184, 119 (1985).
31. C. Massire, C. Gaspin, E. Westhof, *J. Mol. Graphics* **12**, 201 (1994).
32. G. Laughlan *et al.*, *Science* **265**, 520 (1994).
33. Supported by grants from the Deutsche Forschungsgemeinschaft to M.F. and the European Union (grant Biot-CT93-0345) to M.K., E.W., and M.F. M.K. thanks M. Guéron and J. L. Leroy for critical reading of the manuscript. Coordinates have been deposited at the Protein Data Bank (accession number 1KOC and 1KOD) and will be directly accessible on the Web server of the Centre de Biochimie Structurale at http:\\tome.cbs.univ-montp1.fr.

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# Stress-Induced Phosphorylation and Activation of the Transcription Factor CHOP (GADD153) by p38 MAP Kinase

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**CHOP, a member of the C/EBP family of transcription factors, mediates effects of cellular stress on growth and differentiation. It accumulates under conditions of stress and undergoes inducible phosphorylation on two adjacent serine residues (78 and 81). In vitro, CHOP is phosphorylated on these residues by p38 mitogen-activated protein kinase (MAP kinase). A specific inhibitor of p38 MAP kinase, SB203580, abolished the stress-inducible in vivo phosphorylation of CHOP. Phosphorylation of CHOP on these residues enhanced its ability to function as a transcriptional activator and was also required for the full inhibitory effect of CHOP on adipose cell differentiation. CHOP thus serves as a link between a specific stress-activated protein kinase, p38, and cellular growth and differentiation.**

CHOP, also known as growth arrest and DNA damage–inducible gene 153 (GADD153), is expressed in response to various metabolic stresses in all cells tested (*1*). By forming heterodimers with members of the C/EBP family of transcription factors, CHOP influences gene expression as both a dominant-negative regulator of C/EBP binding to one class of DNA targets (*2*) and by directing CHOP-C/EBP heterodimers to other sequences (*3*). Both modes of action are implicated in the effects of CHOP on cellular growth (*4*, *5*) and differentiation (*6*). In addition to inducing CHOP expression, stress increases the ability of CHOP to activate gene expression (*3*). This latter observation prompted us to study the possible stress-induced posttranslational modification of CHOP.

In stressed cells, CHOP is present in two forms, distinguishable by their migration in SDS–polyacrylamide gel electrophoresis (Fig. 1A) (*7*). Isoelectric focusing revealed the form of CHOP with decreased mobility to have a more acidic isoelectric point, suggestive of phosphorylation. To study the possible effects of stress on CHOP phosphorylation independently of the stress-induced increase in protein, we used cells constitutively expressing an epitope-tagged form of CHOP that is distinguishable in size from the endogenous protein. In vivo labeling with [$^{32}$P]orthophosphate followed by immunoprecipitation revealed two to four times as much phosphorylation of tagged CHOP in response to stress with no change in the amount of protein (Fig. 1B). The stress-induced endogenous CHOP was also phosphorylated.

Comparison of the tryptic phosphopeptide maps of the tagged CHOP from stressed and unstressed cells showed most of the inducible phosphorylation to occur on two distinct peptides (Fig. 1C). All known CHOP proteins contain two adjacent serine residues [amino acids 78 and 81 in the mouse sequence (*2*)] in a context that may serve as a substrate for members of the MAP kinase family (Fig. 1E). Members of this family are activated by many of the same insults that induce CHOP expression (*8*) and may therefore participate in the stress-induced phosphorylation of CHOP. Conversion of Ser$^{78}$ or Ser$^{81}$ to Ala led to the selective loss of inducible phosphorylation of one tryptic peptide (Fig. 1C). The minimal residual phosphorylation of the Ala-substituted peptides may have resulted from the presence of other phosphorylated residues. The mutant CHOP proteins exhibited

