A General Role for c-Fos in Cellular Protection against DNA-damaging Carcinogens and Cytostatic Drugs

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ABSTRACT

One of the earliest responses of cells upon exposure to DNA-damaging agents is the induction of c-fos. To elucidate the biological role of Fos expression, we analyzed cells deficient in c-Fos upon treatment with different DNA-damaging agents, including carcinogens and antineoplastic drugs. We show that cells lacking c-Fos are hypersensitive with regard to reproductive cell death, apoptosis, and chromosomal breakage after treatment with agents inducing methylation lesions, bulky adducts, or cross-links in DNA. They were not significantly hypersensitive to ionizing radiation. The activities of various repair enzymes and glutathione S-transferase and the level of proliferating cell nuclear antigen were not altered in c-fos-deficient fibroblasts. Furthermore, the cells were able to remove the main methylation lesions from DNA. c-Fos-deficient cells exhibited a more severe mutagen-induced block to DNA replication and were compromised in the abolition of replication blockage. The data provide compelling evidence that c-Fos/activator protein-1 plays a decisive and general role in cellular defense against genotoxic agents, which require DNA replication to induce chromosomal instability. They are consistent with the hypothesis that impaired recovery from DNA replication inhibition upon mutagen exposure is causally involved in c fos-hypersensitivity.

INTRODUCTION

Mammalian cells respond, upon exposure to DNA-damaging agents, with the activation of preexisting cellular functions and the induction of genes. A very early response of cells upon genotoxic stress is based on the activation of Ras, Raf, and mitogen-activated protein kinase, as well as elements of the Rac and Jun-kinase pathway (1–4), resulting in activation of the heterodimeric (Fos/Jun) and homodimeric (Jun/Jun) transcription factor AP-1 (5) and transcriptional activation of c-fos, c-jun, and, to lesser extent, jun B and jun D (6–8). Downstream targets of induced AP-1 are various genes harboring AP-1 sites in their promoter. They include metallothionein, collagenase, and stromelysin, which respond to AP-1 induction by elevated expression (for review, see Ref. 9). Another pathway subject to induction upon genotoxic stress targets the tumor suppressor protein p53, which becomes stabilized (10) and, in turn, is involved in the induction of p21 and other p53-dependent genes (11, 12).

The AP-1 complex and, thus, c-Fos are involved in a number of basic metabolic processes, such as regulation of cell proliferation (13), differentiation (14), tumorigenesis (15), and possibly also apoptosis (16), making it very likely that a large number of genes are regulated via Fos/Jun. A bulk of data are available showing that c-fos is transcriptionally up-regulated by different factors, such as growth factors, cytokines, and tumor promoters (9), as well as by a diversity of DNA-damaging agents including UV light (17), IR (18), and alkylating agents (8, 19–21). The fact that proteins are activated and genes are induced by different DNA-damaging agents indicates that the observed responses are emergency reactions that serve to increase survival of cells and maintain chromosomal integrity under genomic stress conditions. Clear evidence, however, for this reasonable supposition is available only for a few cases. Thus, induction of the DNA repair gene MGMT in rat liver cells has been proven to result in increase in the cellular capacity of repairing O6-alkylguanine and protection of cells from the mutagenic and genotoxic effects of various nonfunctional alkylating agents (22, 23). Induction of p53 and p21 was shown to elicit G1-S arrest (24) and, thus, may act protectively, at least for treatment with agents with genotoxic activity that is related to DNA replication because more time for prereplicative repair of critical DNA lesions would be available.

Whether activation of the Ras/Raf/mitogen-activated protein kinase pathway targeting the immediate-early genes of the fos and jun family is of biological relevance, e.g., by being involved in cellular defense against genotoxic stress, remained unknown for a long time despite increasing insights gained into the signal transduction mechanisms. A significant step toward an understanding of the physiological role of immediate-early-induced functions upon genotoxic stress was the finding that cells lacking c-Fos are hypersensitive to the cytotoxic, DNA breakage-inducing, and clastogenic effects of UV-C irradiation (25, 26), indicating that c-Fos plays a role in cellular defense. However, whether the hypersensitivity of c-Fos-deficient cells is a unique peculiarity observed only after exposure to UV-C or whether it is a general response to DNA-damaging agents remained an unanswered question. Here, we report that cells deficient in c-Fos are hypersensitive to a broad spectrum of chemical mutagens, including tumor-therapeutic agents that differ in their mode of action and DNA-damaging properties but are all bound on DNA replication in eliciting genotoxic effects. On the basis of the finding that c-Fos-deficient cells display a general mutagen-hypersensitive phenotype pertaining to S phase-dependent agents and the end points cell death, apoptosis and induced genomic instability, we conclude that c-Fos/AP-1 induction is part of a general defense of cells aimed at increasing cellular survival and maintaining the chromosomal integrity upon genotoxic exposures.

