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Activation of AP-1 and of a Nuclear Redox Factor, Ref-1, in the Response of HT29 Colon Cancer Cells to Hypoxia

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Many solid tumors contain substantial fractions of hypoxic cells which are relatively resistant to both radiation therapy and certain cytotoxic drugs. We have previously shown that exposure of human HT29 cells to hypoxic conditions results in the overexpression of certain enzymes involved in the detoxication of xenobiotics, including NAD(P)H:(quinone acceptor) oxidoreductase (DT)-diaphorase, and γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione synthesis. This hypoxic effect on DT-diaphorase was shown to involve both transcriptional induction and altered message stability. We have investigated the effects of hypoxia on elements in the promoter region of DT-diaphorase. Electrophoretic mobility shift assays demonstrate the induction of a binding activity to the AP-1 response element of DT-diaphorase. Supershift assays suggest that this binding is due to AP-1 nuclear factors and that members of the *jun* family are induced to a greater degree than *fos* by hypoxia. Analysis of the kinetics of transcription factor expression indicates that the expression of c-*jun* and *junD* is induced during hypoxic exposure; mRNA levels fall during reoxygenation. Induction of *fos* on the other hand is not as florid during hypoxia (5-fold) and is most pronounced (17-fold) 24 h after the restoration of an oxic environment. Thus, the hypoxic response of DT-diaphorase expression is mediated in part through AP-1, initially by a *jun*-related mechanism and then by the involvement of *fos*. The affinity of transcription factors for the AP-1 binding site depends on the redox state of a cysteine residue located close to the DNA-binding region of both Fos and Jun. A nuclear protein, Ref-1, maintains the reduced state of Fos and Jun and promotes binding to AP-1. Nuclear extracts of HT29 cells exposed to hypoxia show markedly increased Ref-1 protein content. Elevation of *ref-1* steady-state mRNA levels occurs as an early event following induction of hypoxia and persists when cells are restored to a normally oxygenated environment. Nuclear run-on analysis demonstrates that induction of transcription is the mechanism of *ref-1* mRNA elevation. Electrophoretic mobility shift assays and immunodepletion assays were used to further define the interaction of Ref-1 with specific AP-1-binding proteins under hypoxic conditions. These data demonstrate that the induction of detoxicating enzyme expression in HT29 cells exposed to hypoxia results from the induction of both transactivating factors that bind to the AP-1 element and of redox proteins that enhance their affinity for this element.

Exposure of mammalian cells to environmental stress triggers specific genetic responses that are likely to have evolved to protect the cell from permanent damage or death (19). The identity of the genes that are induced depends on the nature of the stress to which the cell is exposed. Heat shock, UV radiation, heavy metal ions, oxidative stress, and hypoxic stress each induce a particular set of genes, although there is substantial overlap among them (25). Some responses such as those to heat shock and UV radiation have been extensively studied, and the regulatory mechanisms involved are beginning to be elucidated (18, 23, 29, 34). However, relatively little is known about the molecular basis for the response to hypoxic stress.

Hypoxia has long been recognized as a determinant of the resistance of tumors to cytotoxic chemotherapy and radiation (6, 24). Hypoxic cells are some threefold-less sensitive than oxic cells to X-irradiation, a condition that has been attributed to diminished formation and altered half-times of reactive oxygen radicals in an hypoxic environment (2, 5). A similar degree of resistance has been observed for many cytotoxic agents, particularly those which form radicals intracellularly (40). The common solid tumors, which are most resistant to chemotherapy, have substantial hypoxic fractions in vivo (30). Direct measurements in human tumors have confirmed these findings (20), and markers of hypoxia suitable for broad clinical use have been described (22). Most cytotoxic drugs are detoxified through bioreductive processes or through conjugation to glutathione. We have previously shown that exposure of colon tumor cells to hypoxia results in an increase in the activity of the two-electron bioreductive enzyme NAD(P)H:(quinone acceptor) oxidoreductase (DT)-diaphorase and in the cellular content of glutathione (31). These findings prompted the further investigation of mechanisms of hypoxic cell resistance to cytotoxic drugs.