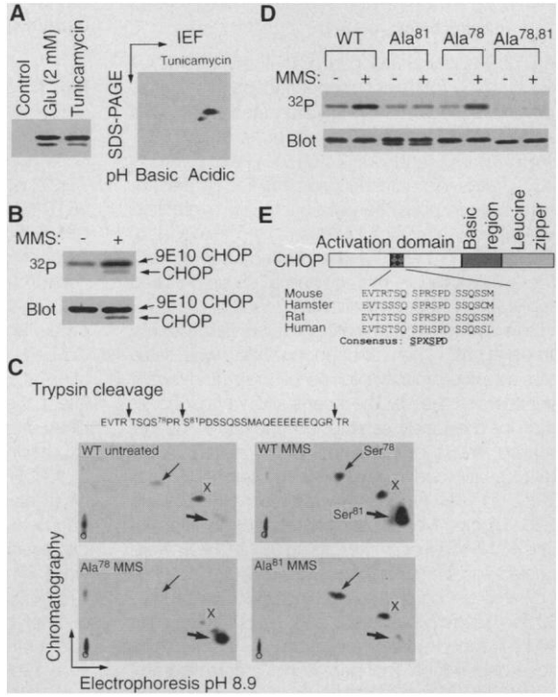


**Fig. 1.** Stress-induced phosphorylation of CHOP on Ser$^{78}$ and Ser$^{81}$. (**A**) Two isoforms of CHOP are present in stressed cells. NIH 3T3 cells were cultured in a medium with a low concentration of glucose [(Glu), 2 mM, 16 hours] or treated with tunicamycin (25 μg/ml, 4 hours). CHOP, detected by protein immunoblotting with the 9C8 monoclonal antibody (*6*), migrates as a doublet with 11% SDS–polyacrylamide gel electrophoresis (SDS-PAGE). The form with less mobility is more acidic on isoelectric focusing (IEF). (**B**) Endogenous CHOP and Mycepitope–tagged CHOP [9E10 CHOP (*5*)] from [$^{32}$P]orthophosphate-labeled NIH 3T3 cells (500 μCi/ml, 5 hours) that were treated with the stress-inducing alkylating agent MMS (100 μg/ml, 3 hours) were immunoprecipitated with 9C8. Autoradiography (top) and protein immunoblotting with rabbit antiserum to CHOP (bottom) are shown. (**C**) Wild-type (WT) and mutant CHOP, immunoprecipitated from treated or untreated NIH 3T3 cells with the antibody to 9E10 (*17*), were digested with trypsin and the phosphopeptides separated by electrophoresis and thin-layer chromatography and visualized by autoradiography (*18*). The circles mark the origin of the run. The arrows mark the position of the two peptides that underwent inducible phosphorylation upon MMS treatment. The peptide marked "X" was constitutively phosphorylated. The predicted sequence of the tryptic phosphopeptides, with small arrows denoting the tryptic cleavage sites, is shown above the autoradiograms (*19*). (**D**) Comparison of the in vivo phosphorylation of wild-type and Ala substitution mutants of CHOP from untreated cells and cells treated with MMS. Autoradiography (top) and CHOP immunoblot (bottom) are shown. (**E**) Schematic diagram of the CHOP protein. The region containing the stress-inducible phosphorylation sites is stippled, and the peptide sequence of this area, from four mammalian species, shows the conservation of context of Ser$^{78}$ and Ser$^{81}$ (*19*).

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substantially less total phosphorylation in both the basal and induced state, with marked attenuation in the case of the $Ala^{81}$ substitution. Replacing both $Ser^{78}$ and $Ser^{81}$ with Ala abolished nearly all phosphorylation of CHOP (Fig. 1D).