MATERIALS AND METHODS

Cells and Culture Conditions

The cell lines f20 (c-fos+/+), f1 (c-fos+/-), and f10 (c-fos-/-) are spontaneously immortalized 3T3-like mouse fibroblasts that were derived from either wild-type or c-fos knockout mice (provided by Dr. E. F. Wagner, Research Institute of Molecular Biology, Vienna, Austria). The establishment and growth characteristics of these lines have been described previously (27). Several other c-fos+/- and c-fos-/- cell lines (BK4+/+, BK4-/-, BK5-/-, and others not shown here) that were independently established in our laboratory displayed basically the same response as the lines indicated above.4 For the sake of comparison with previously published data with UV-C, we performed most of the experiments shown here with the lines f20, f1, and f10. Primary cell lines were derived from wild-type (strain C67BL/6J) and c-fos

knockout mice (strain C57BL/6-j-fos) purchased from The Jackson Laboratory (Bar Harbor, ME). Newborn mice were sacrificed, and tissue mostly from skin was minced by a scissor and incubated with PBS containing 0.25% trypsin, 100 μg/ml gentamicin, and 2.5 μg/ml fungizone for at least 4 h with gentle rotation. Thereafter, the tissue homogenate was washed 3 times with complete medium and seeded in 10-cm dishes (6 ml of medium each). After reaching confluence, the fibroblastid cells were passaged (1:3) or stored as a stock.

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Drug Treatments and Irradiation

MNNG, MMS, melphanal, and NQO were from Sigma Chemical Co.; BPDE, which is the activated form of benzo(a)pyrene (28), was a kind gift of Dr. A. Seidel (Institute of Toxicology, Mainz, Germany). MNNG and BPDE were solubilized with DMSO, NQO with ethanol, and melphanal with 1% HCl, and they were all diluted with distilled water to the desired stock concentration and stored in batches at −80°C. Immediately before use, the mutagenic stock solution was thawed, and the agents were added directly to the medium of exponentially growing cells. If not otherwise stated, cells were incubated with the agents for 60 min in the CO2 incubator. Thereafter, the medium was aspirated, and fresh medium was added. Treatment with IR occurred by irradiation of cells attached onto the plates with γ-rays emitted by a 137Cs source.

Survival Experiments

For determination of clonogenic cell survival, 600–800 cells were plated per 6-cm dish and treated 6 h later with different concentrations of the mutagens. After 60 min of incubation, the medium was changed, and cells were incubated for 10 days in the CO2 incubator. Colonies were fixed with methanol, stained with Giemsa crystal violet, and counted. Cell survival was expressed in relation to the mock-treated control, which was included in each experiment in duplicate. The plating efficiency of controls was in the range of 20–40%.

Determination of Dead Cells

The viability of cells was determined by trypan blue dye exclusion. After treatment of cells with the corresponding agents, they were trypsinized at the indicated time points, and both adherent and nonadherent cells were pooled. The cell suspension was centrifuged, and the pellet was resuspended in the same volume of trypan blue dye (0.1% in PBS). The percentage of trypan blue dye-permeable cells, which represents the dead cell fraction, was determined by counting with a hemocytometer.

Determination of Apoptotic Cells

For detecting apoptotic cells, two methods were applied, which gave basically the same results: quantitative fluorescence microscopy (29) and flow cytometry (30). For quantitative fluorescence microscopy, cells were seeded at a density of 1 × 10⁵ per 6-cm dish (containing coverslips) and allowed to grow for 36 h. The cells were then incubated with various concentrations of BPDE for 1 and fixed after 48 h in methanol/acetic acid (3:1) for 20 min. Coverslips were dried and stained with Hoechst 33258 (0.5 μg/ml in PBS) for 20 min. At least 500 cells were scored for the incidence of apoptotic bodies, i.e., nuclear changes such as shrinkage and fragmentation of chromatin resulting in the occurrence of nuclear bodies, which are generally accepted to be signs of apoptosis (31). For microscopic evaluation, we used a BX-FLA fluorescence attached microscope equipped with an U-MWB cube filter (Olympus Optical, Hamburg, Germany). For measuring the apoptotic fraction by flow cytometry, which is based on the fact that a DNA content below the G1 level (hypodiploid) indicates apoptosis (30), about 10⁶ cells were seeded per 6-cm dish, grown for 36 h, and treated with the different chemical agents. At the indicated time points, cells were harvested by trypsinization, and adherent and nonadherent cells were pooled and fixed in 70% ethanol at 4°C for 30 min. Before the analysis, cells were washed with PBS and then incubated with RNase A (0.1 mg/ml) and propidium iodide solution (20 μg/ml) for 15 min in the dark. The samples were analyzed by 488-nm laser excitation on a FACSort flow cytometer (Becton Dickinson, San Jose, CA). The data obtained from at least 10,000 cells each were analyzed using CellQuest software (Becton Dickinson).

Determination of Chromosomal Aberration Frequencies

Exponentially growing cells were treated with the mutagens for the indicated time periods. Thereafter, the medium was changed, and the cells were postincubated in the CO2 incubator. Two h before fixation, colcemid was added at a final concentration of 50 ng/ml. Cells were harvested by trypsinization, treated with hypotonic solution (75 mm KCl), and fixed with methanol: acetic acid (3:1). Slides were prepared according to conventional techniques, and chromosomes were stained with Giemsa. Per measure point, 100 well-spread metaphases were evaluated under the microscope. The following aberration types were scored: chromatid and isochromatid breaks, dicentrics, triradials, quadradrials and other types of complete and incomplete reunited translocations, intercalary deletions, and fragmentations. Cells exhibiting more than six aberrations were considered metaphases with multiple aberrations. Gaps were scored but not considered genuine aberrations and, thus, not included into the final evaluation.

Enzyme and DNA Repair Assays

MGMT Activity. MGMT activity was determined from cell extracts by measuring the transfer of radioactivity from a DNA substrate containing radioactively labeled O6-methylguanine to protein, as described previously (32). In brief, about 10⁷ cells were sonicated, and the protein concentration was determined as described (33). Two hundred μg of cell extract were incubated with a total of 40,000 cpm of [3H]MNU-labeled template (32) for 90 min at 37°C. Thereafter, the protein was precipitated, the DNA degraded by boiling with perchloric acid, the protein solubilized in NaOH, and the radioactivity in the protein determined by scintillation counting. MGMT activity was expressed as fmol of MGMT per mg of protein.

