of break formation.

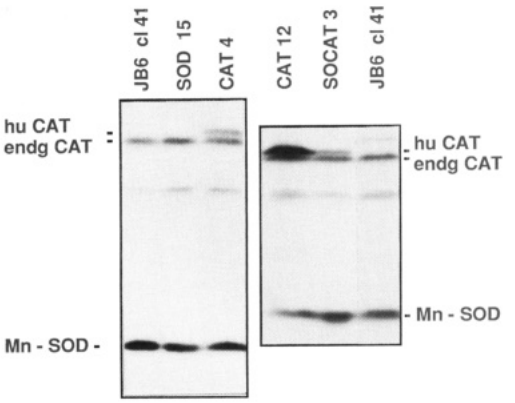

FIGURE 4: Immunoblots for CAT and Mn-SOD in sonicates of the parent JB6 clone 41 and its transfectants. Boiled cellular sonicates were separated on 12.5% SDS–PAGE and electrotransferred to a PVDF membrane. CAT and Mn-SOD proteins were determined by the reaction with a mixture of rabbit anti-human CAT antibodies and rabbit anti-human Mn-SOD antibodies. The immune complexes were made visible by the reaction with ^{125}I-labeled goat anti-rabbit immunoglobulin G, followed by autoradiography (for experimental details, see Materials and Methods).

Growth Properties and DNA Synthesis Capacity of Antioxidant Gene Transfectants in the Presence and Absence of Oxidant Stress. Growth curves were determined for the parent clone 41 and the antioxidant gene transfectants. Monolayer cultures were established by plating 10^5 cells into 10-cm Petri dishes and cell numbers counted after different lengths of growth. The results shown on Figure 6A are for routine culture conditions in an atmosphere of 5% CO_2. With the exception of CAT 4 with a slightly higher growth rate, the parent clone and the transfectants possess similar growth potentials. Addition of 15 or 25 μg/mL xanthine to the culture medium did not affect growth. Panels B and C of Figure 6 show growth curves under oxidative stress generated extracellularly by X/XO, which produces a mixture of $O_2^{\bullet-}$ and H_2O_2. Two doses were used in order to magnify strain differences between the SOD transfectants and the parent strain (Figure 6B) and the CAT transfectants and the parent strain (Figure 6C), respectively. Monolayer cultures were treated 2 days after plating when they reached a density of (4–6) × 10^5 cells per 10-cm Petri dish. After 6 h 15/1.5 μg/mL X/XO (initial rate of $O_2^{\bullet-}$ production of 0.45 nmol/min) had destroyed 81% of the parent clone, 68% of SOCAT 3, 89% of SOD 15, and 94.4% of SOD 3. While the surviving cells of the parent clone and SOCAT 3 possessed normal growth potential, the remaining cells of SOD 15 and SOD 3 failed to grow. Our results indicate that the increase in Cu,Zn-SOD in SOD 15 and SOD 3 resulted in sensitization to oxidative stress and that this sensitization was eliminated in SOCAT 3 by a compensatory increase in CAT. A large difference in sensitivity between the parent and CAT transfectants becomes apparent at a higher dose of X/XO of 25/2.5 μg/mL. Under these conditions 93% of the parent cells were killed within 6 h as compared to 65% for CAT 12 and 51% for CAT 4. Cells which survived the initial toxicity possessed normal growth properties. Qualitatively similar results were obtained at low doses for the survival of colony-forming ability although the quantitative differences between the six cell strains were less

