Induction of CCAAT/Enhancer-binding Protein (C/EBP)-homologous Protein/Growth Arrest and DNA Damage-inducible Protein 153 Expression during Inhibition of Phosphatidylcholine Synthesis Is Mediated via Activation of a C/EBP-activating Transcription Factor-responsive Element*

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The gene for the proapoptotic transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible protein 153 (CHOP/GADD153) is induced by various cellular stresses. Previously, we described that inhibition of phosphatidylcholine (PC) synthesis in MT58 cells, which contain a temperature-sensitive mutation in CTP:phosphocholine cytidylyltransferase (CT), results in apoptosis preceded by the induction of CHOP. Here we report that prevention of CHOP induction, by expression of antisense CHOP, delays the PC depletion-induced apoptotic process. By mutational analysis of the conserved region in the promoter of the CHOP gene, we provide evidence that the C/EBP-ATF composite site, but not the ER stress-responsive element or the activator protein-1 site, is required for the increased expression of CHOP during PC depletion. Inhibition of PC synthesis in MT58 cells also led to an increase in phosphorylation of the stress-related transcription factor ATF2 and the stress kinase JNK after 8 and 16 h, respectively. In contrast, no phosphorylation of p38 MAPK was observed in MT58 cultured at the nonpermissive temperature. Treatment of MT58 cells with the JNK inhibitor SP600125 could rescue the cells from apoptosis but did not inhibit the phosphorylation of ATF2 or the induction of CHOP. Taken together, our results suggest that increased expression of CHOP during PC depletion depends on a C/EBP-ATF element in its promoter and might be mediated by binding of ATF2 to this element.

Phosphatidylcholine (PC) constitutes the major portion of cellular phospholipids in eukaryotic cells. PC serves as a major structural building block of biological membranes, and PC has also been recognized as an important source for signaling molecules (1–3). In eukaryotic cells, PC is made primarily through the CDP-choline pathway in which CTP:phosphocholine cytidylyltransferase (CT) is the key enzyme (4). CT localizes mainly to the endoplasmic reticulum (ER) and nucleus (5). CT activity in cells is regulated primarily by association with membrane lipids; translocation between ER, cytoplasm, and nucleus (6–10); and gene expression (8, 9, 11, 12). Inhibition of PC biosynthesis leads to perturbations in PC homeostasis, which eventually influences cell proliferation and cell death (13–15). Treatment of cells with inhibitors of the enzymes of the CDP-choline pathway results in an inhibition of cell growth and increased apoptosis (16–18). Likewise, in MT58, a mutant Chinese hamster ovary (CHO) cell line containing a thermo-sensitive mutation in CT (19–21), inhibition of PC synthesis causes apoptosis (14). The death of MT58 cells reveals a link between PC homeostasis and apoptosis. In a previous study, we have shown that MT58 cells, incubated at the nonpermissive temperature, express a high level of C/EBP-homologous protein (CHOP), also known as growth arrest and DNA damage-inducible protein 153 (GADD153), preceding apoptosis (22). CHOP encodes a nuclear, proapoptotic, bZIP transcription factor of the CCAAT/enhancer-binding protein (C/EBP) transcription factor family, which is often induced by cellular stress (23–25). CHOP activity is regulated by both changes in expression level and post-translational events, such as phosphorylation (24, 26, 27). Upon phosphorylation, CHOP dimerizes with other C/EBP transcription factors, and these stable heterodimers are capable of recognizing novel DNA targets (24, 26–30). In general, CHOP induction in cells is often associated with perturbations that culminate in the induction of stress in the ER, the so-called unfolded protein response (31–34). The classical ER stress response results in transcriptional activation of genes coding for the ER chaperone Bip (also known as GRP78), CHOP and caspase 12 (34–38), and inhibition of protein synthesis by phosphorylation of eIF2a by the ER-resident protein kinase PERK (39). However, CHOP induction in PC-depleted MT58 cells was not accompanied by any of these classical ER stress response events (22). Therefore, the route to induction of CHOP by PC depletion seems distinct from the ER stress signaling cascade, but the exact pathway is still not identified.