The elevation of DT-diaphorase activity following hypoxia was shown to result both from transcriptional induction and increased message stability. While catalytic enzyme activity did not increase for some hours following the hypoxic exposure, induction of mRNA transcription began within 2 h of establishing the hypoxic environment (31). Studies on the DT-diaphorase promoter region in rats (16) and humans (28) have identified elements that mediate response to xenobiotics and to antioxidants. The human, but not the rat, antioxidant response element contains an AP-1-binding sequence (17). Nuclear factor binding to this element has been identified following treatment of HepG2 hepatoma cells with β-naph-

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thaflavone (17, 28), and the selective induction of Jun binding to AP-1 is an early event in the mammalian UV response (14).

The affinity of various transcription factors involved in stress responses (including AP-1, NF-κB, and factors binding to the serum response element) for their cognate DNA sequences depends on the redox state of specific cysteine residues. For example, in Fos and Jun the critical cysteine residue is located in the DNA-binding domain (1). Consequently, chemical oxidation or modification of this residue inhibits DNA-binding domain (1). Consequently, chemical oxidation or modification of this residue inhibits DNA-binding activity (1). Mutation of the cysteine residue to a serine augments the transforming potential and DNA binding of Fos in vivo (32). A bifunctional protein, Ref-1, that, in the absence of chemical reducing agents, maintains the reduced state of these cysteines, thereby enhancing DNA binding, has been identified (44, 46). Ref-1 also possesses an endonuclease activity involved in the repair of DNA lesions caused by oxidative damage (33, 46). However, the redox and repair activities are encoded by distinct regions of the protein and at least in vitro can function independently of each other (43, 45). Interestingly, in analyzing the potential role of AP-1 binding in the response to hypoxia, we found that the alteration in Ref-1 expression, like that of AP-1, is an early event in this response.

In this paper, we present data that implicate nuclear factor binding to the AP-1 response element in the response of colon tumor cells to hypoxia. The findings suggest that the selective induction of Jun-Jun homodimers is an early event in this response. Potentiation of the interaction is suggested by the finding that marked induction of transcription of the redox factor Ref-1 is also an early event. These results identify the AP-1 binding site as an important component of the response to hypoxia.

MATERIALS AND METHODS

Cell culture. The human colon adenocarcinoma cell line HT29 was grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. The cells were grown in 500-ml glass milk dilution bottles (Corning Glass works, Corning, N.Y.) at 37°C in 5% CO_2–95% air. Cells were passaged weekly and have a doubling time of 20 h.

Hypoxic exposure. Exposure of cells to a hypoxic environment was performed as described previously (31). Single-cell suspensions were plated at high density (1.5×10^6 cells per bottle) and allowed to reach 30 to 40% confluence within 1 to 2 days. Flasks for hypoxic exposure were sealed with rubber stoppers and insufflated through steel needles with O_2-poor (<1 part per 10 billion) N_2 for 2 h. Following removal of the gas supply, the flasks remained sealed for a further 6 h. The hypoxic exposure was terminated by replacing the rubber stoppers with conventional caps. Cells were harvested at various times during and after the hypoxic exposure. Following an 8-h exposure to hypoxia, HT29 cells are 86% viable as measured by trypan blue exclusion. Oxic cells were harvested in parallel as controls.

Isolation and analysis of RNA. Total cellular RNA (20 μg) was isolated by a single-step acid guanidium isothiocyanate-phenol-chloroform extraction procedure (9), subjected to electrophoresis in a 1% agarose–2.2 M formaldehyde gel, transferred onto nylon membranes (Magna NT; MSI, Westboro, Mass.), and hybridized to ^{32}P-labeled DNA probes prepared by multiprimer labeling (Amersham) with a specific activity of $>1.5 \times 10^8$ cpm. The probes employed were as follows: (i) the 1.4-kb *Eco*RI insert of a human DT-diaphorase cDNA probe from the pDTD41 plasmid (47); (ii) the 1.8-kb *Bam*HI-*Eco*RI insert of a human c-*jun* probe containing a 1.0-kb cDNA and 0.8-kb 3′-untranslated sequence purified from a Bluescript SK(+) plasmid (3); (iii) the 3.1-kb *Xho*I-*Nco*I fragment of a human c-*fos* DNA purified from the pc-fos-1 plasmid (13); (iv) the 1.0-kb *Xho*I fragment of a human *junD* DNA from a pBS *junD* plasmid (36); (v) the 1.4-kb *Eco*RI insert of *ref-1* cDNA from the pCB6+ -Ref-1 plasmid (46); and (vi) the 2.0-kb *Pst*I insert of a chicken β-actin cDNA from plasmid pA1 (10). Hybridization was carried out overnight at 42°C in a solution containing 50% formamide, 5× SSPE (1× SSPE is 180 mM NaCl, 10 mM sodium phosphate, 1 mM Na_2 EDTA, pH 8.0), 2× Denhardt's reagent (2× Denhardt's reagent consists of 0.02% bovine serum albumin and 0.02% polyvinylpyrrolidine in 0.2% Ficoll), and 0.1% sodium dodecyl sulfate (SDS). The washing conditions were as follows: 20 min at room temperature in 1× SSC–00.1% SDS, 20 min at 55°C in 0.2× SSC–0.1% SDS, and 20 min at 55°C in 0.1× SSC–0.1% SDS. Autoradiography was carried out at −70°C for 3 to 7 days. The blots were subsequently stripped and reprobed. The intensity of β-actin labeling was used in normalizing values to provide a measure of the relative expression of the mRNA of interest.