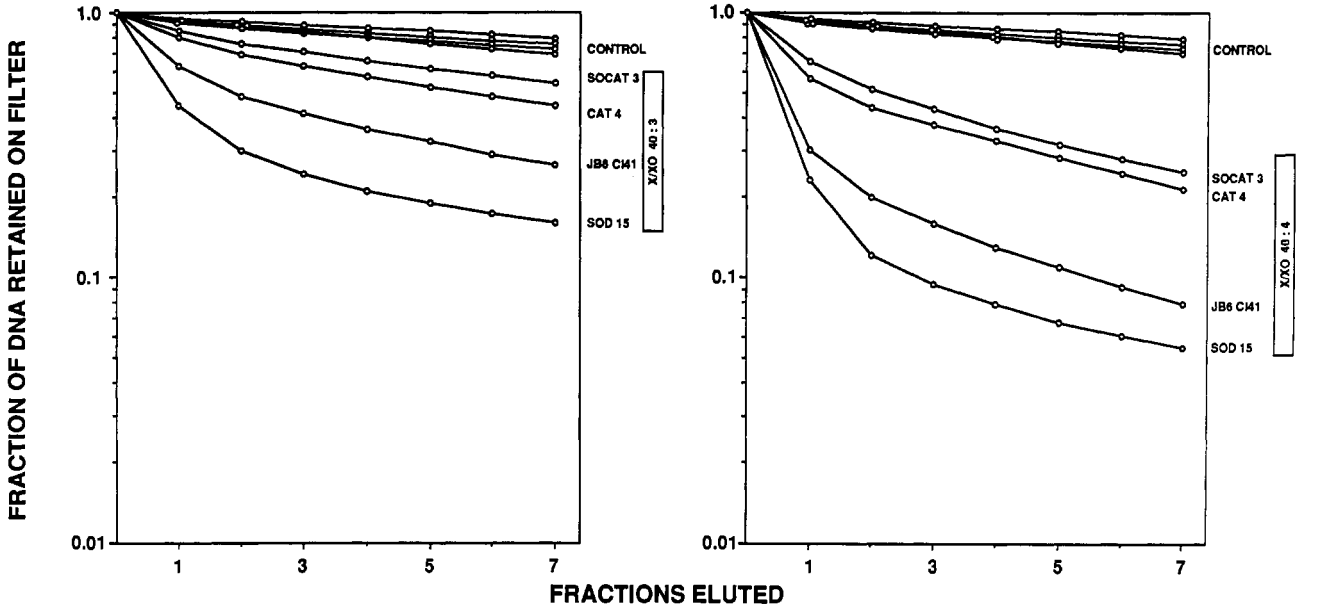

FIGURE 5: Active oxygen induced DNA strand breakage in the parent JB6 clone 41 and the transfectants SOD 15, CAT 4, and SOCAT 3. Monolayer cultures were labeled in their DNA with [^{14}C]thymidine. Before treatment with 40/3 μg/mL X/XO (left side) or 40/4 μg/mL X/XO (right side), respectively, fresh medium supplemented with 8% fetal calf serum was added. Following treatment for 30 min the medium was removed, and the cells were scraped and collected on the filters used for alkaline elution.