The present study was designed to identify possible transcription-activating pathways that induce CHOP expression during inhibition of PC synthesis. The CHOP promoter region...
contains several elements, responsive to various stresses. Transcriptional induction of the CHOP gene in response to ER stress is mediated by a defined DNA element, the ER stress-responsive element (ERSE) (30). This ERSE-containing region in the human CHOP promoter, comprising nucleotides −104 to −75, is required for both constitutive and ER stress-induced expression. Other important stress-responsive elements in the promoter of the CHOP gene are an AP-1 site (40) and a C/EBP-ATF composite site (41–43). The AP-1 site, located between nucleotides −250 and −225 of the human CHOP promoter, has been described to have a significant role in transcriptional activation of CHOP during oxidative stress and DNA damage (40). UV, arsenite, and H$_2$O$_2$ treatment of cells stimulate c-Fos and c-Jun phosphorylation and the formation of heterodimeric c-Jun/c-Fos complexes. Binding of the c-Jun/c-Fos heterodimer to the AP-1 site results in transcriptional activation of CHOP (40). c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (p38 MAPK), both belonging to the family of stress-activated MAP protein kinases, were identified as being responsible for the phosphorylation of c-Jun during CHOP induction (27, 44). Furthermore, both kinases were described to play a role in the post-translational phosphorylation of CHOP upon activation during stress (26, 27). The C/EBP-ATF composite site has been described as regulating the expression of CHOP during arsenite treatment as well (43). Furthermore, a C/EBP-a role in the post-translational phosphorylation of CHOP upon activated MAP protein kinases, were identified as being responsive to CHOP expression during PC depletion. Furthermore, we found that JNK is active in MT58 cells at the nonpermissive temperature, but this stress kinase seems not to be involved in the induction of CHOP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ham’s F-12 medium, fetal bovine serum, and calcium-free phosphate-buffered saline (PBS) were purchased Invitrogen, and culture dishes and flasks were from Nunc Inc. (Rochester, NY). SP600125 JNK/SAPK kinase inhibitor and SB203580 p38 MAPK inhibitor were obtained from Tocris (Bristol, UK). Penicillin, streptomycin, G418 neomycin, trypsin/EDTA solution, and all other chemicals were from Sigma. Polyclonal anti-actin, polyclonal anti-phospho-ATF2, polyclonal anti-GADD153 (CHOP), and monoclonal anti-phospho-c-Jun were provided by Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Polyclonal antibodies against JNK/SAPK kinases, phospho-JNK/SAPK kinases, and phospho-p38 MAPK were purchased from New England Biolabs (Hertfordshire, UK). The Supersignal chemiluminescent substrate kit (ECL) for detection of proteins on immunoblots and the Coomassie® Plus Protein assay reagent kit were supplied by Pierce.

**Generation of Antisense CHOP-expressing MT58 Cells**—For the construction of an antisense CHOP expression vector, an 847-bp DNA fragment, containing the CHOP gene was excised from BamHI from pD-X vector containing the cDNA insert for hamster CHOP (kindly donated by Dr. N. Holbrook) and subcloned into BamHI-digested pDNA3.1 expression vector. Clones were checked for the proper antisense orientation by digestion with HindIII and PvuII. Positive clones were sequenced to confirm the construction of an antisense CHOP-containing expression vector, or empty pDNA3.1 vector using Lipofectamine reagent (Invitrogen). Individual neomycin-resistant colonies were selected with 600 μg/ml G418 in Ham’s F-12 medium. Single colonies were picked and grown as individual cell lines in the presence of 300 μg/ml G418. Positive clones were identified by Western blotting for CHOP protein after induction by shifting cells to the nonpermissive temperature.

**Cell Culture**—Wild-type CHO-K1, MT58 (15, 20, 22), and CT-expressing MT58 (46) cell lines were cultured in Ham’s F-12 medium supplemented with 7.5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Stable cell lines expressing antisense CHOP vector constructs were generated in Ham’s F-12 medium supplemented with 7.5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml G418. All cells were maintained in 80-cm² flasks at 33 °C, 5% CO$_2$, and 90% relative humidity and subcultured twice a week, and media were changed every 2–3 days.