MPG Activity. MPG activity was determined as described previously (34), with the slight modification that the methylpurines 7-methylguanine and 3-methyladenine liberated from the DNA substrate were separated by HPLC (Bio-Rad HPLC System) using a Whatman SCX column (250 mm) and a gradient buffer system (20 mm NH4COOH [pH 4.0]-10% methanol and 200 mm NH4COOH [pH 4.0]-5% methanol) and quantitated by scintillation counting.

APE Activity. APE activity was determined by measuring the cleavage of an oligonucleotide duplex containing a single apurinic site upon incubation with cell extract protein. Cell extracts and the preparation of the duplex occurred as described previously (35). The conversion of the radioactively labeled duplex to the cleavage products was quantified by autoradiography and scanning of the autoradiograms.

PCNA. For preparation of cell extracts, treated and untreated cells were collected by trypsinization and washed with PBS. Cells were then resuspended in a hypotonic lysis buffer [10 mm Tris-HCl (pH 7.4), 2.5 mm MgCl2, 0.5 mm Nonidet P-40, and 1 mm phenylmethylsulfonyl fluoride] and incubated for 8 min on ice to release unbound protein. After centrifugation, the soluble and insoluble DNA-bound fraction of PCNA was recovered as described (36). For Western blot analysis, samples with 40 μg of protein each were resolved on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Nonspecific binding was blocked by immersing the filters in 5% nonfat dry milk and 0.1% Tween-20 in PBS overnight. Thereafter, the filters were incubated with mouse anti-PCNA (Ab-1)/PC10 antibody (Calbiochem, Cambridge, MA; diluted 1:300 in blocking solution) for 2 h and thereafter with a sheep antimouse antibody coupled to horseradish peroxidase (Amersham; diluted 1:5000) for 45 min. The protein-antibody complexes were visualized by enhanced chemiluminescence (Amersham) according to the manufacturer’s protocol.

GST Activity. The amount of GST in cell extracts was determined as previously reported (37).

Methylation Lesions in DNA. Cells in subconfluent state were treated with [3H]MNU (Amersham; specific activity, 18.9 Ci/mmol) at a final concentration of 0.5 mm. The total activity of labeled mutagen per 10-cm plate was 300 μCi in 5 ml of medium. After 60 min of incubation with gentle agitation at 37°C in 7% CO2, cells were rinsed with PBS and either harvested by trypsinization or postincubated for 6 and 12 h in the incubator. Cells were lysed and DNA was isolated using QIAmp Blood Kit columns according to the
manufacturer's protocol (Qiagen). DNA was hydrolyzed by heat treatment with 0.1 n HCl (30 min at 70°C), and methylpurines were separated by HPLC onto a partisil SCX column (Alltech) using 40 mM ammonium formiate (pH 3.5)-10% methanol for elution. The amount of guanine in the eluate, which was measured by OD260, served as an internal standard for the amount of DNA subjected to HPLC analysis. The amount of N7-methylguanine and O6-methylguanine was determined by liquid scintillation counting of the HPLC fractions containing the adducts.

Measurement of DNA Synthesis

For measuring DNA replication in nontreated and mutagen treated cells, two methods were used: [3H]thymidine incorporation and BrdUrd pulse-labeling followed by flow cytometry. For [3H]thymidine incorporation, exponentially growing wild-type and c-Fos-deficient cells that were not treated (control) or treated with the agents for 1 h were pulse-labeled with [3H]thymidine (1 μCi/ml medium, specific activity of 25 Ci/mmol; Amersham) by incubating them for 20 min at 37°C. Thereafter, cells were rinsed two times with ice-cold PBS and harvested by trypsinization. An aliquot of the cell suspension was used for determination of cell number using a hemocytometer. Plates were kept on ice until the cells were collected onto GF/C filters and carefully rinsed with 5% ice-cold trichloroacetic acid and finally 70% and absolute ethanol. The radioactivity incorporated into trichloroacetic acid-insoluble material was determined by scintillation counting. The incorporation of [3H]thymidine was calculated as radioactivity per 10⁶ cells and expressed in relation to the non-mutagen-treated control. For BrdUrd pulse-labeling, at various times after treatment of exponentially growing cells with the mutagen, the cells were pulse-labeled for 20 min with 10 μM BrdUrd (Sigma). After being washed twice with cold PBS, cells were harvested by trypsinization. The pellet was resuspended in PBS and then fixed in ice-cold 70% ethanol overnight at −20°C. DNA denaturation was performed in 2 n HCl at 37°C for 30 min. After being washed twice with PBS, the cell pellet was resuspended in PBT buffer (PBS with 1% BSA and 0.5% Triton X-100) containing anti-c-Fos antibody (Transduction Laboratories) and fluorescein isothiocyanate-conjugated antibody (Becton Dickinson; dilution 1:10). DNA-quantitation was achieved by staining with propidium iodide (20 μg/ml) given together with RNase (0.1 mg/ml) to the cells, which were then incubated for 30 min in the dark at room temperature. Samples were stored at 4°C until analysis in a FACSort (Becton Dickinson). For evaluation of histograms and quantification of cell populations, a computer-based program (CellQuest; Becton Dickinson) was used.