Nuclear run-on assay. Nuclei from hypoxia-treated or oxic control cells were prepared as described by Celano et al. (8). The cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped into buffer A (20 mM Tris HCl [pH 7.4], 10 mM NaCl, 3 mM Mg Cl_2) following which they were made 0.1% by volume with Nonidet P-40. Cells were vortexed, and the plasma membrane was lysed in a sterile Dounce homogenizer on ice. The nuclei were pelleted at $1,000 \times g$ for 10 min at 4°C, washed in cold buffer A, and counted. The nuclear pellet was resuspended in transcription buffer (35% glycerol, 10 mM Tris HCl [pH 7.5], 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA) and stored at −70°C. Following thawing, the run-on assay was carried out as described by Greenberg (8, 21). The assay was conducted with 10^8 nuclei per reaction mixture in a total volume of 200 μl in transcription buffer with a 4 mM concentration (each) of ATP, GTP, and CTP and 200 μl of [α-^{32}P]UTP (3,000 Ci/mM; Amersham, Arlington Heights, Ill.) at 26°C for 10 min. Nuclei were digested with 10 μl of RNase-free DNase I and 10 μl of 20 mM $CaCl_2$ at 26°C for 5 min. Samples were then treated with 2 μl of proteinase K (10 mg/ml), 15 μl of 10× SET (5% SDS, 50 mM EDTA, 10 M Tris HCl [pH 7.4]), and 5 μl of yeast tRNA (10 mg/ml) at 37°C for 30 min. Nuclear RNA was isolated by the guanidium-phenol-chloroform procedure described above. Finally, the RNA was dissolved in sterile Tris-EDTA with 0.1% SDS. The DNA probes c-*DTD*, c-*jun*, c-*fos*, *ref-1*, and β-*actin* (2 μg of DNA per blot) used in the run-on assay were denatured and blotted onto a prewet nylon slot filter membrane in 6× SSC and allowed to dry at room temperature. The membrane was baked at 80°C for 2 h in a vacuum oven. After prehybridization of the membrane at 42°C for several hours, the [α-^{32}P]-labeled nuclear RNA in 3 ml of hybridization buffer was added to the filter and hybridized for 24 h at 42°C. The filter was washed in 2× SSC–1% SDS at 65°C for 1 h, and then in 0.1× SSC–0.1% SDS at room temperature for 1 h. Autoradiography was performed at −70°C, and quantitation of the results was achieved by densitometric scanning normalized to the signal for β-actin.

Nuclear extract preparation. The nuclear extracts were prepared by the procedure of Dignam et al. (15) as modified by Benjamin and coworkers (4). Cells were harvested as described above. The cells were resuspended in 1.5 volumes of lysis buffer containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 70 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM sodium orthovanadate, 0.4 mM NaF, 0.5 mM phenyl-

methylsulfonyl fluoride, and 1.0 mM dithiothreitol. The mixture was incubated on ice for 20 min and then extracted by adding 1.6 volumes of extraction buffer containing 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 20% glycerol, 1.66 M KCl, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreitol with constant shaking at 4°C for 4 to 5 h. Samples were centrifuged at 55,000 × *g* for 1 h at 4°C. The supernatant was dialyzed at 4°C for 4 to 6 h in 20 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 mM dithiothreitol. Samples were stored at −80°C. Protein content was assayed by the Bradford assay (Bio-Rad, Richmond, Va.).