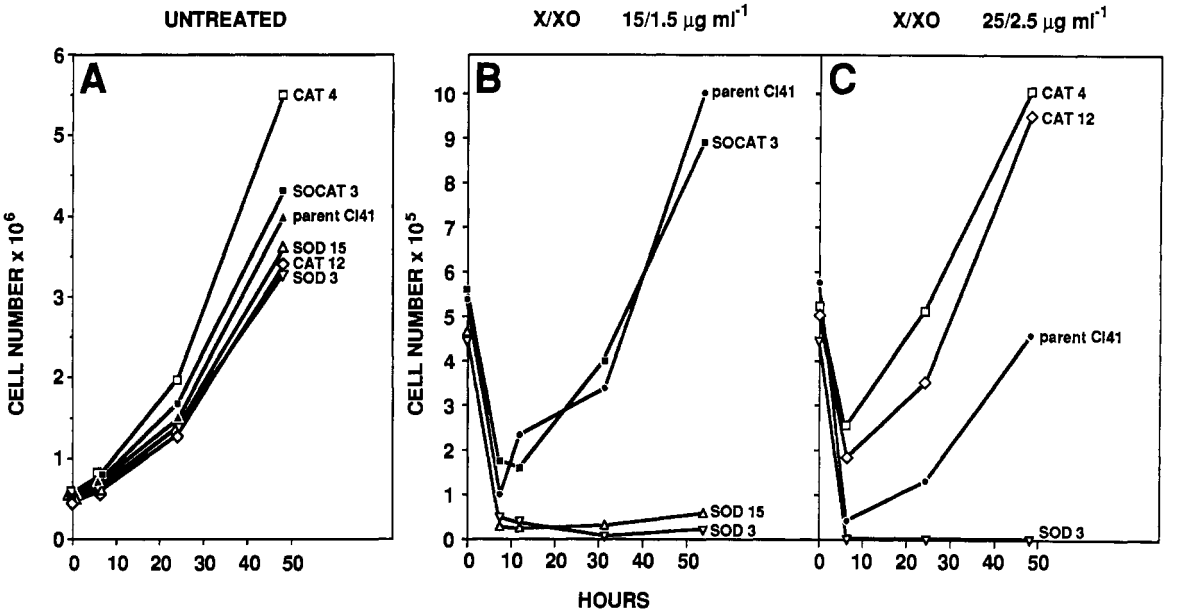

FIGURE 6: Growth curves of the parent JB6 clone 41 and its transfectants under routine culture conditions and following exposure to an extracellular oxidative burst. (A) Monolayer cultures containing (4–6) × 10^5 cells per 10-cm Petri dish were grown in an atmosphere of 5% CO_2 for the indicated lengths of time and cell numbers determined following trypsinization. (B) Monolayer cultures at the indicated cell density were exposed to an extracellular burst of AO produced by 15 μg/mL X and 1.5 μg/mL XO, and growth continued for increasing lengths of time before harvesting and determination of cell numbers. (C) Monolayer cultures at the indicated cell density were exposed to an extracellular burst of AO produced by 25 μg/mL X and 2.5 μg/mL XO, and growth continued for increasing lengths of time before determination of cell numbers (see Materials and Methods).

pronounced than for the growth data reported above (results not shown).

The effect of oxidant stress on the DNA synthesis capacity of the antioxidant gene transfectants was assessed by measuring the incorporation of [^{3}H]thymidine in a 1-h pulse into acid-insoluble material at different lengths of time after exposure to an extracellular oxidative burst. The results were analogous to the growth curves, the relative resistance of the four cell strains being CAT 4 > SOCAT 3 > parent clone 41 ≫ SOD 15. From the protective capacity of intracellular CAT in CAT 4 and CAT 12 and the fact that additional CAT prevented hypersensitivity due to increased levels of Cu,-Zn-SOD in SOCAT 3, we conclude that H_2O_2 is mostly responsible for the cytocidal action of an extracellular burst of $O_2^{\cdot-}$ plus H_2O_2.

Inducibility of c-fos mRNA by Oxidant Stress in Antioxidant Gene Transfectants. The induction of growth-competence-related genes is a necessary prerequisite for growth stimulation. The immediate early gene c-*fos* is a prototype in this regard, and we have shown previously that its transcription is induced in JB6 cells by oxidants (Crawford et al., 1988; Muehlematter et al., 1989). Therefore, we compared the increase in stationary concentration of c-*fos* mRNA following AO treatment in the antioxidant gene transfectants.

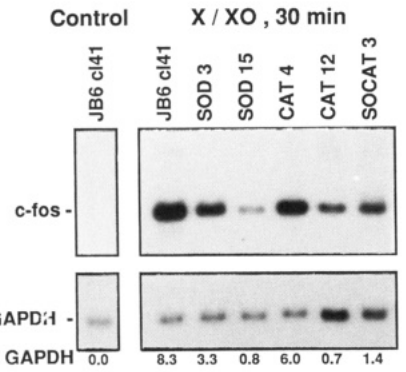

FIGURE 7: Induction of c-*fos* message by an extracellular oxidative burst in the parent JB6 clone 41 and its transfectants. Partially serum-starved monolayer cultures were exposed to AO generated by 20 μg/mL xanthine and 2 μg/mL xanthine oxidase, and growth continued for 30 min before the preparation of total RNA. A labeled transcript of an SP6 recombinant containing in the antisense direction a fragment of v-*fos* DNA was prepared in order to probe the Northern blots. After exposure to an X-ray film the stripped filters were rehybridized with a probe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The autoradiograms of the blots were evaluated by densitometry, and the values for the ratios of c-*fos* mRNA/GAPDH mRNA are listed in the lower portion of the figure.