The above results indicate that the stress-inducible phosphorylation of CHOP is dependent on two serines present in a context favorable for phosphorylation by MAP kinases (*9*, *10*). Members of this family can be divided into three groups: (i) extracellular signal–regulated kinases (ERKs), which are predominantly responsive to extracellular growth signals (*9*, *11*); (ii) stress-activated protein kinases (SAPK-1 or Jun kinase) (*8*, *12*); and (iii) p38 MAP kinase, also called Mpk2 and SAPK-2 (*13*, *14*). The last two groups respond to overlapping sets of stress signals, which include many that induce transcription of *CHOP*. p38 purified from stressed COS-1 cells phosphorylated bacterially expressed CHOP in vitro. Mutant CHOP, bearing the $Ala^{78,81}$ substitution, was not a substrate for p38 (Fig. 2A). CHOP was not a substrate for SAPK-1β and was only weakly phosphorylated on $Ser^{78}$ by ERK2. To explore further the relation between p38 and CHOP, we made use of a recently described, highly specific inhibitor of the enzyme, SB203580 (*15*). The inhibitor led to attenuation of the stress-inducible phosphorylation of CHOP on $Ser^{78}$ and $Ser^{81}$, whereas the activity of the related SAPK-1s was increased (Fig. 2, B and C). Collectively, these findings implicate p38 in the phosphorylation of CHOP in vivo. The ability of methyl methanesulfonate (MMS) to transcriptionally induce CHOP (*1*, *2*) was not inhibited by SB203580. Thus, different stress-induced pathways regulate the transcription of CHOP and its phosphorylation.

CHOP does not homodimerize. When present in cells, it forms stable heterodimers, predominantly with C/EBPβ (*3*). Indistinguishable quantities of C/EBPβ were found to be associated with wild-type and $Ala^{78,81}$ CHOP in both stressed and unstressed cells (Fig. 3A). The DNA binding activity of CHOP, measured through use of a gel mobility-shift assay on a site that binds CHOP-C/EBP heterodimers, was also not affected by the $Ala^{78,81}$ substitution (Fig. 3B). Phosphorylation therefore does not appear to alter the dimerization or DNA-binding properties of CHOP, although stress increases the ability of CHOP to activate transcription (*3*). Known CHOP target sequences also bind C/EBP dimers (*3*), and MAP kinases can phosphorylate the major dimerization partner of CHOP, C/EBPβ (*16*). Therefore, to evaluate possible effects of CHOP phosphorylation on its ability to activate transcription, we used a modified form of CHOP in which the leucine zipper was deleted and the protein fused to the DNA-binding domain of the yeast transactivator Gal4. Because such proteins lack a CHOP dimerization domain

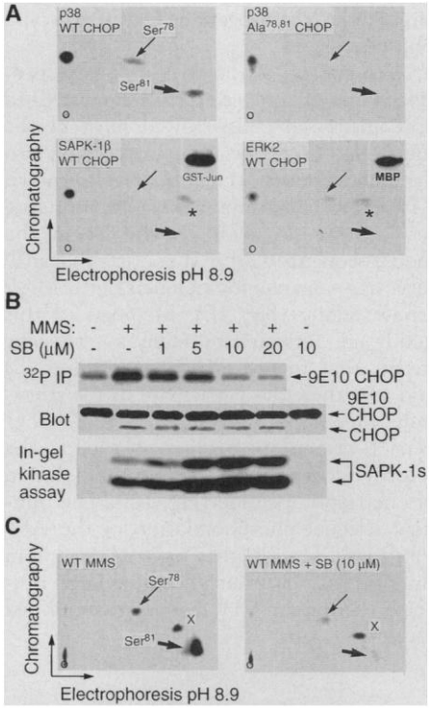


**Fig. 2.** Phosphorylation of CHOP by p38 MAP kinase. (**A**) Tryptic phosphopeptide maps of bacterially expressed CHOP phosphorylated in vitro with purified MAP kinases. Tagged forms of p38 (*13*), SAPK-1β (*20*), and ERK2 (*21*) were expressed in COS-1 cells. Activated kinases, purified by the tag, were used to phosphorylate bacterially expressed wild-type or $Ala^{78,81}$ CHOP with [γ-$^{32}$P]ATP (*22*). The labeled proteins were evaluated as in Fig. 1C. The asterisk marks the position of an unidentified tryptic phosphopeptide that is a result of a cellular kinase activity contaminating the purified enzyme preparations (this phosphopeptide was also present when inactive forms of the recombinant kinases were used). The inserts show phosphorylation of previously established substrates by our purified kinase preparations [GST-Jun for SAPK-1β (*12*, *20*) and myelin basic protein (MBP) for ERK2 (*21*)]. (**B**) A p38-specific inhibitor, SB203580 (SB), blocks MMS-inducible phosphorylation of CHOP. NIH 3T3 cells expressing 9E10 CHOP were labeled with [$^{32}$P]orthophosphate as in Fig. 1B. The indicated concentration of SB203580 was added 30 min before MMS treatment. SB203580 inhibits MMS-induced phosphorylation of CHOP in a dose-dependent manner (top, autoradiogram) with no effect on 9E10 CHOP expression or on the induction by MMS of the endogenous CHOP (middle, CHOP protein immunoblot). In-gel kinase assay of SAPK-1s with GST-Jun as a substrate (*23*) shows that the activity of SAPK-1s is increased (bottom). (**C**) Tryptic phosphopeptide mapping of CHOP from cells treated with MMS in the absence or presence of SB203580 (10 μM) shows that inhibition of CHOP phosphorylation occurs at $Ser^{78}$ and $Ser^{81}$. The peptide marked "X" was constitutively phosphorylated.