Western blotting (immunoblotting). The nuclear extract proteins (20 μg) were loaded on a SDS–12% polyacrylamide gel, electrophoresed, and transblotted to an Immobilon (polyvinylidene difluoride) filter by the procedure described by Towbin et al. (42). After a rinsing with PBS-Tween (0.5% [vol/vol]), the blot was incubated with Ref-1 antiserum (1:4,000 in PBS-Tween) overnight at room temperature on a rotator. The blot was rinsed thrice with PBS-Tween, and incubated with a 1:4,000 dilution of second antibody coupled with calf intestine alkaline phosphatase for 2 h at 37°C on a rotator. The blot was washed thrice with Tween-PBS and once with 50 mM sodium glycine (pH 9.6) and then incubated with phosphatase staining reagents: 0.1 ml of 5-mg/ml 5-bromo-4-chloro-3-indolylphosphate in dimethylformamide, 1.0 ml of freshly prepared *p*-nitroblue tetrazolium chloride in 1 mg of 50 mM sodium glycine (pH 9.6) per ml, and 8.36 ml of 50 mM sodium glycine (pH 9.6), to generate alkaline phosphatase activity. The purple product was developed for 3 to 10 min and then stopped by a washing in H_2O.

Oligonucleotide labeling. The 31-bp DT-diaphorase AP-1 consensus and mutant oligonucleotides were synthesized by the Oligonucleotide Synthesis Facility at the Fox Chase Cancer Center. The AP-1 sequence contained a native consensus sequence -TAGCTCA-. The mutant contains the sequence -TGACTTG-. The sequence of the DT-diaphorase AP-1 oligonucleotide was determined in other studies (26); a 31-bp oligonucleotide located at −442 to −473 in the 5′-flanking region was used. cDNA strands were purified and annealed by standard procedures (38). The double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP by phosphorylation with bacteriophage T4 polynucleotide kinase and then ethanol precipitated to remove the bulk of the unincorporated radioactivity.

Electrophoretic Mobility Shift Assay (EMSA). The nuclear extracts were analyzed for AP-1-binding activity by gel mobility shift assays. The binding reaction mixture containing 10 μg of nuclear extract and 1.8 μg of poly(dI-dC) in a 30-μl final volume of binding buffers containing 20 mM HEPES (pH 7.5), 40 mM KCl, 1.0 mM $MgCl_2$, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.5 mM dithiothreitol. Each reaction mixture contained 15,000 cpm of a 31-bp double-stranded AP-1 consensus oligonucleotide. The reaction mixture was allowed to stand for 25 min at room temperature. After the addition of 5 μl of loading buffer (20% Ficoll, 0.25% bromophenol blue), 15-μl samples were loaded onto a 4% polyacrylamide gel and electrophoresed at 4°C for 2 h at 200 V. The gel was dried under vacuum and exposed to X-ray film overnight at −70°C.

Supershift assay. The nuclear extracts were preincubated with antiserum (1:50 in PBS at 4°C for 2 h before analysis by EMSA as described above. The human anti-c-Jun and anti-c-Fos sera were obtained from Upstate Biotechnology, Inc.; the human anti-JunD serum was obtained as a kind gift of K. Ryder. These sera specifically detect the presence of the corresponding transcription factor and do not interfere with nuclear factor binding.

Immunodepletion assay. Immunodepletion was conducted by a standard procedure (11). The nuclear extract was incubated with Ref-1 rabbit anti-serum at 4°C for 1 h with gentle shaking. After immunocomplexes were cleaned by immunoprecipitation, the antibody-antigen complex was used for SDS-polyacrylamide gel electrophoresis and subsequent silver stain analysis. The cleaned supernatant was used in EMSA for stimulation of AP-1 DNA-binding activity as described above.

RESULTS

Jun and Fos induction by hypoxia. The *cis*-regulatory region of the DT-diaphorase promoter region includes an AP-1-binding site which appears to be the major response element mediating the UV response (14). UV irradiation induces expression of the immediate-early genes c-*jun* and c-*fos*, the former to a greater extent than the latter (14). Previous work in our laboratory indicated that DT-diaphorase enzyme activity was markedly increased by hypoxia (31). To determine whether expression of *jun* and *fos* are involved in DT-diaphorase induction by hypoxic stress, HT29 cells were exposed to hypoxia for 8 h, and total RNA was harvested at various time points after hypoxia. As shown in Fig. 1A, the expression of c-*jun* and *junD* increased approximately 30-fold upon exposure to hypoxia. c-*jun* and *junD* transcripts were significantly elevated at the end of hypoxic exposure, following which levels decreased slowly and had almost returned to baseline at 48 h. c-*fos* expression was also induced by hypoxic exposure but with a different pattern. After the 8 h of hypoxia, c-*fos* mRNA content was elevated approximately fivefold. The expression of c-*fos* then increased gradually to reach a peak at 24 h, after which levels decayed to baseline at 48 h. The maximal induction was 17-fold at 24 h. The differing responses of c-*jun*, *junD*, and c-*fos* to hypoxic exposure is shown clearly by densitometric quantitation of the hybridization signal (Fig. 1B).