Figure 7 shows Northern blots with ratios of densitometer readings relative to the reference gene GAPDH. The following order of decreasing inducibility of c-*fos* (c-*fos*/GAPDH) was observed (after 30 min of AO treatment generated by 20 μg/mL X plus 2.0 μg/mL XO): parent clone 41 (8.3) > CAT 4 (6.0) > SOD 3 (3.3) > SOCAT 3 (1.4) > SOD 15 (0.8) = CAT 12 (0.7). While the absolute values for the ratios of c-*fos*/GAPDH varied, the same order of c-*fos* inducibility for the different strains was observed in several experiments.

Discussion

The cellular antioxidant defense is composed of multiple low molecular weight components and several enzymes. Because these molecules interact, complement, interfere, or even compete with each other and act at different sites in the cell, the elucidation of their respective roles is a difficult task. Our present work focuses on the interrelationship of Cu,Zn-SOD and CAT in the protection of mouse epidermal cells JB6 from an extracellular burst of $O_2^{\bullet-}$ plus H_2O_2. This form of oxidant stress mimics the action of phagocytic leukocytes in inflammation which produce a large amount of AO close to the surface of the target cell (Nathan et al., 1979; Simon et al., 1981; Sinet, 1982). We prepared cells with moderate increases in Cu,Zn-SOD, CAT, or both enzymes because only at moderate increases is a condition created which is physiologically meaningful. SOCAT 3 cells with concomitant increases in both Cu,Zn-SOD and CAT were prepared by consecutive transfections with expression vectors containing different antibiotic selection markers rather than cotransfection. Only this more laborious approach allows the unambiguous evaluation of the effects of the introduction of the second antioxidant enzyme, i.e., Cu,Zn-SOD, on the cellular resistance to oxidative stress.

Clones SOD 3 and SOD 15 contain one to two copies of human Cu,Zn-SOD cDNA and clones CAT 4 and CAT 12 two to three copies of CAT cDNA under the direction of strong transcriptional promoters; the double transfectant SOCAT 3, which is derived from CAT 4, contains in addition to CAT cDNA one to two copies of human Cu,Zn-SOD cDNA. From the Northern blots it is evident that the cDNAs are well expressed in the transfectants. However, the high mRNA concentrations do not result in equal increases in enzyme activities. For example, a ratio of 7 of human Cu,Zn-SOD mRNA over endogenous mouse Cu,Zn-SOD mRNA only results in a 3-fold higher total SOD activity in clone SOD 3. These discrepancies may be due to low stability of the Cu,Zn-SOD mRNAs transcribed from the transfected human cDNAs. This is demonstrated in clone SOD 15 in Figure 2 in an experiment with the transcriptional inhibitor actinomycin D. RNase protection analysis of RNA from CAT 12 and CAT 4 indicates a ratio of 4 and 3, respectively, for mRNA transcribed from the transfected gene relative to mRNA from the endogenous gene. These ratios reflect well the increases in CAT activities in these clones relative to parent cells (see Figure 1 and Table I).

