CMLS, Cell. Mol. Life Sci. 54 (1998) 129–138
1420-682X/98/020129-10 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

EGF-induced programmed cell death of human mammary carcinoma MDA-MB-468 cells is preceded by activation of AP-1

P. Schaerli and R. Jaggi*

Department for Clinical Research, University of Bern, Murtenstrasse 35, CH-3010 Bern (Switzerland), Fax +41 31 632 32 97, e-mail: jaggi@dkf4.unibe.ch

Received 21 July 1997; received after revision 6 November 1997; accepted 6 November 1997

Abstract. MDA-MB-468 is a human mammary adenocarcinoma cell line that overexpresses the epidermal growth factor (EGF) receptor and undergoes programmed cell death (apoptosis) in response to EGF treatment. Programmed cell death was shown to be greatly enhanced when cells were growth-arrested prior to EGF treatment. Apoptosis was characterized by an initial rounding up and detachment of the cells from their substrate starting about 12 h after EGF treatment, followed by chromatin condensation, nuclear fragmentation and oligonucleosomal fragmentation of the DNA at about 24 to 48 h. Cell death was dependent on de novo protein synthesis. We found a rapid induction of c-*fos*, c-*jun* and *junB* at the mRNA level after about 30 min of EGF treatment and a more delayed upregulation of *fosB* and *fra*-1. The *junD* gene was expressed in the absence of EGF, and it was moderately induced within 30 min of growth factor addition. The increase of the different *fos* and *jun* mRNAs were paralleled by an increase of activator protein-1 (AP-1) DNA binding activity. A characterization of the AP-1 complex revealed similar levels of several Fos and Jun proteins. Based on the kinetics of AP-1 accumulation and cell death, it seems likely that AP-1 contributes to the apoptotic cell death of EGF receptor-overexpressing MDA-MB-468 cells.

Key words. AP-1; apoptosis; Fos; Jun; MDA-MB-468 cells; programmed cell death.

Epidermal growth factor (EGF) stimulates growth of a wide variety of normal and malignant cells [1] and in many situations EGF prevents cell death [2–4]. In contrast, a number of studies have revealed involvement of EGF in apoptosis (programmed cell death). Several tumour cells that overexpress the EGF receptor are growth-inhibited and eventually undergo apoptosis upon EGF treatment [5–7], suggesting a relationship between EGF receptor expression and EGF-induced cell fate, such that a low level of EGF receptor expression is associated with growth stimulation by EGF, whereas a high level of receptor expression correlates with growth inhibition and eventually cell death. EGF receptor overexpression is a widespread phenomenon found in different human malignant cells such as tumours of the brain, pancreas, lung, head and neck, bladder and breast [8], and it often coincides with reduced expression of oestrogen receptors [9] and bad prognosis [10]. MDA-MB-468 is a human mammary adenocarcinoma cell line [11] which expresses high levels of normal EGF receptors due to EGF receptor gene amplification and overexpression [12]. Long-term treatment of MDA-MB-468

* Corresponding author.

cells with EGF was previously shown to induce growth arrest and apoptosis [6, 12].

Apoptosis/programmed cell death is an energy-dependent, tightly regulated suicidal process that is paralleled by characteristic morphological and biochemical changes. In the nucleus the chromatin rapidly forms dense crescent-shaped aggregates lining the nuclear membrane, and the DNA is broken apart into fragments of 180 base pairs by nonlysosomal Ca^{2+}/Mg^{2+}-dependent endonucleases [13–16] which appear as a ladder of DNA after electrophoretic separation. DNA fragmentation is accompanied by cell surface convolution and cell degradation into several membrane-surrounded, condensed fragments (apoptotic bodies), which are phagocytozed by macrophages or adjacent cells [17]. Apoptosis occurs spontaneously or in response to chemotherapy in malignant tumours [18], it is essential during embryonic development [19] and it also regulates processes in the adult, for example in the mammary gland after weaning when a majority of secretory epithelial cells are removed by apoptosis in a reductive remodelling process termed 'involution' [20, 21].

The transcription factor AP-1 is a dimeric protein complex existing either as a homodimer of Jun proteins (c-Jun, JunB, JunD) or as a heterodimeric complex of Jun and Fos (c-Fos, FosB, Fra-1, Fra-2). Many reports have documented that AP-1 regulates the expression of genes involved in cellular proliferation. More recently, several direct and indirect lines of evidence have also suggested involvement of Fos and Jun in the regulation of programmed cell death. For example, AP-1 was suggested as a regulator of apoptosis in mouse mammary epithelial cells [22], prostate epithelial cells [23], neurons [24, 25] and in retinal cells [26].

In this study we present evidence that efficient programmed cell death of MDA-MB-468 cells upon EGF treatment requires cellular growth arrest and de novo protein synthesis including AP-1 complexes. Furthermore, we determined the kinetics of induction of different *fos* and *jun* mRNAs and the composition of the resulting AP-1 DNA binding complex which precedes the apoptotic cell death in these cells.

Materials and methods

Cell culture. The human adenocarcinoma cell line MDA-MB-468 was maintained in 50% basal ISCOVE-medium (Seromed, Berlin, Germany) and 50% Dulbecco's modified Eagle's medium containing 4% fetal calf serum at 37 °C and 5% CO_2. MDA-MB-468 cells were plated at a confluency of 20–25% the day before serum withdrawal. Cells were growth-arrested by incubation in medium containing no serum for 2 days. Cell death was induced by the addition of EGF (25 ng/ml) to exponentially growing or growth-arrested cells. Protein synthesis was blocked by the addition of cycloheximide (CHX) (2 μg/ml) to growth-arrested cells 30 min prior to stimulation with EGF. Attached cells were washed, trypsinized, diluted in ice-cold phosphate-buffered saline (PBS) and counted in a cell Coulter Counter (Model Z_{BI}, Coulter Electronics, Luton, England). Three independent plates were counted for each time point.

Staining of apoptotic cells. Growth-arrested MDA-MB-468 cells were treated with EGF for 48 h, and detached cells were centrifuged, applied to a glass slide in PBS and stained with an equal volume of PBS containing RNase A (100 μg/ml) and acridine orange (AO) (5 μg/ml, pH 6.0) or propidium iodide (PI) (1 μg/ml, pH 7.2) for 5 min. Attached cells mostly failed to be stained by AO or PI under similar conditions.