**Plasmids Used for Analysis of the CHOP Promoter—**pGL3 basic plasmids with the −649 to +91, −442 to +91, and −221 to +91 human CHOP promoter regions fused to the luciferase gene were a gift of Dr. P. Fafournoux (29, 42). The mutant CHOP promoter constructs were generated by substitution of nucleotides ACTC of the core sequence in the AP-1 CHOP promoter construct with two 6-bp nucleotides TCGG and AGACACCGGTGGC (42). The mutant AP-1 CHOP promoter construct was generated by substitution of nucleotides ACTC of the core sequence in the AP-1 site into GATA and with the help of a mutagenesis primer, coded by the following nucleotide sequence: 5'-AGACACCGGTGGCAGTTTCTCCCGC-3'. The mutant AP-1 CHOP promoter construct was generated by substitution of nucleotides ACTC of the core sequence in the AP-1 site into CAGA with the help of a mutagenesis primer, nucleotide sequence 5'-GCGCGGCTGCTTGTGAGTTT-3'. The mutant ERSE CHOP promoter construct was generated by changing nucleotides ATAC of the ERSE core sequence into GAAT with the help of a mutagenesis primer, coded by the following nucleotide sequence: 5'-GCGCGGCTGCTTGTGAGTTT-3'. All steps of the mutagenesis reaction were performed according to the manufacturer's instructions. All of the constructs were sequenced before utilization.

**DNA Transfection and Luciferase Assay—**Cells were transfected by using Lipofectamine Plus reagent according to the protocol supplied by Promega. Relative luciferase activity was measured according to the protocol supplied by Promega. Relative luciferase activity was given as a ratio of relative light units (disintegrations/min) to relative β-galactosidase units (O-nitrophenyl β-D-galactopyranoside). All values are the means of at least three independent experiments.

**Electrophoretic Mobility Shift Assay (EMSA)—**For EMSA, nuclear proteins were harvested in principle as described (29). Briefly, confluent cells grown in 185-cm² flasks were washed twice with 20 ml of ice-cold PBS, pH 7.4. Cells were scrapped into 1 ml of PBS and pelleted at 1000 rpm for 5 min. The pellet was resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH, pH 7.9, 4 °C, 1.5 mM MgCl$_2$, 10 mM KC$_2$, 0.6 mM EDTA, and 1 mM dithiothreitol) and incubated for 10 min on ice. The samples were centrifuged for 10 s at 14,000 rpm, and the pellet was washed and resuspended in 20 μl of cold buffer A. The mixture was incubated at 4 °C for 10 min, and the total cell extract (10 μl) was incubated with 2 μl of nuclear extract for 30 min at room temperature. For EMSA, 2 μl of nuclear extract were mixed with 2 μl of 5× EMSA buffer (10 mM HEPES-KOH, pH 8.0, 3.9 mM MgCl$_2$, 185 mM NaCl, 1% Nonidet P-40, 0.5 mM EDTA, and 0.5 mM dithiothreitol). After incubation for 4 h on ice, the samples were subjected to electrophoresis. The gels were stained with silver nitrate, scanned, and analyzed.
resuspended in 80 μl of cold buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 450 mM NaCl, 1.5 mM MgCl₂, 0.6 mM EDTA, and 1 mM dithiothreitol) and incubated on ice for 30 min. After centrifugation (2 min, 4 °C, 14,000 × g) the supernatant was quick frozen at −80 °C and stored at −80 °C. Gel mobility shift assays were performed by incubating 0.5 ng of the C/EBP-ATF site oligonucleotides (5′-AACATGCATCATCCCCGC-3′) labeled with 32P in a 15-μl reaction mixture (47) with 3 μg of nuclear extract for 30 min at room temperature. After this incubation, reaction products were loaded on a 6% polyacrylamide, 0.25× TBE gel and electrophoresed at 350 V for 2.5 h at 4 °C. For supershift analysis, 2 μg of specific antibody was added to the incubation mixture at room temperature 1 h prior to the addition of the labeled probe.

Cell rescue experiments and quantification of apoptosis—Cells were plated at 3 × 10⁵ cells in 60-mm dishes containing 5 ml of medium and incubated at 33 °C or the nonpermissive temperature of 40 °C. At the indicated time points, 20 μl of cell suspension in PBS was lysed with 50 μl of SDS-PAGE sample buffer (final concentration: 62.5 mM Tris, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue, pH 6.8). Cell protein was measured using the CoomassiePlus protein assay reagent kit (Pierce) according to the manufacturer’s instructions, using bovine serum albumin as a standard. After boiling samples for 10 min, 8–10 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose membranes.