Expression of human Cu,Zn-SOD in SOD 15, SOD 3, and SOCAT 3 did not affect significantly CAT activities which remained on the level of the respective parents, i.e., JB6 clone 41 and CAT 4. Similarly, increased CAT activities in CAT 4 and CAT 12 had no major effect on their SOD activities. Judging from the immunoblots shown in Figure 4, neither the expression of human Cu,Zn-SOD nor that of CAT altered the amounts of Mn-SOD protein, and no major differences in Mn-SOD activity were detected in mitochondrial preparations of CAT 4 reltive to the parent clone 41. Similarly, no significant differences were observed in GPx activity for all six cell strains studied in this work. In contrast to our findings, human Cu,Zn-SOD transfectants of mouse L-cells, neuroblastoma cells, and NIH 3T3 fibroblasts possessed increased GPx activities (Ceballos et al., 1988; Kelner & Bagnell, 1990) as did trisomy 21 human fibroblasts with three copies of the Cu,Zn-SOD gene (Sinet, 1982). The concentrations of reduced glutathione were comparable in the parent clone, CAT 4, CAT 12, and SOCAT 3. The fact that they were significantly lower in SOD 3 and SOD 15 may reflect a situation of chronic oxidative stress in these strains.

It is evident from our results that increases in Cu,Zn-SOD and CAT had opposite effects on the sensitivity of JB6 cells to killing, growth inhibition, and the formation of DNA single-strand breaks by an extracellular burst of AO. However, the balance between Cu,Zn-SOD and CAT plus GPx may be more important for sensitivity to AO than the absolute amount of a single antioxidant enzyme. Indeed, at comparable levels of GPx the most sensitive strains SOD 15 and SOD 3 have high ratios of SOD/CAT of 7.2 and 15.8 while the resistant strains CAT 4 and CAT 12 have low ratios of 0.5 and 1.0, respectively (see Table I). Taken together, our data suggest that H_2O_2 represents a major intracellular AO species on the pathways to cytotoxicity and DNA single-strand breakage. However, our results do not imply that DNA is the immediate target for attack by H_2O_2 or its radical derivatives (Cantoni et al., 1989). Sensitization by excess Cu,Zn-SOD appears to be a consequence of overproduction of H_2O_2.

A small extracellular burst of AO can stimulate rather than inhibit the growth of fibroblasts (Zimmerman & Cerutti, 1984; Shibanuma et al., 1988; Murrel et al., 1990) and epidermal cells. In particular, we observed that X/XO stimulated the growth of promotable JB6 clone 41 but not of nonpromotable JB6 clone 30 (Muehlematter et al., 1988). Interestingly, both CAT and Cu,Zn-SOD levels were coordinately increased 2–3-fold on the mRNA and enzyme levels in clone 41 relative to clone 30 (Crawford et al., 1989). At an equal dose, AO induced the growth-competence-related protooncogene c-*fos* more efficiently in the nonpromotable clone 30 (Crawford et al., 1988). The reason for this apparent paradox may be that cells are stimulated to grow when they are protected from excessive AO toxicity as long as a sufficient AO signal remains

to activate the necessary growth pathways. Despite the opposite effects of additional CAT and Cu,Zn-SOD on cytotoxicity by AO, the inducibility of c-*fos* is reduced in both types of transfectants. However, the reasons for the decrease in c-*fos* induction are probably quite different for CAT and Cu,Zn-SOD transfectants. The former are well protected from excessive H_2O_2 toxicity, but at the same time the signal which results in c-*fos* induction is attenuated. We had shown previously that H_2O_2 rather than $O_2^{\bullet-}$ represents the active species for the induction of the translocation to the plasma membrane of protein kinase C (Larsson & Cerutti, 1989) and that CAT rather than SOD inhibited S6 phosphorylation by X/XO in JB6 cells (Larsson & Cerutti, 1988). In contrast, increases in Cu,Zn-SOD levels alone augment the intracellular formation of H_2O_2, and toxic effects on components of the signal transduction pathways may predominate. It should be noted that in the X/XO system used in our work a large amount of $O_2^{\bullet-}$ plus H_2O_2 is generated close to the cell surface. Superoxide may penetrate the membrane sufficiently for dismutation by cytosolic Cu,Zn-SOD. Therefore, enhanced oxidative damage in SOD 15 and SOD 3 may occur preferentially at the plasma membrane. Peroxidatic activity of Cu,Zn-SOD (Hodgson & Fridovich, 1975) may contribute to the peroxidation of membrane lipids. Induction of c-*fos* and other immediate early genes is necessary for the acquisition of growth competence in many types of cells, but it is by no means sufficient for growth stimulation. Oxidants are bound to affect multiple pathways which participate in positive and negative growth regulation. Therefore, it is not astonishing that no simple relationship was observed between the inducibility of c-*fos* and growth response to oxidants for the SOD and CAT transfectants studied in this work.