RESULTS

c-FOS AND CELLULAR PROTECTION

c-Fos-deficient Cells Are Hypersensitive to the Cytotoxic Effect of Chemical Mutagens. To examine the role of c-Fos in the protection of cells against the cytotoxic effect of DNA-damaging agents, c-Fos-deficient (c-fos⁻/⁻) and wild-type (c-fos⁺/⁺) cells were treated with different doses of mutagen and allowed to grow and form colonies. As shown in Fig. 1, c-fos⁻/⁻ cells are clearly more sensitive to the killing effect (reproductive cell death) of a diversity of DNA-damaging agents, such as MNNG, MMS, melphalan, BPDE, and NQO. They are not significantly hypersensitive, however, to inactivation by IR.

c-Fos-deficient Cells Are More Sensitive to Chemical Mutagen-induced Necrosis and Apoptosis. Cytotoxicity, as measured by loss of reproductive ability of the cells, can be due to necrosis and/or apoptosis. To check the sensitivity of c-fos⁻/⁻ cells to these end points, we determined the number of dead cells (defined here as necrotic cells) of an exponentially growing population at various time points after pulse treatment with the agents. As shown in Fig. 2A, after treatment with BPDE, a low frequency of dead cells was observed at 24 h, which increased significantly at 48 and 66 h and remained at this high level 72 h after treatment. Interestingly, with long postincubation periods of 48 h up to 72 h, the c-fos⁻/⁻ population clearly showed higher frequencies of dead cells than the c-fos⁺/⁺ population. Similar results have been obtained with MNNG, MMS, and melphalan (data not shown).

To see whether apoptosis is involved in mutagen-induced cell killing and, notably, c-fos⁻/⁻ hypersensitivity, we determined the proportion of apoptotic cells by flow cytometry on the basis of sub-2n DNA content, which is a reliable marker of apoptosis (30). As exemplified in Fig. 2B, BPDE treatment increased the proportion of apoptotic cells in the c-fos⁻/⁻ population. The quantification of these and other data not shown revealed that BPDE induces apoptosis in a dose-dependent way, and c-Fos-deficient cells exhibited a much higher frequency of apoptosis than normal mouse fibroblasts (Fig. 2C). Basically similar results were obtained by microscopic evaluation of Hoechst 33258-stained cells, which was performed in parallel

Fig. 1. Survival of wild-type (+/+ ) and c-Fos-deficient (−/−) cells treated with various genotoxic agents or IR. The cell strains used were c-fos⁺/⁺ (f1) and c-fos⁻/⁻ (f1 and f10). Data points, means of three independent experiments.

Unpublished data.
Fig. 2. Cell death and apoptosis induced in c-Fos-proficient and c-fos−/− cells treated with BPDE. Cell lines used were c-fos+/+ (120) and c-fos−/− (110). A, frequency of dead cells, as determined by trypan blue dye exclusion, induced by various doses of BPDE at various time points after exposure. B, histogram of wild-type and c-fos−/− cells, as determined by flow cytometry. Control, not BPDE-treated; BPDE, cells treated with 300 nM BPDE for 60 min. Cells were harvested 48 h after exposure. M1, fraction of cells with a DNA content lower than the G1 population. C, quantitation of flow cytometry data showing the yield of apoptotic cells as a function of dose of BPDE at 48 h after treatment. D, yield of apoptotic cells as determined by Hoechst 33258 staining and microscopic fluorescence analysis. Per measure point, 500 cells were evaluated by fluorescence microscopy.

as a control (Fig. 2D). It should be noted that the yield of apoptotic cells was lower than the amount of dead (necrotic) cells measured at the same time points, which was also observed for other chemical mutagens (data not shown).

Increased frequencies of apoptotic cells in the c-fos−/− population were also observed after treatment with other DNA-damaging agents tested, such as MNNG, MMS, and melphalan (Fig. 3). Also in these cases, the frequency of apoptotic cells was highest at the latest time points of measurement after mutagen exposure (i.e., 72 h). Interestingly, after treatment with IR, there was no significant increase in the frequency of apoptotic cells within the assay period, and c-Fos-deficient cells appeared not to be hypersensitive (Fig. 3).

Chromosomal Hypersensitivity of c-Fos-deficient Cells toward S Phase-dependent Agents. The frequency of chromosomal aberrations induced by MNNG is shown in Fig. 4. Both dose-response (Fig. 4, A and B) and recovery time experiments (Fig. 4, C and D) revealed a dramatically enhanced sensitivity of c-fos−/− cells to the chromosome breakage-inducing (clastogenic) effect of the methylating agent. Because aberration frequency was enhanced in c-Fos-deficient cells with all recovery time points after mutagen treatment (Fig. 4, C and D), the conclusion can be drawn that the increased chromosomal sensitivity pertains to the whole cell population, irrespective of the cell cycle stage at the time of treatment. Increased chromosomal sensitivity toward MNNG was also observed in other established c-fos−/− cell lines, an example of which is shown in Fig. 4E for the lines BK4−/− and BK5−/−, which were measured with an independently derived established wild-type line, BK4+/+.

Increased chromosomal sensitivity of c-fos−/− cells was observed after treatment with a diversity of chemical mutagens tested. These are MNNG (shown in Fig. 4), MMS, melphalan, mafosfamide, BPDE, and NQO (Fig. 5). Thus, c-Fos-deficient cells appear to be characterized by general chromosomal hypersensitivity toward chemical mutagens (this paper) and UV-C light (25). It should be noted that chromosomes of c-fos−/− cells were often highly damaged (multiple aberrations per metaphase), which is indicated by the large difference in response between wild-type and c-fos−/− cells, when compared on the basis of aberrations per cell.