The membranes were blocked with 1% Western blocking reagent (Roche Applied Science) for 1 h and exposed to rabbit polyclonal anti-GADD153 (CHOP), anti-phospho-JNK/SAPK kinases, or anti-phospho-p38 MAPK (all diluted 1:1000), mouse monoclonal anti-phospho-c-Jun (dilution 1:250), or goat polyclonal anti-actin (dilution 1:500). Following four washing steps with TBS-Tween (50 mM Tris, 150 mM NaCl, 1% Tween 20, pH 7.5), blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were washed four times with TBS-Tween, and the respective proteins were detected by a reaction on Supersignal West Pico chemiluminescence substrate (Pierce) and exposure to x-ray films.

Results

Inhibition of CHOP expression in MT58 cells postulates the onset of apoptosis—in a previous study, we have shown that inhibition of PC synthesis results in a strong increase of CHOP protein after 16 h, preceding apoptosis (22). Therefore, CHOP could play an important role in the transcriptional activation of apoptotic downstream targets during PC depletion. To investigate this, MT58 cells were stably transfected with a control plasmid or a plasmid containing antisense CHOP cDNA. The transfected cells were exposed for 24 h to the nonpermissive temperature of 40 °C. The antisense CHOP-overexpressing MT58 cells did not express CHOP to the same high level when cultured at 40 °C as observed in control transfected MT58 and previously (22) in nontransfected MT58 cells (see Fig. 1A). Next, we assessed the effect of overexpression of antisense CHOP mRNA in MT58 on apoptosis caused by PC depletion. As shown in Fig. 1B, MT58 cells with antisense CHOP mRNA still were apoptotic, but the onset of apoptosis was delayed by 8–10 h, compared with vector control cells. Similar results were observed for two independent antisense CHOP-expressing MT58 clones (data not shown). The effect of the prevention of CHOP expression on apoptosis in MT58 cells was not caused by an effect on cell growth, since the antisense CHOP and the vector control transfected cells showed similar growth curves at the permissive temperature, as assessed by cell counting (data not shown).

Transcriptional activation of CHOP by PC depletion—To elucidate the regulation of CHOP expression during PC depletion, we analyzed the CHOP promoter. It has been shown that regulation of CHOP expression by various kinds of stress is mediated through the promoter sequence between nucleotide positions −954 and +91 of the 5′ upstream region (30, 41). Serial deletion mutants of the CHOP promoter, fused to the coding region of the luciferase (LUC) reporter gene, were used to identify regions responsive to PC depletion. These constructs were transiently transfected into MT58, the parental CHO-K1, and into MT58 cells, stably transfected with the CTA cDNA. The latter cells were used to exclude differences between MT58 and CHO-K1 cells not related to the inactivation of CT. The transfected cells were incubated for 24 h at 40 °C, and the response to PC depletion was determined by a LUC assay (see Fig. 2). MT58 cells showed increased levels of LUC expression from the −442 and −649 deletion constructs, compared with CHO-K1 or CT-expressing MT58. Upon transfection with the
Deletion analysis of the activation of the CHOP promoter by PC depletion. The plasmids pCHOP-LUC -649, -442, and -221 correspond to the human CHOP promoter region from nucleotides -649, -442, and -221 to +91 fused to the LUC gene. CHO-K1, MT58, and CT-expressing MT58 cells were transiently transfected with these plasmids along with the plasmid pCMV-βgal as described under “Experimental Procedures.” 48 h after transfection, cells were shifted to 40 °C for 24 h and in the case of CHO-K1 were incubated without or with 1 μM tunicamycin (as a positive control). Subsequently, cells were harvested and assayed for luciferase and β-galactosidase activity. Results are given as relative luciferase activity per nmol of O-nitrophenyl β-d-galactopyranoside (ONPG) hydrolyzed. Shown are the means ± S.E. of five independent experiments.

To further investigate the role of the AP-1 and C/EBP-ATF site, luciferase constructs were used containing two copies of the AP-1 (jun2TRE), E4-ATF, or C/EBP-ATF site placed immediately upstream of a thymidine kinase promoter (29). These constructs were transfected into CHO-K1, MT58, and CT-expressing MT58 cells and incubated at 40 °C for 24 h. The adenovirus E4ATF binding site did not induce an increase in LUC expression in response to PC depletion (see Fig. 3B). A very low level of inducible promoter activity was observed with the proximal AP-1 binding site, compared with the controls, but this response was severalfold lower than the induction of luciferase from the C/EBP-ATF construct in response to PC depletion. Together, these findings suggest that the up-regulation of CHOP transcription when cells are faced with a shortage in PC is mediated, at least in part, by the C/EBP-ATF site.