DNA fragmentation assay. After treatment detached and/or attached cells were pelleted and resuspended with 10 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA pH 8.0. The DNA was isolated by the addition of SDS (0.5%), proteinase K (0.2 mg/ml) and incubation for 2 h at 50 °C, extracted with Tris-phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). The DNA was precipitated and dissolved in 10 mM Tris, 1 mM EDTA pH 8.0 at 4 °C. The DNA was incubated with RNase A (1 μg/ml) at 37 °C for 1 h and 15 μg of DNA were separated electrophoretically on a 1.2% agarose gel in 0.5× TBE (25 mM Tris base, 25 mM boric acid, 0.5 mM Na_2EDTA).

RNA analysis. Total RNA was prepared using guanidinium thiocyanate [27]. Ten micrograms of total RNA were separated electrophoretically, transferred to nitrocellulose and probed with random-prime labelled cDNA fragments corresponding to homologous c-*fos*, *fos*B, *fra*-1, c-*jun*, *jun*B and *jun*D as described [28]. RNA was prepared from two independent series of EGF stimulations.

Nuclear protein extraction and bandshift analysis. Cells were pelleted and resuspended in buffer A (10 mM Hepes-potassium hydroxide pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing protease inhibitors (5 ng/ml leupeptin, 50 ng/ml aprotinin, 5 ng/ml antipain, 5 ng/ml chymostatin, 0.5 ng/ml pepstatin, 1 mM 6-aminohexanoic acid, 1 mM β-mercaptoethanol, 1 mM DTT, 0.3 mM PMSF). Four hundred microlitres of buffer A were added to a cell pellet derived from two 14-cm plates. Cells were homogenized with a polytron on ice for 10 s at 20,000 rpm, and nuclei were centrifuged at 4700 rpm for 5 min at 4 °C. The nuclei were gently resuspended in 50 μl of buffer C (20 mM Hepes-potassium hydroxide

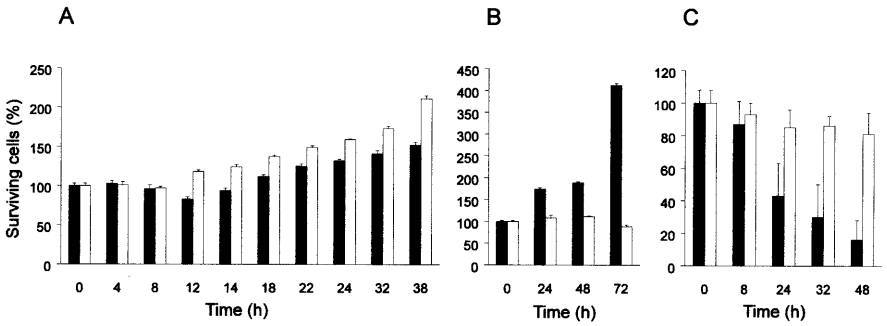

Figure 1. Growth arrest and EGF-induced cell death of MDA-MB-468 cells. (*A*) Exponentially growing cells were cultivated with 25 ng/ml EGF (black) or without EGF (white) for the indicated periods of time. (*B*) Arrest of proliferation by serum withdrawal. Cells were maintained in serum-containing medium (black) or in serum-free medium (white) for the indicated periods of time. (*C*) Cells were growth-arrested by serum starvation for 2 days in medium lacking fetal calf serum before treatment with 25 ng/ml EGF (black) or serum-free medium alone (white) for the indicated periods of time. Cells were counted in a Coulter Counter, and the percentages of surviving cells are depicted. The error bars represent standard deviations from three independent plates.

pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 5 ng/ml leupeptin, 50 ng/ml aprotinin, 5 ng/ml antipain, 5 ng/ml chymostatin, 1 mM 6-aminohexanoic acid, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT), 0.3 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 ng/ml pepstatin) and centrifuged at 13,000 rpm for 30 min at 4 °C. Protein concentration was determined using the Bio-Rad protein assay. Extracts were shock-frozen and stored at −70 °C.

Bandshift reaction. Double-stranded oligodeoxynucleotides were end-labelled with polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany). The oligodeoxynucleotides had the following sequences: 5′-AAGCATGAGTCAGACAC-3′ (AP-1, coding-strand [29]); 5′-CTTCTTAATTTGCATACCCTCA-3′ (Oct-1, noncoding strand [30]). For bandshift analysis 5 µg of extract in 5 µl of buffer C was added to 3 µl of bovine serum albumin (BSA) (2 µg/µl), 2 µl of poly-[d(I-C)] [1 $u_{O.D.260}$/25 µl] and 14 µl of 2× buffer D (10 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 1.5 mM DTT, 15% glycerol, 0.1% NP-40). The samples were incubated on ice for 15 min, 2 µl of labelled oligodeoxynucleotides was added and the incubation proceeded for 15 min. Three microlitres of loading buffer (0.06% bromophenol blue, 20% Ficoll 400 in 1× buffer D) were added, and the protein/DNA complexes were separated electrophoretically on a 6% native polyacrylamide gel in 0.25× TBE at 170 V and 4 °C. For antibody competition assays antibodies were added for 15 min after the incubation with oligodeoxynucleotides. The following affinity-purified antibodies (Santa Cruz Biotechnology, Santa Cruz, California) were used: αc-Fos: rabbit polyclonal IgG (sc-52); αFra-1: rabbit polyclonal IgG (sc-183); αFra-2: rabbit polyclonal IgG (sc-604); αFosB: rabbit polyclonal IgG (sc-48); αc-Jun/AP-1: rabbit polyclonal IgG (sc-45); αJunD: rabbit polyclonal IgG (sc-74); αOct-1: rabbit polyclonal serum (a gift of W. Schaffner, Zurich, Switzerland). The impairment of DNA binding in the presence of specific antibodies was further determined by scanning of autoradiographs and densitometric evaluation of the intensities of individual bands using Scanalytics software (MWG, Biotech, Ebersberg, Germany). Bandshift analyses were performed on three independent series of nuclear extracts.