The frequency of aberrations induced by IR in wild-type and c-Fos-deficient cells is shown in Fig. 6. c-fos−/− cells did not display altered aberration rates (percentage of aberrant cells), as compared to...
c-fos+/- cells. The aberration yields (aberrations per cell) were only slightly enhanced at high dose (6 Gy) and early recovery time (8 h). Overall, c-fos+/- cells were not clearly more sensitive than wild-type cells in aberration production upon treatment with IR.

c-fos-/- Primary Embryonal Fibroblasts Display Chromosomal Hypersensitivity to Chemical Mutagens but not IR. Most of our previous work (25) and this study with c-fos-/- and c-fos+/- cells were performed with established, spontaneously immortalized 3T3-like cell lines. In addition to the lines used in these studies, we also observed mutagen hypersensitivity in other lines independently established in our laboratory from c-fos knockout mice (Fig. 4). However, despite these reproducible findings, one cannot entirely exclude the effects of secondary changes. Because immortalization and long-term cultivation are often accompanied by genetic, notably karyotypic changes that might affect the cellular response to mutagens, we were interested to see whether the mutagen hypersensitivity of c-fos-/- is also a peculiarity of nonestablished c-Fos-deficient cells. To test this, the chromosomal stability of primary cultures of c-fos-/- and c-fos+/- cells derived from c-fos knockout and wild-type mice, respectively, were analyzed. As shown in Table 1, primary c-fos-/- fibroblasts derived from newborn mice displayed a significantly enhanced level of chromosomal aberrations after melphalan, BPDE, and MMS treatment. They were also hypersensitive to the clastogenic effect of UV-C but not IR, as compared to the corresponding c-fos+/- cells. Together with the fact that all established c-fos-/- lines we have analyzed were mutagen hypersensitive (except to IR), the data obtained with primary fibroblasts allow us to conclude that chromosomal hypersensitivity upon exposure to UV light and chemical DNA-damaging agents is an inherent and general property of cells lacking c-Fos.

Expression of Repair Enzymes and Adduct Removal. c-fos-/- cells are remarkably hypersensitive to alkylating agents. Because DNA repair, notably MGMT and base excision activity, affects the killing and clastogenic response of cells upon alkyltation (38), we determined the expression level of the major enzymes involved in repair of DNA alklylation lesions in wild-type and c-Fos-deficient variants. As shown in Table 2, MGMT was expressed in all c-fos-/- lines tested to about the same level as in c-fos+/- cells. Thus, the cell lines analyzed are phenotypically Mex- . Also, there was no difference in the expression level of MPG. Another key enzyme in the repair of N-alkylation DNA lesions is APE. As shown in Fig. 7, A and B, APE activity was the same in wild-type and c-fos-/- cells. Because PCNA has been shown to be involved in DNA repair (39), we also checked the level of both DNA-bound and nucleoplasmic PCNA and found it to be expressed at comparable level in c-fos-/- and wild-type cells (Fig. 7C). Also, treatment of G1-arrested cells with BPDE (and UV-C) increased the amount of DNA-bound PCNA to the same extent in wild-type and c-Fos-deficient cells, as detected by flow cytometry (data not shown). Furthermore, there was no difference in the GST level between c-fos-/- and c-fos+/- cells (Table 2), making differences between the lines in mutagen activation or detoxification unlikely. This was confirmed by the fact that the initial DNA methylation level was the same in wild-type and c-Fos-deficient cells (26 N7-methylguanines and 2.8 O°-methylguanines per 10^5 guanines after treatment with 0.5 mm [3H]MNU). Moreover, there was no significant difference in the rate of removal from DNA of these main methylation lesions (data not shown). In summary, hypersensitivity of c-fos-/- cells to alkylating agents cannot be explained on the basis of overall DNA repair capacity and glutathione-based detoxification.

DNA Replication Inhibition. The effect of DNA-damaging agents on DNA replication in wild-type and c-fos-/- cells is shown in Fig. 8. Melphalan and MNNG inhibited DNA replication, as measured by [3H]thymidine incorporation, in a dose-dependent way. Clearly, c-Fos-deficient cells were more sensitive than wild-type cells (Fig. 8, A and B). Cells have an ability to recover from the initial mutagen-induced block to DNA replication. Interestingly, c-Fos-deficient cells exhibited a delay in recovery, when compared to c-fos+/- cells, which is shown for MNNG in Fig. 8, C and D. If cells were pulse-treated with the mutagen (for 60 min) and then allowed to recover before pulse-labeling with BrdUrd, the proportion of cells that incorporated BrdUrd was significantly reduced at 2 h of recovery, compared to the non-mutagen-treated control (Fig. 8C). This initial replication block-
Fig. 4. Chromosomal sensitivity of wild-type and c-Fos-deficient cells treated with MNNG. A and B, aberration frequency as a function of dose (recovery time, 19 h); C and D, aberration frequency as a function of time after MNNG exposure (20 μM, 60 min). Cell lines used were c-fos "+" (120) and c-fos "−" (11). E, MNNG-induced aberrations (10 and 20 μM, 60 min treatment) in three independently derived established cell lines: BK4+/+ (wild-type) and BK4−/− and BK5−/− (both c-Fos-deficient). Note that for the two doses of MNNG, two different c-fos lines have been used. Differences between c-fos "−" and wild-type are highly significant (P < 0.001).