The Transcription Factor ATF2 Is Activated by Phosphorylation during PC Depletion—A transcription factor that was identified to bind C/EBP-ATF during CHOP activation is ATF2 (50). To identify whether this transcription factor is activated during PC depletion, CHO-K1 and MT58 cells were incubated for various time points at 40 °C, and whole cell extracts were prepared for Western blotting using phospho-specific antibodies against ATF2. The results demonstrate that the temperature shift in MT58 led to an increase of phosphorylated ATF2 after 8 h until 24 h (see Fig. 4). Incubation of wild-type CHO-K1 at 40 °C for 24 h had no effect on the level of phosphorylated ATF2. The AP-1 binding transcription factor C-Jun was only increased after 24 h in MT58 when cultured at 40 °C (data not shown).

To determine whether ATF2 transcription factor exhibits affinity for the C/EBP-ATF sequence, we performed EMSA in the presence of specific antibodies recognizing ATF2 or phosphorylated ATF2 (see Fig. 4B). A radiolabeled C/EBP-ATF double strand oligonucleotide from -313 to -295 of the human CHOP proximal promoter was used as a probe. Experiments were performed using nuclear extracts from wild-type CHO-K1 and MT58 cells incubated at the nonpermissive temperature of 40 °C. As described by Bruhat et al. (29), a major specific DNA-protein complex is detected after incubation of CHO-K1 and MT58 nuclear extracts with 32P-labeled C/EBP-ATF probe in the absence of specific antibody (left arrow in Fig. 4B). Antibodies against...
ATF2 and phospho-ATF2 supershifted the C/EBP-ATF-bound complex only with the nuclear extracts of MT58 cells (right arrow in Fig. 4B). In contrast, nuclear extracts of MT58 cells incubated with the c-Jun antibody did not result in a supershift. These results indicate that the binding of phosphorylated ATF2 to the C/EBP-ATF site might be involved in the up-regulation of CHOP transcription during PC depletion.

Inhibition of PC Synthesis Induces the Phosphorylation of JNK Kinase—JNK and p38 MAPK were described as being responsible for the phosphorylation of c-Jun during CHOP transcription activation. JNK was also suggested to play a role in ATF2 phosphorylation. To determine whether JNK or p38 MAPK is involved in the CHOP induction, caused by PC depletion, we assessed the effect of inhibition of PC synthesis on the phosphorylation status of these kinases. Phosphorylation of JNK and p38 MAPK is necessary for their activity in signaling cascades in response to cellular stresses. Culturing MT58 cells at 40 °C resulted in an increase in the phosphorylation state of JNK at 16 and 24 h (see Fig. 5A). In contrast, CHO-K1 cells did not contain any phosphorylated JNK. The phosphorylation of JNK at 40 °C observed in MT58 cells was clearly correlated with the defect in PC synthesis as MT58 cells overexpressing CT/H9251 showed no increase in phosphorylated JNK (see Fig. 5B). Incubation of CHO-K1 or MT58 cells at the nonpermissive temperature did not result in the phosphorylation of p38 MAPK, although these cells were capable of phosphorylating p38 in response to sorbitol treatment (see Fig. 5C). Taken together, these results show that active JNK is present in

![Figure 3](http://www.jbc.org/)
MT58 cells incubated at the nonpermissive temperature and therefore may play a role in the response of these cells to PC depletion.

**JNK Is Involved in the Apoptotic Process of PC-depleted Cells but Not in the Induction of CHOP**—It has been shown that the JNK inhibitor, SP600125, specifically inhibits activation of JNK in response to stress stimuli (47, 48). To investigate the role of JNK in the apoptosis of PC-depleted cells, we determined whether MT58 cells, incubated at the nonpermissive temperature, could be rescued by adding SP600125 to the medium at various time points. In the presence of SP600125, only 10 ± 2% of the MT58 cells became apoptotic after 48 h at the nonpermissive temperature (see Fig. 6A). In contrast, 60 ± 5% of the MT58 cells died in the absence of the JNK inhibitor. MT58 cells grown at 40 °C could be rescued almost completely if SP600125 was added within 8 h and partly when it was added within 16 h (see Fig. 6A). The addition of the inhibitor at 24 h or later did not prevent the apoptotic process. In contrast, the addition of p38 MAPK inhibitor SB203580 was not capable of rescuing MT58 cells from apoptosis during incubation at 40 °C (see Fig. 6B). These results suggest that the activation of JNK kinase takes place before 16 h, and its effect is an early event in the apoptotic process of MT58 cells, depleted of PC.