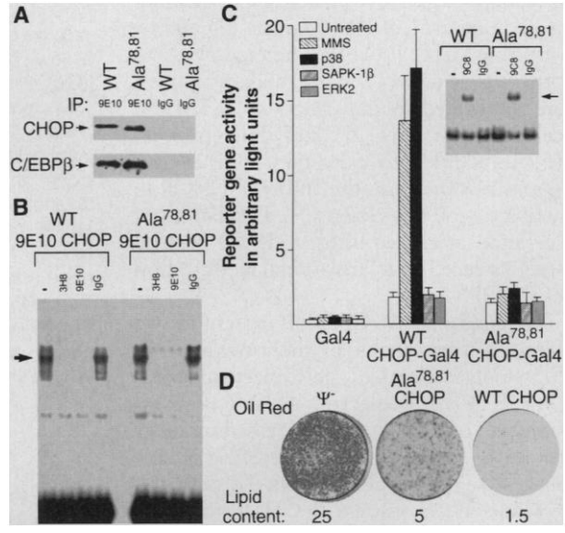


**Fig. 3.** Functional consequences of CHOP phosphorylation by p38. (**A**) Phosphorylation at $Ser^{78}$ and $Ser^{81}$ does not affect the dimerization of CHOP with C/EBPβ. Complexes of 9E10-tagged wild-type and $Ala^{78,81}$ CHOP from NIH 3T3 cells were immunoprecipitated with 9E10 or nonimmune mouse immunoglobulin G (IgG) and immunoblotted with rabbit antisera to CHOP (top) or C/EBPβ (bottom). (**B**) Phosphorylation at $Ser^{78}$ and $Ser^{81}$ does not affect the ability of CHOP to bind DNA. Nuclear extracts from cells expressing wild-type or $Ala^{78,81}$ 9E10 CHOP were used in a gel shift of a labeled CHOP-binding DNA fragment (*3*). Both proteins form indistinguishable complexes that were disrupted by antibodies to CHOP (3H8) and to the tag (9E10) but not by control IgG. (**C**) CHOP has a stress and p38-inducible transactivation domain. Wild-type or $Ala^{78,81}$ CHOP was fused to the yeast Gal4 DNA-binding domain (*24*). NIH 3T3 cells were transfected with the indicated combinations of a reporter plasmid driven by two Gal4 binding sites (UASp59-luciferase, 5 μg per plate), CHOP-Gal4 expression vectors (5 ng per plate), and kinase expression vector (5 ng per plate). Where indicated, the cells were treated with MMS (100 μg/ml) for 1.5 hours, 12 hours before harvest for luciferase assay. Shown are the mean and range values of a typical experiment performed in duplicate and reproduced four times. Wild-type and mutant CHOP-Gal4 were expressed in indistinguishable amounts in transfected cells. The inset shows a gel shift of a labeled Gal4 binding-site oligonucleotide by extracts from cells transfected with wild-type and mutant CHOP-Gal4 proteins. The arrow points to the complex supershifted with the 9C8 antibody to CHOP. (**D**) $Ala^{78,81}$ CHOP is attenuated in its ability to inhibit adipocytic differentiation of 3T3-L1 cells. Pools of cells stably transfected with empty SRα retrovirus [$\Psi^-$ (*25*)] or virus expressing either wild-type or mutant CHOP were induced to differentiate in vitro to adipocytes (*26*). The differentiation process was monitored by staining the fixed plates with the lipophilic dye Oil Red (*27*).