Our results imply that H_2O_2 is the major damaging species as well as the trigger for pathophysiological reactions for an extracellular burst of AO produced by X/XO (Simon et al., 1981; Link & Riley, 1988). Similarly, the toxic effects of activated inflammatory leukocytes can be mostly attributed to H_2O_2 (Nathan et al., 1979; Simon et al., 1981; Thomas et al., 1986). Evidently epidermal cells such as JB6 are poorly protected from a large extracellular flux of H_2O_2 in part because CAT is located mostly in peroxysomes and the contribution of cytosolic SeGPx is only minor (Michiels et al., 1988). Any further increase in H_2O_2 by excess Cu,Zn-SOD only aggravates toxicity. Reduction of Fe^{III} by $O_2^{\bullet-}$ which might be diminished by SOD does not appear to contribute in a major way to toxicity (Kyle et al., 1988). It should be appreciated that the contributions of CAT and Cu,Zn-SOD to antioxidant defense could be quite different under normoxic conditions or when AO is generated intracellularly. For example, in most cell systems a moderate increase in cytoplasmic SOD was protective against redox-cycling drugs such as paraquat (Elroy-Stein et al., 1986; Krall et al., 1988).

ACKNOWLEDGMENTS

We thank Dr. A. Baret for supplying antibodies against human catalase and manganese superoxide dismutase and Drs. Y. Groner, R. Gravel, and T. Hashimoto for cDNAs of human Cu,Zn-superoxide dismutase and human and rat catalase, respectively.

Registry No. SOD, 9054-89-1; CAT, 9001-05-2; GPx, 9013-66-5; reduced glutathione, 70-18-8.

REFERENCES

Beauchamp, B., & Fridovich, I. (1971) *Anal. Biochem. 44*, 276–287.

Bloch, C., & Ausubel, F. (1986) *J. Bacteriol. 168*, 795–798.

Cantoni, O., Sestili, P., Cattabeni, F., Bellomo, G., Pou, S., Cohen, M., & Cerutti, P. (1989) *Eur. J. Biochem. 182*, 209–212.

Ceballos, I., Delabar, J., Nicole, A., Lynch, R., Hallewell, R., Kamoun, P., & Sinet, P. (1988) *Biochim. Biophys. Acta 949*, 58–64.

Cerutti, P. (1985) *Science 227*, 375–381.

Cerutti, P., & Trump, B. (1991) *Cancer Cells 3*, 1–7.

Cerutti, P., Fridovich, I., & McCord, J., Eds. (1988) *Oxy-Radicals in Molecular Biology and Pathology*, pp 493–507, Alan R. Liss, Inc., New York.

Chen, C., & Okayama, H. (1987) *Mol. Cell. Biol. 7*, 2745–2752.

Chirgwin, J., Przbyla, A., MacDonald, R., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Clairborne, A. (1985) Catalase activity, in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R., Ed.) pp 283–284, CRC Press, Boca Raton, FL.

Cotgreave, I., & Moldeus, P. (1986) *J. Biochem. Biophys. Methods 13*, 231–249.

Crawford, D., Zbinden, I., Amstad, P., & Cerutti, P. (1988) *Oncogene 3*, 27–32.

Crawford, D., Amstad, P., Yin Foo, D., & Cerutti, P. (1989) *Mol. Carcinog. 2*, 136–143.

Elroy-Stein, O., Bernstein, Y., & Groner, Y. (1986) *EMBO J. 5*, 615–622.

Furuta, S., Hayashi, H., Hijikata, M., Miyazawa, S., Osumi, T., & Hashimoto, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 313–317.