Figure 4 shows the chromosomal sensitivity of wild-type and c-Fos-deficient cells treated with MNNG. The data were obtained by analyzing the frequency of aberrations as a function of dose (recovery time, 19 h) and time after MNNG exposure (20 μM, 60 min). Three independently derived established cell lines were used: BK4+/+ (wild-type) and BK4−/− and BK5−/− (both c-Fos-deficient). The MNNG-induced aberrations for the two different c-fos lines were compared, and the differences were highly significant (P < 0.001).

Decrease in DNA replication upon mutagen treatment can be due to inhibition of the rate of DNA synthesis and/or blockage of entry of cells into S phase. The question of whether either one or both of the mechanisms were involved was studied in an independently performed series of experiments. Cells were pulse-labeled with BrdUrd for 20 min and then treated with the mutagen for 1 h. The progression of the BrdUrd-labeled population through the cell cycle was monitored by bimodal fluorescence-activated cell sorting analysis. As postincubation time increased, the proportion of BrdUrd-labeled fraction in wild-type cells decreased, reaching nearly control level at 8 h of recovery. For c-fos "−" cells, the proportion of BrdUrd-labeled fraction increased with postincubation time but clearly remained at a lower level, compared to the wild type. A quantitative evaluation of histograms showed a significant increase in the proportion of BrdUrd-labeled fraction in wild-type cells over time, indicating that c-fos mutant cells have the ability to overcome the initial block to DNA replication, but resumption of replication is clearly delayed, as compared to that in wild-type cells. We should note that with the doses of the mutagens used, immediate-early cytotoxic effects (as measured by vital staining up to 8 h after treatment) were not observed. Therefore, the effect of the agents on DNA replication cannot be explained on the basis of cell killing.

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Figure 5 shows the aberration frequencies in wild-type and c-Fos-deficient cells treated with MMS, melphalan, mafosfamide, BPDE, and NQO. Exponentially growing cells were treated with the indicated concentrations of the agents for 60 min (except for mafosfamide, which was chronically applied) and harvested 19 h later (colcemid treatment, 17-19 h) for chromosomal preparation. Cell lines were c-fos "+" (120) and c-fos "−" (11 and 110). For each agent, both aberration rates (as %) and aberration yields (aberrations per cell) are shown.
Table 2 Activity of MGMT, MPG, and GST in c-Fos-proficient (+/+) and c-Fos-deficient (–/–) cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGMT (fmol/mg of protein X min)</th>
<th>MPG (fmol/mg of protein X min)</th>
<th>GST (nmol/mg of protein X min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fos+/+ (T20)</td>
<td>55</td>
<td>17</td>
<td>81</td>
</tr>
<tr>
<td>c-fos–/– (T10)</td>
<td>71</td>
<td>17</td>
<td>87</td>
</tr>
<tr>
<td>c-fos–/– (f1)</td>
<td>61</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Primary +/+</td>
<td>170</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>Primary –/–</td>
<td>160</td>
<td>15</td>
<td>93</td>
</tr>
</tbody>
</table>

a f1, f10, and T20 are established cell lines (passages 40–60). Nonestablished (primary) fibroblasts were out of the first passage. For activity measurements see “Materials and Methods.”

b ND, not determined.

Table 1 Chromosomal sensitivity of primary fibroblasts derived from wild-type and c-fos knockout (c-fos–/–) mice

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose</th>
<th>Aberrations (%)</th>
<th>Aberrations/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>wild-type</td>
<td>c-fos–/–</td>
</tr>
<tr>
<td>Melphalan</td>
<td>8 µM</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>BPDE</td>
<td>125 nm</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>MMS</td>
<td>1 mM</td>
<td>52</td>
<td>84</td>
</tr>
<tr>
<td>UV-C</td>
<td>20 J/m²</td>
<td>46</td>
<td>87</td>
</tr>
<tr>
<td>IR</td>
<td>4 Gy</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

Exponentially growing cells out of the first passage were treated for 60 min with the agents. After 17 h of recovery, cells were incubated for 2 h with colcemid and harvested for chromosomal preparation.

b Difference between wild type and c-fos–/– is highly significant (P < 0.001).

revealed by the dot plots shown in Fig. 9, without mutagen treatment, most of the BrdUrd-labeled fraction of cells had passed S phase within 4 h of postincubation (compare control 0 h and 4 h after BrdUrd labeling), whereas the mutagen-treated cells (MNNG, 4 h) were severely blocked within S phase. It should be noted that in this pulse-chase experiment, a significant difference in S phase fraction between wild-type and c-fos–/– cells treated with the mutagen was not obvious, which is due to the fact that cells were labeled with BrdUrd prior to the treatment with the genotoxic agent, and therefore, despite differences in recovery from inhibition of replication (Fig. 8D), they were still detected as being in S phase. Similar results were obtained with UV-C and BPDE (data not shown). The results show that the reduced DNA replication of c-Fos-deficient cells after mutagen treatment, as measured by DNA precursor incorporation, is due to inhibition of the rate of replication of cells, which were in S phase at

Fig. 6. Aberrations induced in wild-type (+/+) and c-Fos-deficient (–/–) cells by IR. The cell lines used are c-fos+/+ (T20) and c-fos–/– (T10). A and B, aberration frequency as a function of dose (recovery time, 19 h); C and D, aberration frequency as a function of time after irradiation; exponentially growing cells attached onto the plates were irradiated with γ-rays (dose: 4 Gy). The difference in aberration yield (aberrations per cell) between wild-type and c-fos–/– cells for 6 Gy (B) and 6 h of recovery (D) was statistically significant.