To distinguish between transcriptional and posttranscriptional mechanisms, we estimated the rate of RNA synthesis by using the nuclear run-on assay (Fig. 2). Consistent with the steady-state mRNA expression findings, a marked increase in the rate of transcription of RNA encoding c-*jun* and c-*fos* was observed. The time course of the induction of transcription was also consistent: transcription of c-*jun* occurred early, while that of c-*fos* was delayed and less extensive than that of c-*jun*. The run-on assay indicated a persistently high rate of transcription of c-*jun* in the face of declining steady-state mRNA levels at 24 h: a shorter half-life of the c-*jun* message may accompany reoxygenation. Induction of the target enzyme DT-diaphorase is also delayed. Therefore, hypoxia appears to stimulate in particular the transcription of c-*jun*, which may mediate the transcriptional effects of hypoxia on other genes.

Induction of AP-1 binding by hypoxic exposure. To determine the characteristics of Jun and Fos complex binding to the AP-1 motif in hypoxic cells, we performed EMSA with the AP-1 binding site located in the DT-diaphorase promoter region. Induction of factor binding was found as an early event immediately after hypoxic exposure, and binding activity was present continuously up to 24 h (Fig. 3). The AP-1-binding activity was effectively inhibited by an excess of unlabeled AP-1 oligonucleotide but not by a mutated oligonucleotide (Fig. 3).

The degree and nature of the response to the binding of dimers to the AP-1 element may relate both to the nature of the complex induced and to its affinity for binding. To characterize further the proteins binding to the AP-1 site under

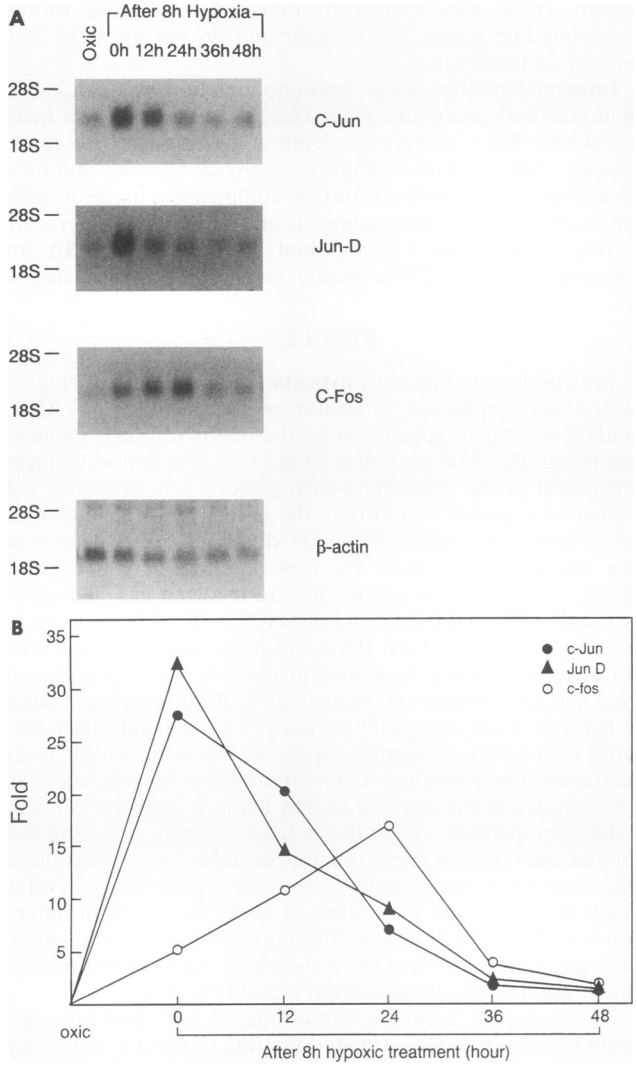

FIG. 1. Induction of expression of immediate-early gene mRNA of HT29 colon adenocarcinoma cells by hypoxia. (A) Northern blot analysis of HT29 cells treated by 8 h of hypoxia. Total cellular RNA (15 μg) was isolated and hybridized to the ^{32}P-labeled c-*jun*, *junD*, and c-*fos* probes. Hybridization to the β-actin probe as an internal control demonstrated equal loading of RNA. (B) Comparison of the induction kinetics of c-Jun, JunD, and c-Fos by hypoxia. The autoradiogram shown in panel A was quantitated by laser densitometry, and the relative induction of c-Jun, JunD, and c-Fos relative to the level of the β-actin signal was determined.