Results

EGF-induced programmed cell death in MDA-MB-468 cells. Several reports have demonstrated that MDA-MB-468 cells can be growth-inhibited by EGF and eventually undergo programmed cell death. For example, the percentage of cells harvested after 7 days of EGF treatment in serum-containing medium was reduced to 80% as compared with 310% in untreated cells [31]. We observed a moderate decrease in cell number after 12 h of EGF treatment (fig. 1A). This EGF-induced effect was only transient, and EGF-treated cells gradually continued to grow over the next 38 h. Armstrong et al. [6] determined programmed cell death at first after 4 days of EGF treatment, and they described a slight increase in cell number after 1–2 days. Apoptosis clearly predominated after 6 days, when about a 75% decrease in cell number was measured. We investigated whether the early effect of EGF-mediated cell death seen at 12 h can be potentiated by cultivating the cells in medium devoid of growth factors. Figure 1B shows that serum withdrawal rapidly led to growth arrest that was obvious already after 24 h. While the number of cells gradually decreased within the next 3 days, cells grown in serum-containing medium continued to proliferate

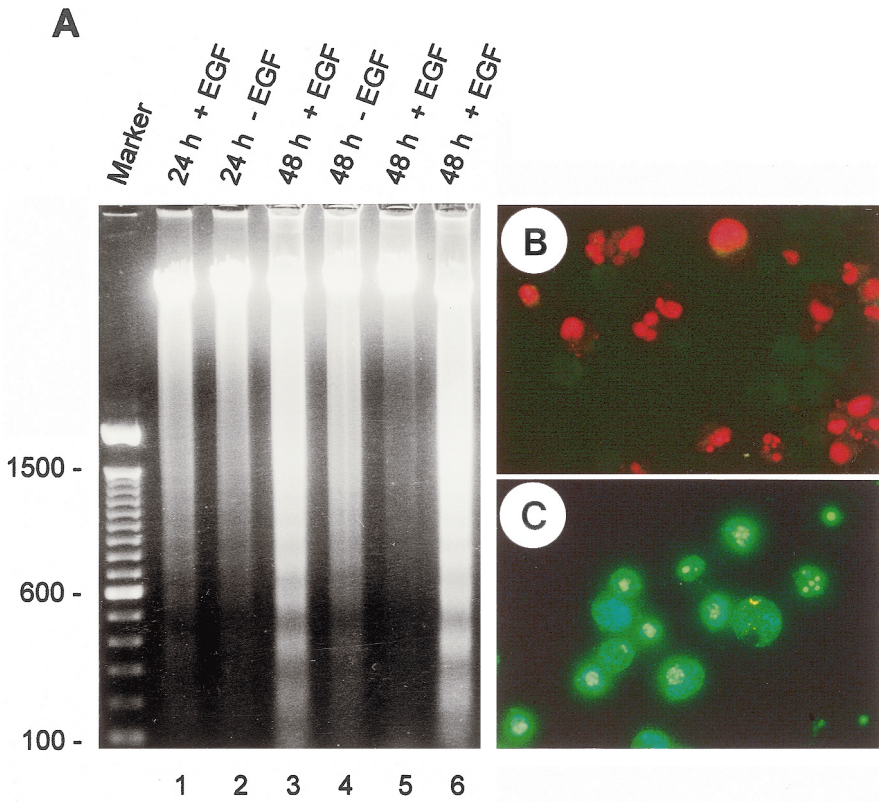

Figure 2. DNA fragmentation and morphology of EGF-treated cells. (*A*) Growth-arrested cells were treated with 25 ng/ml EGF for 24 (lane 1) or 48 h (lanes 3, 5 and 6), and the DNA was isolated from adherent cells (lane 5), detached cells (lane 6) or from both cells together (lanes 1–4). Fifteen micrograms of DNA were separated electrophoretically. Cells grown under identical conditions but in the absence of EGF were processed in parallel (lanes 2 and 4). Detached cells were stained with propidium iodide (*B*) or acridine orange (*C*) and visualized by fluorescence microscopy 48 h after treatment with EGF.

exponentially with a generation time of about 35 h (fig. 1B). Treatment of these growth-arrested cells with EGF resulted in massive cell death as measured by the decrease in the number of cells within 2 days (fig. 1C). After 2 days of EGF treatment only about 20% of the original number of cells survived, whereas about 80% of untreated cells survived in the absence of EGF.

Cell death is accompanied by oligonucleosomal DNA fragmentation and nuclear condensation. Oligonucleosomal DNA fragmentation was previously described as a hallmark of apoptosis. It is caused by Mg^{2+}/Ca^{2+}-dependent endonucleases which produce a ladder of nucleosomal oligomers. No or only very minor internucleosomal cleavage was detected in untreated serum-starved cells and in cells treated with EGF for 24 h (fig. 2A, lanes 1 and 2). However, massive DNA fragmentation was observed after 48 h of EGF treatment (lane 3). Minor DNA fragmentation also occurred in untreated cells under these serum-free conditions, probably due to spontaneous cell death (lane 4). Interestingly, DNA fragmentation upon EGF treatment was restricted to the fraction of cells which had lost their adherence to the plate and which were detached from the surface (lane 6). Adherent cells never showed oligonucleosomal fragmentation (lane 5 and data not shown). These results indicate that DNA fragmentation occurs after detachment of the cells from the plate.

Another characteristic feature of apoptosis is the condensation and fragmentation of nuclei accompanying the fragmentation process of the genome. These morphological changes can be visualized microscopically by staining nucleic acids with propidium iodide (PI) or acridine orange (AO). Untreated quiescent cells showed normal morphology and no spontaneous condensation or fragmentation of nuclei. Most of these cells were not stained by PI, probably as a result of intact membranes which inhibited penetration of the stain (data not shown). EGF treatment for 24 h resulted in a strong increase in the number of detached cells, but only a few of them were stained with PI or showed apoptotic features (data not shown). After 48 h of EGF treatment most of the detached cells exhibited condensation and fragmentation characteristic of apoptotic nuclei and could be stained efficiently with PI (fig. 2B) or AO (fig.

2C). Some nuclei stained by PI (fig. 2B) appeared discrete and were not dispersed into apoptotic bodies. Staining by PI mostly depends on disrupted membranes which are generated during both necrosis and apoptosis. Some of the PI-stained nuclei may therefore reflect cells that are dying by necrosis rather than apoptosis. In fact, cell death by necrosis regularly occurs in vivo, e.g. during postlactational involution when the mammary gland is reorganized and a mass of secretory epithelial cells is eliminated (Vallan and Jaggi, unpublished observations).