Fig. 7. APE activity and PCNA level in wild-type and c-fos–/– cells. A, APE activity assay was performed with nuclear extracts. The reaction was stopped after 0, 5, 15, and 25 min of incubation and DNA fragments were separated by gel electrophoresis. Upper and lower bands, the uncleaved 35-mer and the converted IS-mer radiolabeled oligonucleotides, respectively. At zero time, the oligonucleotide was mixed with cell extract and the reaction was stopped immediately after the incubation. B, quantification of radioactively labeled substrate converted by APE cleavage as a function of time. Data are from densitometric analysis of the autoradiogram shown in A. C, amount of PCNA determined by Western blot analysis. Exponentially growing cells were not treated (control) or treated with BPDE for 30 min, and PCNA was extracted from soluble (Lanes S) and unextractable (Lanes U) fraction. +/+, wild-type cells; –/–, c-Fos-deficient cells.
induction of genomic instability, as monitored by the formation of chromosomal aberrations (i.e., chromosomal breaks and different types of translocations). Interestingly, the agents also induced, with significantly elevated frequency, apoptosis in c-fos<sup>−/−</sup> fibroblasts. This indicates that c-Fos expression is required not only for protection from mutagen-induced chromosomal damage but also cell death, which results from apoptosis. To our knowledge, this is the first report showing induction of apoptosis in c-Fos-deficient fibroblasts by alkylating agents, other classes of chemical mutagens, and tumor-therapeutic drugs and the protection against it by an inducible cellular function.

Apoptosis increased in frequency with postexposure incubation time and was found to be rather a late event. Thus, the highest yields of apoptotic cells were detected at 72 h after mutagen treatment, when cells had passed more than one replication cycle. Contrary to this, chromosomal aberrations were induced with highest frequency in the first posttreatment replication cycle. This was shown here for MNNG in wild-type and c-fos<sup>−/−</sup> cells (with a peak at 18—24 h after treatment; Fig. 4) and is well known from other mutagens and Mex<sup>+</sup> cell types (40—42). An interesting speculation would be that chromosomal

the time of treatment, and not to blockage of entry of cells from G<sub>1</sub> into S.

**DISCUSSION**

In this study, we provide evidence that c-Fos plays a general role in cellular defense against genotoxic agents. It is based on the finding that cells deficient in c-Fos are hypersensitive to a variety of chemicals that react with DNA, such as methylating compounds (MMS and MNNG), DNA cross-link-inducing drugs (maphosfamide and melphalan), and carcinogens inducing bulky adducts in DNA (BPDE and NQO). The hypersensitivity pertained to both the end point cytotoxicity, which was measured by vital staining and colony formation, and
c-fos' and wild-type cell lines used in this study. Furthermore, in our previous repair studies with UV-C, we have found no significant alterations in c-fos"/" cells that could account for their hypersensitivity. Thus, in the dose range of UV-C that induced with elevated frequency chromosomal aberrations and cytotoxic effects in c-fos"/" cells (up to 20 J/m²), the extent of DNA repair synthesis was the same in c-fos"/" and wild-type cells (25). Similar results with UV-C have been obtained independently by another group (26). Furthermore, in this work, we have compared the activity of various enzymes decisive in repair of alkylated bases induced by MNNG and MMS. One of them is MGMT, which repairs the premutagenic and pretoxic lesion O⁶-methylguanine and thus is decisively involved in determining the killing and clastogenic response of cells to methylating agents (38). MGMT was found to be expressed to almost the same level in all c-fos"/" and wild-type cell lines used in this study. Furthermore, MPG and APE, which are involved in excision repair of alkylated lesions and thus affect the level of resistance to alkylating agents (34, 35, 44–46), are also expressed to the same level in these lines. In accordance with this is the rate of removal from DNA of the main methylation lesions N⁷-methylguanine and O⁶-methylguanine, which was not significantly different in wild-type and c-fos"/" cells. Also, there was no change in the level of GST, which is involved in defense against several chemical agents (47). It should be noted that simple methylating agents neither require metabolic activation nor are subject to membrane-related transport processes.

If DNA repair is not compromised, how then is c-Fos involved in killing protection and maintaining the stability of the genome after genotoxic exposures? It is important to note that the agents c-fos"/" cells are hypersensitive to elicit genotoxic effects in an S phase-dependent manner, i.e., DNA replication is required to transform critical DNA damage into chromosomal aberrations (for review of S phase-dependent and -independent agents, see Refs. 40–42). The molecular process by which a particular type of DNA damage (e.g., O⁶-methylguanine or N-methylpurines) is converted into chromosomal aberrations by means of semiconservative DNA replication is not known, although various hypotheses have been proposed (48). Importantly, DNA replication is commonly inhibited immediately after mutagen exposure (49–52). As previously proposed (25, 53), the mutagen-induced blockage of DNA replication could play a causal role in aberration production and induction of genotoxicity by increasing the probability of nuclease attack at stalled, nuclease-sensitive replication forks. In fact, c-fos"/" cells exhibit more severe mutagen-induced immediate DNA replication inhibition, which was shown for UV-C (25, 26) and chemical mutagens (this paper). Thus, a reasonable hypothesis explaining c-fos"/" hypersensitivity would be that the inability of c-fos"/" cells to abrogate the block to replication at appropriate time results in nuclease attack at stalled replication forks. This would give rise to increase of DNA double-strand breakage and, finally, chromosomal breakage and rearrangements.