and bind DNA through the heterologous Gal4 peptide, the confounding effects of the stress-induced modification of the dimerization partners of CHOP is avoided. MMS treatment or overexpression of p38 markedly activated a reporter gene driven by Gal4 binding sites only when the CHOP-Gal4 chimeric protein was present (7.9 and 10 times as much, respectively); overexpression of SAPK-1β or ERK2 was without effect. The effect of MMS and p38 appeared to be dependent on CHOP phosphorylation because in cells expressing CHOP-Gal4 with an $Ala^{78,81}$ substitution, reporter gene expression was not enhanced by these stimuli (Fig. 3C).

Overexpression of CHOP leads to attenuated adipocytic differentiation of 3T3-L1 cells (6). This effect is dependent on the ability of the protein to dimerize and bind DNA. We compared the ability of wild-type and of $Ala^{78,81}$ CHOP to inhibit adipocytic differentiation in 3T3-L1 cells. Both proteins inhibited differentiation; however, cells expressing wild-type CHOP showed less lipid accumulation than cells expressing the $Ala^{78,81}$ substitution mutant (Fig. 3D). Thus, we conclude that $Ser^{78}$ and $Ser^{81}$ are necessary for the full biological activity of CHOP.

Under favorable conditions, CHOP is not present in cells. Stress leads to accumulation of CHOP in the nucleus. Our results indicate that stress also leads to phosphorylation of the protein and that this modification results in enhanced transcriptional activation by CHOP. The CHOP accumulation in response to stress is apparently dependent on the activity of cellular kinases because it can be inhibited by the broad-spectrum kinase inhibitors 2-aminopurine and H7 (*1*). However, the signaling pathway that activates CHOP phosphorylation is distinct from the one that leads to CHOP expression; an inhibitor of p38 does not block CHOP expression (Fig. 2B). Perhaps the potent ability of CHOP to modify cell growth and differentiation requires careful modulation beyond that provided by the mechanisms that control expression of the protein. The stress-activated p38 appears to serve a specific role in this fine-tuning of CHOP activity.