Günzler, W., & Flohé, L. (1985) Glutathione peroxidase, in *Handbook of Methods for Oxygen Radical Research* (Greenwald, R., Ed) pp 285–290, CRC Press, Boca Raton, FL.

Hodgson, E., & Fridovich, I. (1975) *Biochemistry 14*, 5299–5303.

Kelner, M., & Bagnell, R. (1990) *J. Biol. Chem. 265*, 10872–10875.

Kohn, K., Erickson, L., Ewig, R., & Friedman, C. (1976) *Biochemistry 15*, 4629–4637.

Korneluk, R., Quan, F., Lewis, W., Guise, K., Willard, H., Holmes, M., & Gravel, R. (1984) *J. Biol. Chem. 259*, 13819–13823.

Krall, J., Bagley, A., Mullenbach, G., Hallewell, R., & Lynch, R. (1988) *J. Biol. Chem. 263*, 1910–1914.

Kyle, M., Nakae, D., Sakaida, I., Micadei, S., & Farber, J. (1988) *J. Biol. Chem. 263*, 3784–3789.

Larsson, R., & Cerutti, P. (1988) *J. Biol. Chem. 263*, 17452–17458.

Larsson, R., & Cerutti, P. (1989) *Cancer Res. 49*, 5627–5632.

Link, E., & Riley, P. (1988) *Biochem. J. 249*, 391–399.

Melton, D., Krieg, P., Rebagliati, M., Maniatis, T., Zinn, K., & Green, M. (1984) *Nucleic Acids Res. 12*, 7035–7056.

Michiels, C., Raes, M., Azchary, M. D., Delaive, E., & Remacle, C. (1988) *Exp. Cell Res. 179*, 581–589.

Muehlematter, D., Larsson, R., & Cerutti, P. (1988) *Carcinogenesis 9*, 239–245.

Muehlematter, D., Ochi, T., & Cerutti, P. (1989) *Chem.-Biol. Interact. 71*, 339–352.

Murrel, G., Francis, M., & Bromley, L. (1990) *Biochem. J. 265*, 659–665.

Nathan, C., Silverstein, S., Brükner, S., & Cohn, Z. (1979) *J. Exp. Med. 149*, 100–113.

Natvig, D., Imlay, K., Touati, D., & Hallewell, R. (1987) *J. Biol. Chem. 262*, 14697–14701.

Norris, K., & Hornsby, P. (1990) *Mutat. Res. 237*, 95-106.
Paoletti, F., Aldinucci, D., Mocali, A., & Caparrini, A. (1986) *Anal. Biochem. 154*, 536-541.
Peterson, G. (1977) *Anal. Biochem. 83*, 346-356.
Phillips, J., Campbell, S., Michaud, D., Charbonneau, M., & Hilliker, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2761-2765.
Scott, M., Meshnik, S., & Eaton, J. (1987) *J. Biol. Chem. 262*, 3640-3645.
Sherman, L., Dafni, N., Lieman-Hurwitz, J., & Groner, Y. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5465-5469.
Shibanuma, M., Kuroki, T., & Nose, K. (1988) *Oncogene 3*, 17-21.
Simon, R., Scoggin, C., & Patterson, D. (1981) *J. Biol. Chem. 256*, 7181-7186.
Sinet, P. (1982) *Ann. N.Y. Acad. Sci. 396*, 83-94.
Thomas, M., Shirley, P., Hedrick, C., & DeChatelet, L. (1986) *Biochemistry 25*, 8042-8048.
Van Beveren, C., van Straaten, F., Curran, T., Müller, T., & Verma, I. (1983) *Cell 32*, 1241-1255.
Zimmerman, R., & Cerutti, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2085-2087.