Our results indicate that the process of cell death in MDA-MB-468 cells can be chronologically divided into several distinct phases: Untreated quiescent cells remain attached to the substrate and do not stain with PI, indicating normal integrity of cell membranes. EGF treatment for 24 h results in detachment of cells from the substrate, and only a few cells can be stained with PI. Interestingly, most of these stained cells show no apoptotic features at this stage. EGF treatment for 48 h results in a majority of cells which are stained with PI. Most of these stained cells have an apoptotic phenotype. These results suggest that DNA fragmentation and nuclear condensation are relatively late events and are only obvious after 48 h of EGF treatment.

Requirement for de novo protein synthesis in EGF-induced cell death. Examples have been described where all the components required for programmed cell death are constitutively present in the cell and only need to be biochemically activated. In other cell death pathways the necessary components are actively synthesized upon cell death induction [32]. Which of these pathways is effective in EGF-mediated programmed cell death of MDA-MB-468 cells was determined by measuring the number of surviving cells upon EGF treatment for 30 h in the presence and absence of cycloheximide. In the presence of cycloheximide the number of surviving cells was about twofold higher than in its absence (fig. 3), suggesting that de novo protein synthesis was at least in part necessary for cell death to occur. Our results indicate that programmed cell death in this system is an active process which depends on the induction of a new set of genes.

Induction of gene expression upon EGF and/or serum treatment. Many genes have been identified which are expressed during programmed cell death and which might actively contribute to this process. In several systems the expression of various *fos* and *jun* proto-oncogenes has been implicated in cell death. We measured the kinetics and complexity of the different members of the *fos* and *jun* families of genes in MDA-MB-468 cells after EGF treatment. *fos* and *jun* genes are immediate early-response genes induced in many different cell types by a wide variety of agents such as mitogens, differentiation factors, pharmacological agents, and also stress and heat. The induction is mostly

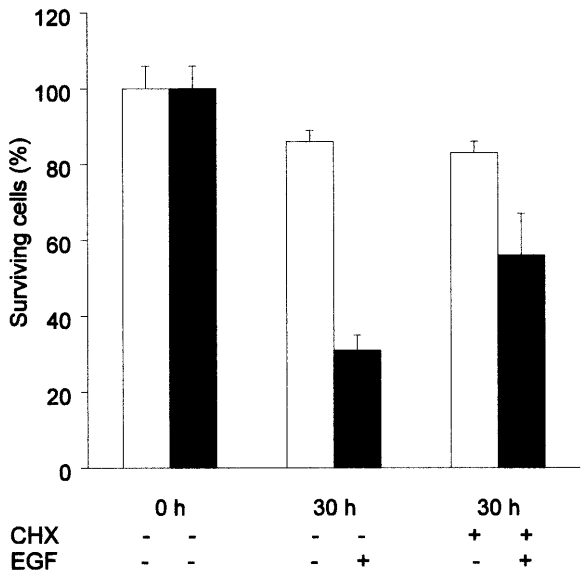

Figure 3. Programmed cell death is dependent on de novo protein synthesis. Growth-arrested MDA-MB-468 cells were treated with 25 ng/ml of EGF, with 2 μg/ml of cycloheximide (CHX) or with a combination of both as indicated. Cells were counted in a Coulter Counter, and the percentages of surviving cells are depicted. The error bars represent standard deviations from three independent plates.

rapid and transient, and it occurs at the level of transcription. A rapid and transient induction of c-*fos*, c-*jun* and *junB* mRNA was observed upon EGF treatment of quiescent MDA-MB-468 cells (fig. 4). The expression of these genes was apparent within 0.5 h after EGF addition, reaching maximum levels by 1–2 h, and c-*fos* mRNA was essentially undetectable at 4, 8 and 16 h. The expression of c-*jun* and *junB* was also transient, but mRNAs were still detected after 8 and 16 h. Induction of *fosB* and *fra*-1 was slightly delayed reaching maximum levels by 1–2 h (*fosB*) and 2–4 h (*fra*-1). The level of *fosB* mRNA rapidly decreased within 4 h, while *fra*-1 mRNA was maintained until 8–16 h of treatment. The level of *junD* expression was detectable in untreated cells; it was induced after 0.5 h and then remained relatively constant for 8–16 h. The prolonged expression of *junD* is in contrast to the findings of Armstrong et al. [6], and the difference may be a consequence of the serum-free growth conditions used in our system. Stimulation of quiescent cells with growth factors by the addition of fetal calf serum resulted in a very similar accumulation of all the various *fos* and *jun* genes but no apparent cell death (data not shown). A combination of EGF and serum also stimulated the expression of *fos* and *jun* genes, again with very limited cell death (data not shown).