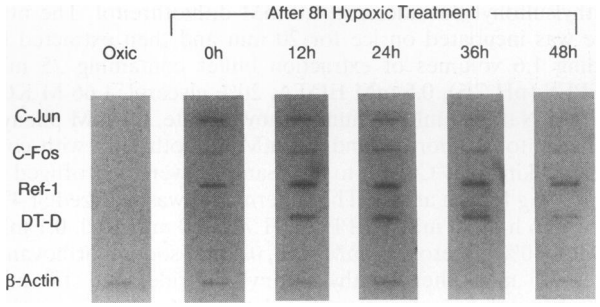

FIG. 2. Time course of nuclear run-on assay of the c-*jun*, c-*fos*, *ref-1*, and DT-diaphorase genes in hypoxia-treated and untreated human HT29 colon cells. RNA transcripts from hypoxia-treated and untreated HT29 nuclei hybridizing to c-Jun, c-Fos, Ref-1, DT-diaphorase II (DT-D), and β-actin cDNA inserts are shown.

hypoxic conditions, we used supershift assays with antibodies specific to Fos, Jun, and JunD. The results (Fig. 4) show that the dimers binding to the AP-1 site at 24 h following exposure to hypoxia are composed of members of both the *jun* and *fos* families. Both Jun- and JunD-containing dimers are observed. Jun-Fos heterodimers demonstrate a less intense signal, but additional studies are required to elucidate possible selective factor binding.

Ref-1 stimulates the DNA-binding activity of AP-1 transcription factors under hypoxia. Redox regulation of Fos-Jun DNA-binding activity is mediated through a conserved cysteine residue present in the DNA-binding domain, which is flanked by a sequence of basic amino acids (KCR) (44, 46). Preliminary experiments (46) indicate that Ref-1 might stimulate the DNA-binding activity of several transcription factors including AP-1 and NF-κB. To investigate the role of Ref-1 in the response of DT-diaphorase to hypoxia, we examined the expression of Ref-1 in cells exposed to hypoxia. Hypoxic exposure increased *ref-1* steady-state levels of mRNA (Fig. 5). As with c-*jun* and *junD*, the elevation was an early event, preceding that of DT-diaphorase. The mechanism of *ref-1* overexpression was investigated by using the nuclear run-on assay (Fig. 2). Pronounced induction of *ref-1* was an early event which occurred with a time course parallel to that of c-*jun*.

Using a specific Ref-1 antibody, we analyzed further the Ref-1 protein content of HT29 cells under these conditions, by

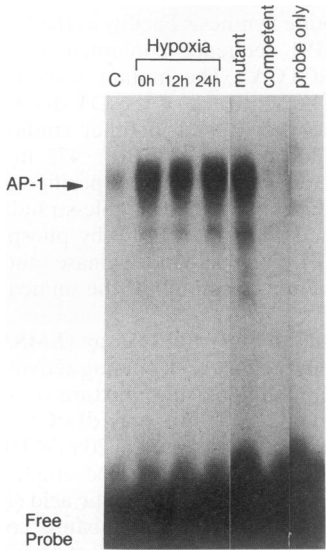

FIG. 3. EMSA of AP-1-binding activity in protein extracts from cultured human HT29 cells exposed to oxic or hypoxic conditions and harvested 24 h after restoration of an oxic environment in the latter. EMSAs were performed by using a ^{32}P-labeled synthetic double-stranded oligonucleotide containing consensus DT-diaphorase AP-1 as the probe. The hypoxia-inducible DNA-protein complexes formed after expression of the cells to hypoxia are indicated (arrow). The unbound (free) probe in the gel is indicated at the bottom. Stress-inducible DNA-binding activity was abolished by competition with a 100-fold molar excess of unlabeled natural AP-1 oligonucleotide (lanes competitor) but not by an identical concentration of oligonucleotide containing a mutant DT-diaphorase AP-1 (lanes mutant).