Tyrosine and Threonine Phosphorylation of an Immunoaffinity-Purified 44-kDa MAP Kinase†

Jean-Claude Scimeca, Robert Ballotti, Tien T. Nguyen, Chantal Filloux, and Emmanuel Van Obberghen*
INSERM U145, Faculté de Médecine, 06034 Nice Cédex, France
Received May 28, 1991; Revised Manuscript Received July 22, 1991

ABSTRACT: We have approached the functioning of a MAP kinase, which is thought to be a "switch kinase" in the phosphorylation cascade initiated from various receptor tyrosine kinases including the insulin receptor. To do so, antipeptide antibodies were raised against the C-terminal portion of ERK1 (extracellular signal-regulated kinase 1), a protein kinase belonging to the family of MAP kinases. With these antipeptide antibodies, we observed the following: (i) a 44-kDa protein can be specifically recognized both under native and denaturing conditions; (ii) a 44-kDa phosphoprotein can be revealed in ^{32}P-labeled cells; its phosphorylation is stimulated by insulin, sodium orthovanadate, and okadaic acid; (iii) a MBP kinase activity can be precipitated, which phosphorylates MBP on threonine residues, and which is stimulated by insulin, sodium orthovanadate, okadaic acid, and fetal calf serum; (iv) this MBP kinase activity appears to be correlated with the in vivo induced phosphorylation of the 44-kDa protein. We next studied the in vitro phosphorylation of this 44-kDa/ERK1-immunoreactive protein. A time- and manganese-dependent phosphorylation was stimulated by the in vitro addition of sodium orthovanadate. Phosphoamino acid analysis of the in vitro phosphorylated 44-kDa protein revealed both threonine and tyrosine phosphorylation. Importantly, this in vitro phosphorylation of MAP kinase results in activation of phosphorylation of added MBP substrate. As a whole, our data indicate that the 44-kDa phosphoprotein identified by our antipeptide antibodies very likely corresponds to a MAP kinase. The observation that the immunoaffinity-purified 44-kDa MAP kinase exhibits in vitro phosphorylation on threonine and tyrosine residues suggests that this MAP kinase possesses both a threonine and tyrosine kinase autophosphorylation activity or that one or more MAP kinase kinase(s) copurify(ies) with the 44-kDa MAP kinase.

A growing list of hormones and growth factors initiate their biological effects by interacting with their specific transmembrane receptors, which carry protein tyrosine kinase activity (Ullrich & Schlessinger, 1990; Yarden & Ullrich, 1988). One of the major goals of the current research focused on cell metabolism and growth concerns the eludication of the mechanism by which these hormones and growth factor receptors stimulate pleiotropic cell responses. For insulin, it is now well established that the earliest post-binding event identified so far is activation of the receptor tyrosine kinase, with ensuing receptor tyrosine autophosphorylation (Gammeltoft & Van Obberghen, 1986; Rosen, 1987). Concurrent with this insulin receptor phosphorylation, the state of phosphorylation of a series of cellular proteins changes, and this concerns for the most part proteins phosphorylated on serine and/or threonine residues (Czech et al., 1988; Pelech et al., 1987; Rosen, 1987). One of the most pressing problems to be solved in this context is the identification of the serine/threonine protein kinases and phosphatases, which are the missing components in the phosphorylation cascade initiated from the insulin receptor tyrosine kinase.

MAP kinase is a serine/threonine kinase transiently activated in many cell types by a variety of extracellular signals such as insulin, EGF,[1] PDGF, FGF, NGF, or TPA (Erickson

† This research was supported by funds from INSERM, Université de Nice, and Association pour la Recherche sur le Cancer (Grant 6760). J.-C. Scimeca is recipient of a fellowship from Ligue Nationale Française Contre le Cancer, Fédération des Comités Départmentaux, Comité Départemental du Var. Dr. T. T. Nguyen is recipient of a postdoctoral fellowship from the Medical Research Council of Canada.
* Corresponding author.

[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; MAP kinase, mitogen-activated protein kinase; TPA, *O*-tetradecanoylphorbol 13-acetate; MBP, myelin basic protein; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PMSF, phenylmethanesulfonyl fluoride; C peptide, ERK1 peptide/sequence 356-367; TLC, thin-layer chromatography; ERK1, extracellular signal-regulated kinase 1; PNPP, *p*-nitrophenyl phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/91/0430-9313$02.50/0