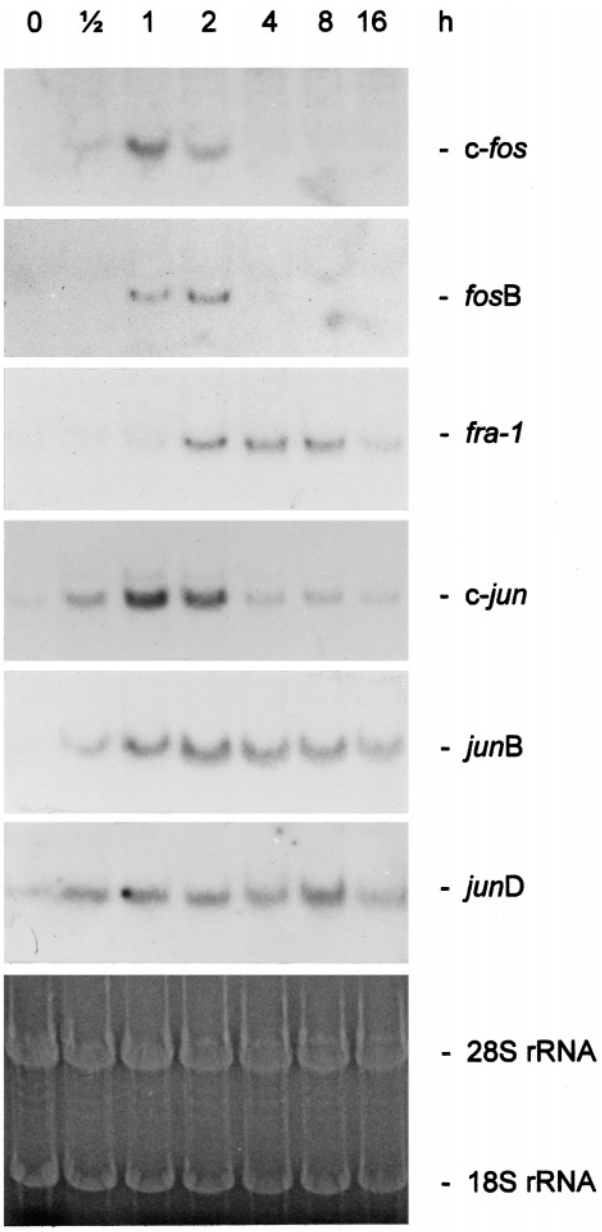

Figure 4. Northern blot analyses depicting the level of expression of *fos* and *jun* genes after stimulation of growth-arrested MDA-MB-468 cells with 25 ng/ml of EGF for the indicated periods of time. Ten micrograms of total RNA per lane were separated electrophoretically, transferred to nitrocellulose membranes and hybridized with ^{32}P-labelled DNA probes specific for c-*fos*, *fos*B, *fra*-1, c-*jun*, *jun*B and *jun*D as indicated. 28S rRNA and 18S rRNA were visualized by acridine orange staining and are shown as loading controls.

EGF-induced formation of AP-1 DNA-binding complexes. Whether the various *fos* and *jun* mRNAs are translated and whether the respective proteins participate in AP-1 complex formation and DNA binding was determined by bandshift analysis (fig. 5). Bandshift analysis using an oligodeoxynucleotide specific for AP-1 showed a basal level in quiescent cells and a substantial increase in AP-1 DNA binding within 0.5 h of EGF treatment which persisted until at least 8 h of treatment (fig. 5B). The AP-1 DNA binding activity reached a maximum by 4 h. In order to reveal the composition of AP-1 at different time points of EGF treatment, Fos and Jun proteins involved in DNA binding were determined by antibody interference assays. Binding of the antibody to AP-1 results in inhibition of AP-1 binding to DNA or to the formation of a larger complex with reduced mobility (supershift). The interference assays were performed with specific antibodies to individual Fos and Jun proteins. αc-Fos antibodies competed with DNA binding mainly at 1 to 4 h. The inhibition was further analysed by semiquantitative densitometry (for details see 'Materials and methods'). In the presence of αc-Fos a reduction of AP-1 DNA binding of about 20% at 1–2 h and 40% at 4 h was determined (fig. 5A). Fra-1 DNA binding was competed with αFra-1 antibodies at 2, 4 and 8 h (fig. 5A) by about 25%. The relatively late detection of Fra-1 in the AP-1 complex is in close agreement with the mRNA data (fig. 4). A significant fraction of the AP-1 complex contained Fra-2, as a reduction of about 40% was measured 2, 4 and 8 h after EGF treatment (fig. 5B). No significant participation of FosB in the AP-1 complex could be measured by antibody interference (fig. 5B). The composition of the AP-1 DNA-binding complex was further characterized after 4 h of EGF treatment by the simultaneous addition of all four Fos-specific antibodies (αc-Fos, αFosB, αFra-1 and αFra-2) to the assay which efficiently prevented AP-1 DNA binding (fig. 5B) and resulted in the formation of a supershifted complex migrating more slowly in the gel. This result indicates that Fos proteins are major components of the AP-1 DNA binding complex, and several Fos members seem to contribute to a similar extent to the formation of AP-1. The AP-1 complexes were similarly tested for the presence of c-Jun and JunD. A partial competition of JunD could be observed at all time points of EGF treatment (fig. 5C). For c-Jun no consistent impairment of DNA binding could be obtained with this antibody interference assay. The addition of a nonrelated antibody (αBcl-2) had no effect on AP-1 DNA binding at 4 and 8 h (fig. 5A). The decrease seen at 2 h was not consistently observed and most likely reflects an experimental variation.

As a control the DNA binding activity of the ubiquitously expressed transcription factor Oct-1 was measured in the same nuclear extracts. Oct-1 is involved in the regulation of H2b and small nuclear RNA genes [33]. As can be seen from fig. 5D Oct-1 DNA binding activity did not change within 8 h of EGF treatment. The specificity of DNA binding was confirmed by preincubation with αOct-1 antibodies prior to DNA binding resulting in very efficient inhibition of Oct-1 DNA binding (fig. 5D).