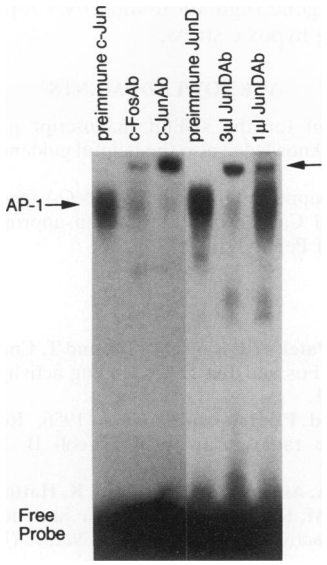

FIG. 4. Supershift assay of DT-diaphorase AP-1-binding activity in HT29 nuclear extracts of hypoxia-treated HT-29 cells harvested 24 h after restoration of an oxic environment. Preincubation of 10 μg of nuclear extract from HT29 cells treated with hypoxia with preimmune c-Jun (control), JunD (control), and anti-c-Fos, anti-c-Jun, and anti-JunD sera before EMSA resulted in a supershifted band (arrow) consistent with the formation of DNA-protein-antibody (Ab) complexes.

Western blot (Fig. 6). Consistent with the Northern (RNA) analysis (Fig. 4), marked overexpression of Ref-1 protein was evident at the end of the hypoxic exposure. However, despite persistently elevated mRNA, protein content decayed upon reintroduction of the cells to an oxic environment. Ref-1 levels did not return to those of baseline oxic cells, even at 72 h.

To verify the functional role of Ref-1 protein in enhancing factor binding to the AP-1 response element under these conditions, we examined nuclear extracts of hypoxic HT29 cells at the end of an 8-h hypoxic exposure. In an EMSA it could be

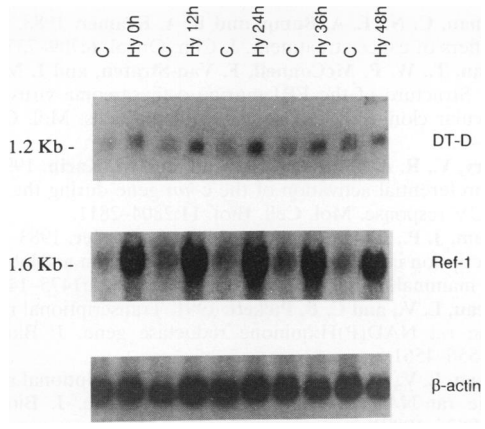

FIG. 5. Effects of hypoxia on expression of DT-diaphorase (DT-D) and Ref-1 genes in human HT29 cells. Shown is total RNA from control (lanes C) and hypoxic (lanes Hy) cells hybridized to human DT-diaphorase and Ref-1 cDNA probes, respectively. A β-actin probe was used as a control. The times indicated above are the duration of incubation under oxic conditions after an 8-h hypoxic treatment.

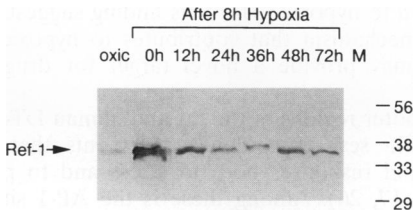

FIG. 6. Western blot analysis of Ref-1 protein in HT29 cells exposed to 8 h of hypoxia and restored to an oxic environment. A total of 10 μg of cell protein isolated from control and treated HT29 cells were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-Ref-1 sera. Lane oxic, control cell extract. M, molecular size (in kilobases) markers.

shown that factor binding to a human DT-diaphorase-derived AP-1 element was induced by hypoxia (Fig. 7). After removal of Ref-1 from the nuclear extract by immunodepletion with a specific anti-Ref-1 antibody, the AP-1-binding activity was dramatically reduced in a manner dependent on the amount of anti-Ref-1 antibody used. This finding implicates Ref-1 in the AP-1-mediated response of HT29 cells to hypoxia.