To elucidate the role of JNK in CHOP expression, MT58 cells were pretreated with SP600125 before shifting to 40 °C. JNK inhibitor-treated MT58 cells showed an elevated CHOP expression at 16 and 24 h, comparable with CHOP expression levels of untreated MT58 cells (see Fig. 7A). The CHOP induction observed in MT58 cells is not an effect of the inhibitor, because SP600125-treated wild-type K1 cells, incubated at 33 °C, or SP600125-treated wild-type K1 cells, incubated at 33 or 40 °C, did not show induction of CHOP expression. Furthermore, ATF2 phosphorylation is also not depending on JNK. JNK inhibitor-treated MT58 cells showed an elevated level of phospho-ATF2 at 8, 16, and 24 h, comparable with phospho-ATF2 levels of untreated MT58 cells (see Fig. 7B). In conclusion, we have evidence that JNK plays a role in the apoptotic processes of PC-depleted cells but is not required for the induction of CHOP or the phosphorylation of ATF2.

**DISCUSSION**

Inhibition of PC biosynthesis induces apoptosis by an unknown pathway. In previous work, we reported that inhibition of PC synthesis induces the expression of the proapoptotic, ER stress-related protein CHOP but does not result in a canonical ER stress response (22). We demonstrate here that the induction of CHOP plays a significant role in the induction of apo-
ptosis, caused by PC depletion, since inhibition of CHOP expression by antisense CHOP mRNA significantly delays the onset of the terminal execution phase of the apoptotic process. This suggests that CHOP expression is an early upstream event in the signaling cascade initiated in response to PC depletion, eventually leading to apoptosis. However, prolonged inhibition of PC synthesis still results in apoptosis in CHOP antisense MT58 cells, implying that other stress pathways not involving CHOP are activated as well.

CHOP is a transcription factor that is induced by cellular stress, especially ER stress. However, CHOP induction has also been observed in apoptotic pathways, independent of the ER stress response. For instance, amino acid limitation, especially leucine and arginine, up-regulates CHOP expression via a response element, known as the AARE (29, 41). Here we report that a cis DNA sequence located upstream from the transcription start site (−442 to −211) is involved in the activation of CHOP expression during PC depletion. Evidence is provided that a C/EBP-ATF site in this region is responsible for the increased CHOP expression during PC depletion. The C/EBP-ATF composite site present in the CHOP promoter is highly conserved between humans and hamsters (49). The C/EBP-ATF composite site in the CHOP promoter is a part of the AARE (29, 41). The C/EBP-ATF composite site in the CHOP promoter is a part of the AARE and can interact with specific members of the C/EBP and ATF/CREB transcription factor families (29, 43, 50). All members of these families contain a DNA binding domain consisting of a cluster of basic amino acids and a leucine zipper region (b-ZIP domain) (51). They can form homodimers or heterodimers through their leucine zipper regions (52, 53). The group of Fafournoux (29) reported that binding of ATF2 to the C/EBP-ATF composite site of the AARE is essential for the transcriptional activation of CHOP by amino acid deprivation. Our present results demonstrate that the transcription factor ATF2 is phosphorylated at an early stage in MT58 cells at the nonpermissive temperature, preceding CHOP expression. In contrast, the transcription factor c-Jun is phosphorylated after 24 h, indicating that it is not involved in the regulation of CHOP expression. Furthermore, phospho-ATF2 was shown to bind to the C/EBP-ATF composite site oligonucleotide in EMSA assays. Therefore, we suggest that CHOP expression during PC depletion seems to be mediated, at least in part, by binding of phosphorylated ATF2 to the C/EBP-ATF site.