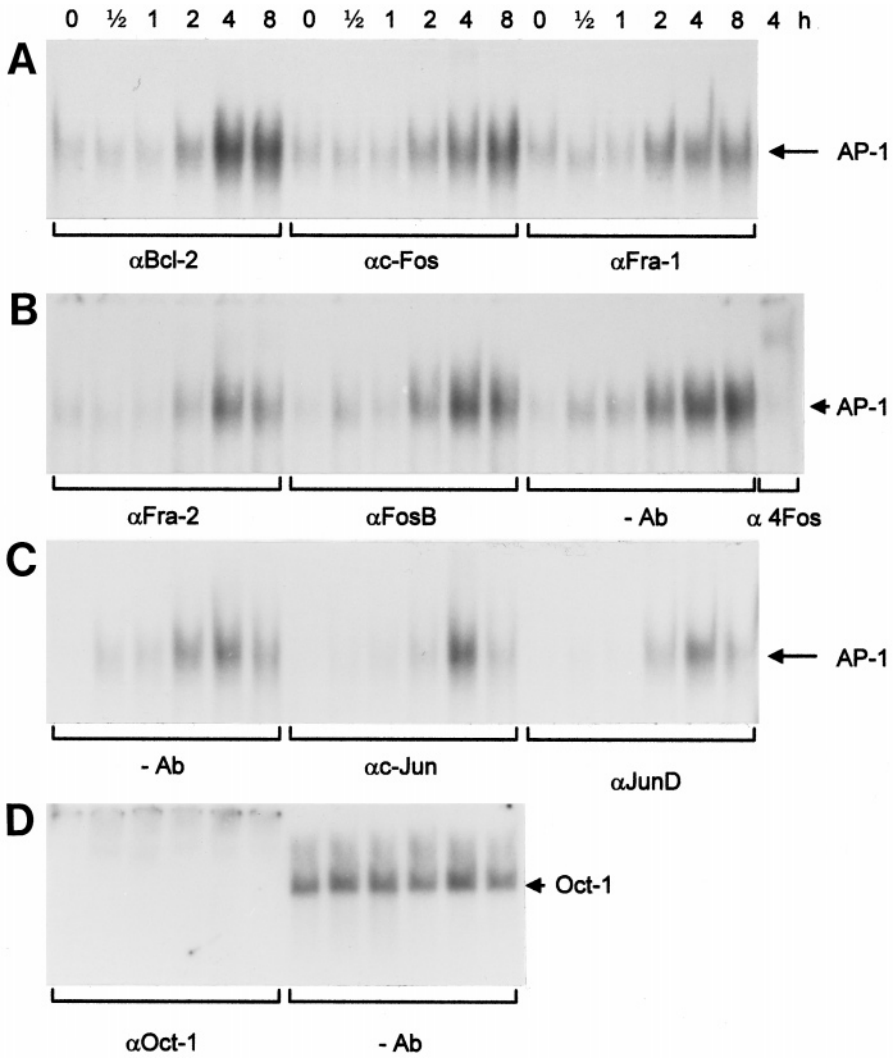

Figure 5. DNA binding activities of AP-1 and Oct-1. Growth-arrested cells were treated with 25 ng/ml of EGF for the indicated periods of time and nuclear proteins were extracted. Five micrograms of nuclear proteins were assayed for DNA binding with ^{32}P-end-labelled double-stranded oligodeoxynucleotides containing a consensus binding site for AP-1 (*A*, *B*, *C*) or Oct-1 (*D*). The composition of the AP-1 DNA binding complex was determined by antibody interference assays using specific antibodies directed against c-Fos or Fra-1 (*A*), Fra-2 or FosB (*B*), or c-Jun or junD (*C*) as described in 'Materials and methods'. A nonrelated αBcl-2 antibody was used as control. The specificity of Oct-1 DNA binding was confirmed with polyclonal rabbit serum raised against Oct-1 (*D*). Protein/DNA complexes were resolved by gel electrophoresis and visualized by autoradiography. Only the areas containing protein/DNA complexes are shown. The results of one out of three independent analyses are shown.

Discussion

The present study confirms that EGF receptor-overexpressing MDA-MB-468 human breast cancer cells undergo limited cell death upon EGF treatment. Cell death was greatly potentiated when cells were growth-arrested by serum deprivation before treatment. Cell death was characterized by rounding up of the cells that was followed by detachment from the substrate. Oligonucleosomal DNA fragments and nuclear fragmentation and condensation, which are characteristic of apoptotic cell death, appeared relatively late during EGF treatment and were only detected in detached cells floating in the culture medium. Apoptosis was shown to be at least in part dependent on active protein synthesis, and cell death was preceded by induction of *fos* and *jun* gene expression and AP-1 DNA binding activity. Antibody interference assays showed that various Fos and Jun proteins are contained at similar levels in the DNA binding AP-1 complex. The DNA binding of the ubiquitously expressed Oct-1 transcription factor did not decrease within the same period of investigation, confirming that the changes in AP-1 DNA binding activity

occurred while basic nuclear processes were not or not yet affected by the process of cell death.

The fact that programmed cell death was greatly enhanced in growth-arrested cells as compared with exponentially growing cells suggests that growth factors provided by the serum prevented cells from undergoing EGF-mediated apoptosis or, alternatively, that serum starvation itself predisposed cells to apoptosis. Serum deprivation is a powerful mechanism by which cells can be arrested in G_1, and in some cell types this was shown to be sufficient to induce programmed cell death [34]. In MDA-MB-468 cells, serum withdrawal alone led to growth arrest and did not promote cell death. We present evidence that EGF treatment of serum-deprived, growth-arrested cells greatly enhances programmed cell death, raising the question by what mechanism growth arrest contributes to cell death. It is possible that the addition of EGF to growth-arrested MDA-MB-468 cells induces a strong growth-promoting signal, most probably through activation of the Ras/ERK1/2 MAPK pathway which in the absence of additional, essential growth-promoting signals fails to induce cell cycle progression and proliferation, and instead provokes a situation of strong conflicting signals forcing the cells into programmed cell death. In fact, addition of growth factors (serum) alone or in combination with EGF was paralleled by a similar induction of *fos* and *jun* gene expression and stimulation of proliferation rather than cell death (data not shown). The expression of *fos* and *jun* genes probably contributes to progression of the cells from a G_0-like state to an early G_1 phase of the cell cycle during both processes. It would seem that at this stage the cells retain the capacity either to progress in the cell cycle and to proliferate or to enter a programme that leads to cell death. It may be noteworthy that short EGF pulses of 1–8 h to quiescent cells had consequences very similar to those of continuous exposure to EGF for 48 h (data not shown). This may imply that MDA-MB-468 cells are committed to cell death during an early phase of EGF treatment, but cell death will only become apparent 24–48 h later.

Several reports document that serum withdrawal results in a profound and progressive elevation in endogenous levels of ceramide that leads to arrest in G_0/G_1 [35]. In fact, ceramide has also been shown to be an important regulatory participant in programmed cell death induced by TNFα and Fas ligand [36, 37] by stimulating sphingomyelinase, which in turn catalyses the degradation of sphingomyelin to ceramide. Increasing numbers of studies are revealing the physiological role of ceramide signalling. In several cell lines ceramide was shown to activate JNK [c-Jun NH_2-terminal protein kinase, also termed 'stress-activated protein kinase' (SAPK)] [38–40]. Since c-Jun was identified as a downstream target of ceramide signalling [41], it may be that a JNK-mediated transactivation of c-Jun [42] increases the activity of AP-1. In fact, ceramide-initiated JNK signalling was found to be required in stress-induced apoptosis [40], and AP-1 was shown to be critical for ceramide-induced apoptosis [43]. We showed that EGF-mediated cell death of growth-arrested MDA-MB-468 cells is preceded by strong activation of all known *fos* and *jun* genes including c-*jun*, and it is possible that the growth arrest potentiates the activity of AP-1 via elevated activity of JNK.