DISCUSSION

Human solid tumors, even those less than 1 cm in diameter (i.e., at the limits of clinical detection), may have substantial hypoxic fractions that limit the effectiveness of treatment (6). Hypoxic cells are less sensitive to both radiation and cytotoxic therapy (12, 27, 37, 40, 41). We have previously shown that exposure to hypoxia results in the overexpression of several detoxication enzymes, many of which are associated with resistance to cytotoxic drugs (31). Both transcriptional induction and altered message half-life contributed to elevated steady-state DT-diaphorase mRNA content in colon tumor

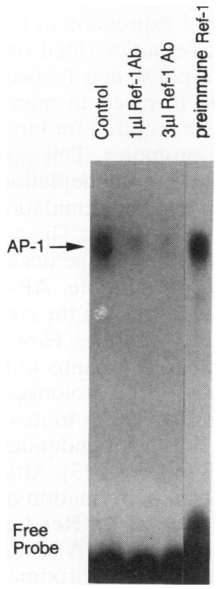

FIG. 7. Effects of immunodepletion of Ref-1 on AP-1-binding activity in the nuclear extract of HT29 cells treated by an 8-h hypoxic exposure. The nuclear proteins were immunodepleted by different amounts of anti-Ref-1 sera as noted. The clear supernatant was used for EMSA to AP-1. The control lane contained preimmune serum.

cells exposed to hypoxia (31). This finding suggests a possible molecular mechanism that contributes to hypoxic cell resistance and may provide a novel target for drug resistance reversal.

The promoter regions of the rat and human DT-diaphorase genes contain several ubiquitous elements that appear to mediate rapid responses both to stress and to proliferative stimuli (16, 17, 26). Among these is the AP-1 site to which binds homodimers of the Jun family and Fos-Jun heterodimers. In human colon tumor cells, *jun* family genes were induced early by hypoxia. Elevation of c-*fos* was a later event based on Northern analysis (Fig. 1B) and on the nuclear run-on analysis (Fig. 2). The later generation of Fos-Jun heterodimers either may alter the kinetics of DT-diaphorase expression or may expand the repertoire of target genes.

The role of the *jun* family in mediating responses to hypoxia was supported further by the demonstration that expression of both c-*jun* and *junD* occurs early in the process. In this aspect, the response to hypoxia is similar to that exerted by DNA damage following UV irradiation or treatment with alkylating agents (19). Additional studies have shown that DNA-damaging agents result in the selective induction of c-*jun* but not *junD* (14). A recent analysis of the response of the c-*jun* promoter to UV irradiation with dimethyl sulfate and DNase I in vivo footprinting suggests that AP-1 sites are already occupied in unstimulated cells (35). Similar observations on the antioxidant response element of the rat quinone reductase gene have been made by Favreau and Pickett (17). However, given that AP-1 family members share similar DNA-binding specificities, these studies cannot determine whether the composition of AP-1 dimers occupying these promoters in unstimulated and stimulated cells is the same. The present work suggests that multiple AP-1 complexes are present in cell extracts derived from cells exposed to hypoxia and that these complexes are capable of interacting with the DT-diaphorase AP-1 element in vitro.

Superimposed on the alterations in the pattern of AP-1 gene expression following hypoxic stress is the rapid induction of *ref-1* transcription and the prolonged expression of the Ref-1 protein. Changes in *ref-1* expression in response to hypoxia or other stimuli have not been described previously. The role of Ref-1 in the hypoxic response may be twofold. Given its redox activity, Ref-1 may be involved in maintaining the reduced state of cysteine residues required for binding of AP-1 proteins to the DT-diaphorase promoter. This possibility is supported by the observation that immunodepletion of Ref-1 from cell extracts, prepared after hypoxic stimulation, inhibits binding to the DT-diaphorase AP-1 element. The continued presence of Ref-1 following its induction may be necessary to maintain the DNA-binding activity of multiple AP-1 proteins that are required at different stages during the course of the transition between the hypoxic and oxic states. However, the involvement of Ref-1 in such interactions remains to be elucidated.

Another possible role for the prolonged expression of Ref-1 following hypoxic exposure relates to its DNA repair function, Ref-1 is identical to HAP-1, an endonuclease that recognizes and cleaves abasic sites in DNA (43). Although it is not known whether hypoxia results in depurination of DNA, it is conceivable that persistent high levels of Ref-1 endonuclease activity are required for the repair of DNA lesions caused by reintroduction of the cells in an oxic environment.

Finally, the induction of an endonuclease activity in rat cells exposed to prolonged, severe anoxia has previously been identified and was postulated to underlie the genomic instability of cancer cells (39). The consequences of constitutive overexpression of Ref-1 have not been described. Thus, Ref-1 may coordinate gene regulation and DNA repair processes in cells undergoing hypoxic stress.

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