In support of this hypothesis is our finding that cells lacking c-Fos are not more sensitive to IR. IR induces chromosomal aberrations via an S phase-independent pathway (42). This is due to its ability to induce DNA double-strand breaks without the involvement of DNA replication, which is in contrast to the other agents applied, including UV-C. Thus, IR induces aberrations in G₂, which UV light and most of the chemical clastogens are unable to do (42, 54). It cannot entirely be excluded that some IR-induced base damage, such as 8-oxo-guanine, might also induce genomic changes in a S phase-dependent manner, but this appears to be a minor component, which may account for the observed very weak response of c-fos"/" cells when aberrations were calculated as aberrations per cell (Fig. 6). Interestingly, c-fos"/" cells were also not more sensitive than the corresponding wild type with respect to induction of apoptosis and cell death by IR, indicating again that chromosomal aberrations, apoptosis, and cell killing are interrelated.

c-Fos is not essential for cellular growth, nor does it appear that the basal level of expression of various AP-1-dependent genes is affected in c-fos-deficient cells (27, 55). However, various AP-1-dependent genes were less induced in c-fos"/" cells by growth factors that stimulate c-fos expression (55). The level of AP-1 activity upon exposure of cells to UV-C is lower in c-Fos-deficient cells (26). This was also found for MMS, which is highly effective in inducing c-fos (8). Although the target genes of AP-1, the products of which may play a role in maintaining genomic stability upon mutagen exposure, are unknown, it is reasonable to suppose that they are expressed at lower level in cells lacking c-Fos. According to the hypothesis proposed above, critical inducible target genes may function in triggering the resumption of DNA replication, which was blocked upon the induction of DNA damage. p53 and p21 appear not to be causally involved in c-fos"/" hypersensitivity, because both are basally expressed and induced by UV-C and chemical mutagens tested at comparable level in primary wild-type and c-fos"/" fibroblasts. Also, PCNA, which is decisively involved in DNA replication (56) and also plays a role in DNA repair (57), was not altered in c-fos"/" cells.

There are various other explanations of the observed c-fos"/" hypersensitivity. Thus, one could argue that c-Fos/AP-1 stimulates the expression of gene products that repress mutagen-induced error-prone (SOS-like) functions. This hypothesis suffers, however, from lack of clear evidence of the existence of error-prone functions in mammalian cell that are involved in mutagen-induced genomic instability and cytotoxicity. One might also speculate that bypass functions are required for circumventing replication-blocking lesions, which are subject to AP-1 regulation. Whatever the mechanism is, the defense function(s) controlled by c-Fos/AP-1 very likely act on DNA level and are basically error-free, thus reducing mutagen-induced genomic instability. In agreement with this are dose-response studies for induction of fos and jun genes and AP-1 on one hand and various genotoxic end points on the other, from which it has been concluded that Fos and Jun are required in defense; they are not obligatory essential for induction of mutations and genomic changes, e.g., by triggering an error-prone mutagenic pathway (8).

In addition to c-Fos, there are other inducible functions that have been shown to be involved in cellular defense. p53 is probably induced by all DNA-damaging agents (10). Due to its ability to prevent entry of cells from G₁ into S, p53 extends the time available for prereplicative repair of critical DNA lesions and possibly activa-

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* A. Ziouka and B. Kaina, unpublished results.

7 S. Haas and B. Kaina, unpublished data.
tion of bypass functions. Other likely candidates of induced functions involved in cellular defense are DNA repair genes. Thus far, however, only two repair genes have been shown to be inducible in mammalian cells by genotoxic stress: MGMT (22, 23) and DNA-polymerase β (58). MGMT induction rendered cells more resistant to a challenge dose of an alkylating agent (22), indicating that this response is of physiological significance. The protective effect of MGMT is limited, however, to simple methylating and chloroethylating N-nitrosoureas and does not pertain to mafosfamide, melphalan, and UV-C (59, 60). Likewise, modulation of DNA polymerase β activity does not affect sensitivity of cells to the toxic and clastogenic effect of UV-C and related agents (61). Thus, compared to the defense elicited by modulation of DNA repair activity, the protection mediated by c-Fos is more general and appears to pertain to all DNA-damaging agents which require DNA replication in order to induce clastogenic effects.

C-Fos-deficient cells were shown here to be more sensitive to the apoptotic, cytotoxic, and chromosomal breakage-inducing effects of mafosfamide and melphalan. Mafosfamide is an analogue of the activated form of cyclophosphamide, which is most often used in tumor therapy. Likewise, melphalan is being used for the treatment of various neoplasia (62). The finding that c-Fos deficiency renders cells more sensitive to various cytostatic drugs bears potential practical implications. If deficiency in c-Fos (or other members of the Fos/Jun family) renders cells more sensitive to cytostatic drugs, it would be desirable to measure, for prognostic purpose, the induced AP-1 level and does not pertain to mafosfamide, melphalan, and UV-C (59, 60). Physiological significance. The protective effect of MGMT is limited, therefore AP-1 induction by antisense techniques and specific inhibitors (63) might increase the sensitivity of tumors to particular chemotherapeutic treatments. It would be an interesting issue of future research to see whether differences do exist in induced expression of AP-1 in various types of tumors and whether deficiency in other components of the AP-1 family of transcription factors also gives rise to hypersensitivity to environmental mutagens and cytostatic drugs.

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A General Role for c-Fos in Cellular Protection against DNA-damaging Carcinogens and Cytostatic Drugs

Bernd Kaina, Simone Haas and Holger Kappes


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