At the present time it is not clear whether additional factors in the serum confer survival, thus counteracting the apoptotic effect of EGF, or whether AP-1 is indeed an essential factor for both pathways in MDA-MB-468 cells. Several reports have claimed that c-Fos and/or c-Jun are essential factors for programmed cell death in vitro. Preston et al. [44] showed that c-Fos plays a causal role in the activation of apoptosis in different cell lines, and Colotta et al. [45] found that c-*fos* and c-*jun* are involved in programmed cell death induced by growth factor deprivation in lymphoid cell lines. In addition, serum-deprived fibroblasts underwent apoptosis when c-Fos was induced, but the cells survived when c-Fos was not induced [46]. AP-1 was also shown to be involved in apoptosis occurring in nerve growth factor (NGF)-deprived neurons. After NGF deprivation c-*jun* was found to be induced in all neurons, whereas c-*fos* was restricted to neurons undergoing chromatin condensation [47]. These neurons could be protected from cell death by the injection of neutralizing antibodies specific for either c-Jun or the Fos family (c-Fos, FosB, Fra-1 or Fra-2), whereas antibodies specific for JunB and JunD had no protective effect [47]. We used antisense oligodeoxynucleotides directed against the coding region of c-*fos* mRNA to treat MDA-MB-468 cells before and during exposure to EGF, and we were able to show a substantial block in c-Fos protein levels in response to treatment (data not shown). However, the kinetics and extent of cell death were not affected by this treatment. It may be insufficient to block the synthesis of a single member of the Fos family in MDA-MB-468 cells, and in agreement with our findings, it was found that antibodies against individual Fos members were only effective in combination in protecting neurons from cell death [47], suggesting that other *fos* members such as *fos*B, *fra*-1 and *fra*-2 are able to replace c-*fos*.

Limited information is available relating c-Fos expression to programmed cell death in vivo. A correlation between cell death and c-Fos expression was found during development when cells from the interdigital web, heart valve and peridermal cells die by apoptosis [46, 48]. In the adult, c-Fos expression was associated with cell death in skin, hair follicles and the ovary [25]. Recently, Hafezi et al. [26] showed that light-induced apoptosis of retinal photoreceptor cells is suppressed in

mice lacking a functional c-*fos* gene, providing direct evidence that c-Fos is an essential mediator of apoptosis in the retina. Furthermore, c-Fos seems to be a mandatory factor for programmed cell death in the mouse prostate upon castration (Feng et al., unpublished observations).

The data presented here suggest that the consequences of elevated levels of transcription factor AP-1 may be complex and depend on several parameters such as the actual composition of the complex, the presence or absence of additional factors and the developmental state of the cell.

Acknowledgements. We thank N. E. Davidson (USA) for providing the MDA-MB-468 cells, W. Schaffner (Switzerland) for the αOct-1 rabbit polyclonal serum, A. Marti for helpful discussions, R. R. Friis and A.-C. Andres for critical reading the manuscript and A. Baltzer for photographic work. This work was supported by the Swiss National Science Foundation, the Bernese Cancer League and the Foundation for Clinical and Experimental Cancer Research (Switzerland).

1 Kawamoto T., Mendelsohn J., Le A., Sato G. H., Lazar C. S. and Gill G. N. (1984) Relation of EGF receptor concentration to growth of human epidermoid carcinoma A431 cells. J. Biol. Chem. **259:** 7761–7766
2 Luciano A. M., Pappalardo A., Ray C. and Peluso J. J. (1994) Epidermal growth factor inhibits large granulosa cell apoptosis by stimulating progesterone synthesis and regulating the distribution of intracellular free calcium. Biol. Reprod. **51:** 646–654
3 Nakajima M., Kashiwagi K., Ohta J., Furukawa S., Hayashi K., Kawashima T. et al. (1994) Nerve growth factor and epidermal growth factor rescue PC12 cells from programmed cell death induced by etopside: distinct modes of protection against cell death by growth factors and a protein-synthesis inhibitor. Neuroscience **176:** 161–164
4 Coles H. R. S., Burne J. F. and Raff M. C. (1993) Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development **118:** 777–784
5 Gulli L. F., Palmer C. K., Chen Y. Q. and Reddy K. B. (1996) Epidermal growth factor induced apoptosis in A431 cells can be reversed by reducing the tyrosine kinase activity. Cell Growth Differ. **7:** 173–178
6 Armstrong D. K., Kaufmann S. H., Ottaviano Y. L., Furuya Y., Buckley J. A., Isaacs J. T. et al. (1994) Epidermal growth factor-mediated apoptosis of MDA-MB-468 human breast cancer cells. Cancer Res. **54:** 5280–5283
7 Brabyn C. J., Franks D. J. and Kleine L. P. (1994) Long-term exposure to epidermal growth factor results in apoptosis in T51B cells. Biochem. Cell Biol. **72:** 429–438
8 Gullick W. J. (1991) Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br. Med. Bull. **47:** 87–98
9 Davidson N. E., Gelman E. P., Lippman M. E. and Dickson R. B. (1987) Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol. Endocrinol. **1:** 216–223
10 Chrysogelos S. A. and Dickson R. B. (1994) EGF receptor expression, regulation and function in breast cancer. Breast Cancer Res. Treat. **29:** 29–40
11 Pathak S., Siciliano M. J., Cailleau R., Wiseman C. L. and Hsu T. L. (1979) A human breast adenocarcinoma with chromosome and isoenzyme markers similar to those of the HeLa line. J. Natl. Cancer Inst. **62:** 263–271
12 Filmus J., Pollak M. W., Cailleau R. and Buick R. N. (1985) MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem. Biophys. Res. Commun. **128:** 898–904
13 Wyllie A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature **284:** 555–556
14 Arends M. J., Morris R. G. and Wyllie A. H. (1990) Apoptosis: the role of the endonuclease. Am. J. Pathol. **136:** 593–608
15 Giannakis C. C., Forbes I. J. and Zalewskiet P. D. (1991) Ca^{2+}/Mg^{2+}-dependent nuclease: tissue distribution, relationship to internucleosomal DNA fragmentation and inhibition by Zn^{2+}. Biochem. Biophys. Res. Commun. **181:** 915–920
16 Peitsch M. C., Polzar B., Stephan H., Crompton T., MacDonald H. R., Mannherz H. G. et al. (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J. **12:** 371–377
17 Ueda N. and Shah S. V. (1994) Apoptosis. J. Lab. Clin. Med. **124:** 169
18 Kerr J. F., Winterford C. M. and Harmon B. V. (1994) Apoptosis: its significance in cancer and cancer therapy. Cancer **73:** 2013–2026
19 Oppenheim R. W., Houenou L. J., Johnson J. E., Lin L. F., Li L., Lo A. C. et al. (1995) Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature **373:** 344–346
20 Walker N. I., Bennet R. E. and Kerr J. F. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. Am. J. Anat. **185:** 19–32
21 Strange R., Li F., Saurer S., Burkhardt A. and Friis R. R. (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development **115:** 49–58
22 Marti A., Jehn B., Costello E., Keon N., Ke G., Martin F. et al. (1994) Protein kinase-A and AP-1 (c-Fos/JunD) are induced during apoptosis of mouse mammary epithelial cells. Oncogene **9:** 1213–1223
23 Buttyan R., Zakeri Z., Lockshin R. and Wolgemuth D. (1988) Cascade induction of c-*fos*, c-*myc* and heat shock 70K transcripts during regression of the rat ventral prostate gland. Mol. Endocrinol. **2:** 650–657
24 Gonzales-Martin C., de Diego I., Crespo D. and Fairen A. (1992) Transient c-*fos* expression accompanies naturally occuring cell death in the developing interhemispheric cortex of the rat. Dev. Brain Res. **68:** 83–95
25 Smeyne R. J., Schilling K., Robertson L., Luk D., Oberdick J., Curran T. et al. (1992) Fos-LacZ transgenic mice: mapping sites of gene induction in the central nervous system. Neuron **8:** 13–23
26 Hafezi F., Steinbach J., Marti A., Munz K., Aguzzi A. and Remé C. E. (1997) Suppression of light-induced apoptosis of retinal photoreceptors in mice lacking *c-fos*. Nature Med. **3:** 346–349
27 Chomczynski P. and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159
28 Jehn B., Costello E., Marti A., Keon N., Deane R., Li F. et al. (1992) Overexpression of Mos, Ras, Src and Fos inhibits mouse mammary epithelial cell differentiation. Mol. Cell. Biol. **12:** 3890–3902
29 Risse G., Jooss K., Neuberg M., Brüller H. J. and Müller R. (1989) Asymmetrical recognition of the palindromic AP1 binding site (TRE) by Fos protein complexes. EMBO J. **12:** 3825–3832
30 LeBowitz J. H., Kobayashi T., Staudt L., Baltimore D. and Sharp P. A. (1988) Octamer-binding proteins from B or HeLa cells stimulate transcription of the immunoglobulin heavy-chain promoter in vitro. Genes Dev. **2:** 1227–1237
31 Church J. G. and Buick R. N. (1988) G-protein-mediated epidermal growth factor signal transduction in a human breast cancer cell line. Evidence for two intracellular pathways distinguishable by pertussis toxin. J. Biol. Chem. **25:** 4242–4246
32 Cohen J. J. (1993) Apoptosis. Immunol. Today **14:** 126–130
33 Fletcher C., Heintz N. and Roeder R. G. (1987) Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell **51:** 773–781