## REFERENCES AND NOTES


1. A. J. Fornace *et al.*, *Mol. Cell. Biol.* **9**, 4196 (1989); S. G. Carlson *et al.*, *ibid.* **13**, 4736 (1993); J. D. Luethy and N. J. Holbrook, *Cancer Res.* **52**, 5 (1992); B. Price and S. Calderwood, *ibid.*, p. 3814; Q. Chen, K. Yu, N. J. Holbrook, J. L. Stevens, *J. Biol. Chem.* **267**, 8207 (1992); S. L. Sylvester, C. M. J. ap Rhys, J. D. Leuthy-Martindale, N. J. Holbrook, *ibid.* **269**, 20119 (1994).
2. D. Ron and J. F. Habener, *Genes Dev.* **6**, 439 (1992).
3. M. Ubeda *et al.*, *Mol. Cell. Biol.*, **16**, 1479 (1966).
4. M. V. Barone, A. Y. Crozat, A. Tabaee, L. Philipson, D. Ron, *Genes Dev.* **8**, 453 (1994).
5. H. Zinszner, R. Albalat, D. Ron, *ibid.*, p. 2513.
6. N. Batchvarova, X.-Z. Wang, D. Ron, *EMBO J.* **14**, 4654 (1995).
7. Treatment of cells with tunicamycin, culture in low concentrations of glucose, and culture in MMS have similar effects on CHOP. For reasons of convenience, MMS was used preferentially in subsequent experiments.
8. J. Kyriakis *et al.*, *Nature* **369**, 156 (1994).
9. R. Davis, *J. Biol. Chem.* **268**, 14553 (1993).
10. ———, *Trends Biochem. Sci.* **19**, 470 (1994).
11. D. Robbins *et al.*, *Adv. Cancer Res.* **63**, 93 (1994).
12. B. Dérijard *et al.*, *Cell* **76**, 1025 (1994).
13. J. Han, J.-D. Lee, L. Bibbs, R. Ulevitch, *Science* **265**, 808 (1994).
14. J. Rouse *et al.*, *Cell* **78**, 1027 (1994).
15. J. C. Lee *et al.*, *Nature* **372**, 739 (1994); A. Cuenda *et al.*, *FEBS Lett.* **364**, 229 (1995).
16. T. Nakajima *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2207 (1993).
17. G. I. Evan, G. K. Lewis, G. Ramsay, J. M. Bishop, *Mol. Cell. Biol.* **5**, 3610 (1985).
18. Electrophoresis was at pH 8.9, and thin-layer chromatography was in isobutyric acid buffer, as described [W. Boyle, P. van der Geer, T. Hunter, *Methods Enzymol.* **201**, 110 (1991)].
19. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. I. Sánchez *et al.*, *Nature* **372**, 794 (1994).
21. J.-H. Her *et al.*, *Biochem. J.* **296**, 25 (1993).
22. Wild-type and mutant CHOP were expressed in *Escherichia coli* as $His^6$-tagged fusion proteins and purified by affinity chromatography on a $Ni^{2+}$ nitrilotriacetic acid–agarose column (Qiagen).
23. M. Hibi, A. Lin, T. Smeal, A. Minden, M. Karin, *Genes Dev.* **7**, 2135 (1993).
24. The DNA-binding domain of Gal4 (amino acids 3 through 147) was amplified by polymerase chain reaction and ligated in frame with wild-type or mutant murine CHOP complementary DNA (cDNA) at the unique Nhe I site of the mutant cDNA, thus deleting the CHOP dimerization domain. The chimeric cDNA was transferred to the pCDNA-Amp mammalian expression plasmid.
25. A. J. Muller *et al.*, *Mol. Cell. Biol.* **11**, 1785 (1991).
26. H. Green and O. Kehinde, *Cell* **1**, 113 (1974).
27. Lipid content was estimated by measuring the integrated intensity of the red signal in scanned images of the plates and is presented in arbitrary units (Fig. 3D). Shown is a typical result of an experiment performed in duplicate and reproduced three times. Equal expression of the wild-type and mutant proteins was observed by immunocytochemistry and immunoblotting (X.-Z. Wang, unpublished data).


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# G Protein–Mediated Neuronal DNA Fragmentation Induced by Familial Alzheimer's Disease–Associated Mutants of APP


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Missense mutations in the 695–amino acid form of the amyloid precursor protein ($APP_{695}$) cosegregate with disease phenotype in families with dominantly inherited Alzheimer's disease. These mutations convert valine at position 642 to isoleucine, phenylalanine, or glycine. Expression of these mutant proteins, but not of normal $APP_{695}$, was shown to induce nucleosomal DNA fragmentation in neuronal cells. Induction of DNA fragmentation required the cytoplasmic domain of the mutants and appeared to be mediated by heterotrimeric guanosine triphosphate–binding proteins (G proteins).


Alzheimer's disease (AD) is characterized pathologically by the presence of senile plaques and neurofibrillary tangles as well as extensive neuronal loss in the brain (*1*). Senile plaques are extracellular deposits whose major constituent, β-amyloid (Aβ), is cleaved from the transmembrane precursor APP (*2*). Alternative splicing of transcripts from a single gene results in at least 10 isoforms of APP (*3*), of which $APP_{695}$ is preferentially expressed in neuronal tissues. V642I, V642F, and V642G—three missense mutations in which $Val^{642}$ in the transmembrane domain of $APP_{695}$ is replaced by Ile, Phe, or Gly, respectively—have been associated with dominantly inherited familial AD (FAD) (*4*). These mutations cosegregate with the AD phenotype (*5*) and account for most, if not all, of the evidence for linkage of AD to chromosome 21 (*4*). Overexpression of such APP mutants mimics the neuropathology of AD in transgenic mice (*6*).

Recent studies of mice in which the APP gene has been disrupted (*7*) have suggested an essential role for normal APP in neuronal development and function. To elucidate the function of $APP_{695}$ and its mutants, we used F11 cells (*8*), hybrids of a primary rat dorsal root ganglion neuron and a mouse