The trans-activating capacity of ATF2 is depending on phosphorylation of N-terminal residues Thr<sup>69</sup>, Thr<sup>71</sup>, and Ser<sup>90</sup> by stress-activated protein kinases (54–56). JNK and p38 MAPK were implicated in the phosphorylation of transcription factors involved in CHOP expression (27, 44). JNK is present in an active, phosphorylated state in MT58 cells incubated at 40 °C, and treatment of MT58 cells with the specific JNK inhibitor SP600125 can rescue these cells from apoptosis. Therefore, JNK activity could be responsible for the phosphorylation of ATF2 during PC depletion and the consequent induction of CHOP. However, we still observed CHOP induction and ATF2 phosphorylation in MT58 cells at the nonpermissive temperature that were treated with SP600125. These results suggest that JNK is not essential for the induction of CHOP expression and ATF2 phosphorylation. Other kinases, besides JNK and p38 MAPK, have been identified as being capable of ATF2 activation. Induction of growth arrest by treatment of cells with the tumor promoters 12-O-tetradecanoylphorbol-13-acetate

![Figure 6](http://www.jbc.org/)

**FIG. 6.** SP600125 JNK inhibitor rescues cells from PC depletion-induced apoptosis. MT58 cells were treated with the JNK inhibitor SP600125 (top) or the p38 MAPK inhibitor SB203580 (bottom). To investigate whether these inhibitors can rescue MT58 cells from apoptosis, subconfluent cells were shifted to 40 °C. SP600125 (final concentration 40 μM) and SB203580 (final concentration 20 μM) were added at the indicated time points to the cells and refreshed every 24 h afterward. Cells were harvested 48 h after shifting cells to 40 °C, and apoptosis was assessed as described under “Experimental Procedures.” Open bars, wild-type K1; black bars, MT58. Shown are the means ± S.E. of three independent experiments.
and Saikosaponin a depends on ATF2 phosphorylation by ERK, another MAPK family member (57). ATF2-dependent positive regulation of the human insulin gene requires Ca2+/calmodulin-dependent protein kinase IV. This kinase phosphorylates ATF2 also on Thr73, in combination with Thr69 and Thr71 (58). Protein kinase A is known to phosphorylate ATF2 on Ser62 in response to cyclic AMP (59). Moreover, a recently identified, nuclear Ser-Thr kinase, vaccinia-related kinase-1 is described to phosphorylate ATF2 on Ser62 and Thr71, thereby stabilizing the ATF2 protein, which results in its activation (60). ATF2 is likely to integrate many types of cellular signals that might reach the transcription factor by different kinases. The group of Lazo (60) reported that phosphorylation of ATF2 by two kinases, vaccinia-related kinase-1 and JNK, on different amino acids can have an additive effect on the activation of transcription. This dual activation may cooperate with different signals if all of them are acting at suboptimal conditions or be exclusive if any of them reaches a maximum effect. Therefore, we cannot exclude the possibility that in this study ATF2 might also be phosphorylated by JNK in combination with another kinase. In that case, inhibition of JNK with SP600125 would still result in ATF2 phosphorylation and CHOP induction by this other kinase. Furthermore, JNK could be involved in phosphorylation of CHOP rather than its induction. Both JNK and p38 MAPK are capable of phosphorylating CHOP on Ser78 and Ser81 in response to stress (26, 44). Phosphorylation of CHOP is required for enhanced transcriptional activation of its downstream targets (45). On the other hand, the JNK signaling could also be independent of CHOP signaling. The mechanisms by which JNK induces apoptosis are as yet largely unknown and might be dependent on the cell system (61). Since MT58 will be faced with several insults during prolonged PC depletion by the reduction of membranes, these insults will evoke early and late stress responses. CHOP induction seems to play a role in the early response, whereas JNK could be responsible for late events. This is in agreement with the fact that we observe phosphorylation of c-Jun after 24 h.

In conclusion, we have shown that the induction of the pro-apoptotic CHOP gene by PC depletion is more similar to its induction in response to amino acid limitation compared with glucose deprivation, since it involves the C/EBP-ATF response element and presumably activation of ATF2 rather than the ER stress pathway. The latter (ER stress) pathway was shown to be involved in the apoptotic response to cholesterol loading in macrophages, a treatment that was suggested to cause a misbalance between cholesterol and phospholipids in biological membranes (62). Since PC depletion is also likely to cause a change in the phospholipid/cholesterol ratio, it is interesting to note that both membrane-disturbing treatments cause an induction of CHOP, but by different mechanisms. Defining the precise upstream cascade of molecular events that result in CHOP expression, ATF2 phosphorylation, and JNK activation by inhibition of PC synthesis will be an important contribution to the understanding of phospholipid homeostasis in mammalian cells.

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