34 Howard M. K., Burke L. C., Mailhos C., Pizzey A., Gilbert C. S., Lawson W. D. et al. (1993) Cell cycle arrest of proliferating neuronal cells by serum deprivation can result in either apoptosis or differentiation. J. Neurochem. **60:** 1783–1791
35 Jayadev S., Liu B., Bielawska A. E., Lee J. Y., Nazaire F., Pushkareva M. Y. et al. (1995) Role for ceramide in cell cycle arrest. J. Biol. Chem. **270:** 2047–2052
36 Hannun Y. A. and Obeid L. M. (1995) Ceramide: an intracellular signal for apoptosis. Trends Cell Biol. **20:** 73–77
37 Obeid L. M., Linardic C. M., Karolak L. A. and Hannun Y. A. (1993) Programmed cell death induced by ceramide. Science **259:** 1769–1771
38 Cuvillier O., Pirianov G., Kleuser B., Vanek P. G., Coso O. A., Gutkind S. et al. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature **381:** 800–803
39 Kyriakis J. M., Banerjee P., Nikolakaki E., Dai T. A., Rubie E. A., Ahmad M. F. et al. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature **369:** 156–160
40 Verheij M., Bose R., Lin X. H., Yao B., Jarvis W. D., Grant S. et al. (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature **380:** 75–79
41 Ballou L. R., Laulederkind S. J., Rosloniec E. F. and Raghow R. (1996) Ceramide signalling and the immune response. Biochim. Biophys. Acta **1301:** 273–287
42 Dérijard B., Hibi M., Wu I. H., Barrett T., Su B., Deng T. L. et al. (1994) Jnk1 – a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:** 1025–1037
43 Sawai H., Okazaki T., Yamamoto H., Okano H., Takeda Y., Tashima M. et al. (1995) Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J. Biol. Chem. **270:** 27326–27331
44 Preston G. A., Lyon T. T., Yin Y., Lang J. E., Solomon G., Annab L. et al. (1996) Induction of apoptosis by c-Fos protein. Mol. Cell Biol. **16:** 211–218
45 Colotta F., Polentarutti N., Sironi M. and Mantovani A. (1992) Expression and involvement of c-*fos* and c-*jun* protooncogenes in programmed cell death induced by growth factor deprivation in lymphoid cell lines. J. Biol. Chem. **267:** 18278–18283
46 Smeyne R. J., Vendrell M., Hyward M., Baker S. J., Miao G. G., Schilling K. et al. (1993) Continuous c-*fos* expression precedes programmed cell death in vivo. Nature **363:** 166–169
47 Estus S., Zaks W. J., Freeman R. S., Gruda M., Bravo R. and Johnson E. M. Jr. (1994) Altered gene expression in neurons during programmed cell death: identification of c-*jun* as necessary for neuronal apoptosis. J. Cell Biol. **127:** 1717–1727
48 Schilling K., Luk D., Morgan J. I. and Curran T. (1991) Regulation of a *fos*-lacZ fusion gene: a paradigm for quantitative analysis of stimulus-transcription coupling. Proc. Natl. Acad. Sci. USA **88:** 5665–5669