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Identification of novel stress-induced genes downstream of *chop*

Xiao-Zhong Wang, Masahiko Kuroda, John Sok, Nikoleta Batchvarova, Robin Kimmel, Peter Chung, Helene Zinszner and David Ron[1]

Skirball Institute of Biomolecular Medicine, the Departments of Medicine, Cell Biology and the Kaplan Cancer Center, NYU Medical Center, New York, NY 10016, USA

[1]Corresponding author
e-mail: ron@saturn.med.nyu.edu

CHOP (GADD153) is a small nuclear protein that dimerizes avidly with members of the C/EBP family of transcription factors. Normally undetectable, it is expressed at high levels in cells exposed to conditions that perturb protein folding in the endoplasmic reticulum and induce an endoplasmic reticulum stress response. CHOP expression in stressed cells is linked to the development of programmed cell death and, in some instances, cellular regeneration. In this study, representational difference analysis was used to compare the complement of genes expressed in stressed wild-type mouse embryonic fibroblasts with those expressed in cells nullizygous for *chop*. CHOP expression, in concert with a second signal, was found to be absolutely required for the activation by stress of a set of previously undescribed genes referred to as DOCs (for downstream of CHOP). *DOC4* is a mammalian ortholog of a *Drosophila* gene, *Tenm/Odz*, implicated in patterning of the early fly embryo, whereas *DOC6* encodes a newly recognized homolog of the actin-binding proteins villin and gelsolin. These results reveal the existence of a novel CHOP-dependent signaling pathway, distinct from the known endoplasmic reticulum unfolded protein response, which may mediate changes in cell phenotype in response to stress.

Keywords: endoplasmic reticulum/gene targeting/subtractive hybridization

Introduction

Cellular stress activates intracellular signaling pathways that converge on a sizable number of transcription factors leading to alterations in the program of gene expression. Significant progress has been made in characterizing the stress-induced changes in activity of specific transcription factors and in linking these changes to upstream signaling pathways (for examples, see Montminy *et al*., 1990; Schreck *et al*., 1992; Karin and Hunter, 1995; Treisman, 1996). However, identification of target genes that are regulated *in vivo* through the activity of specific stress-activated transcription factors has not been quite as rapid. Physiological redundancy between signaling pathways and functional redundancy among members of transcription factor families presumably contribute to this difficulty. However, a detailed understanding of the cellular response to stress requires the development of methods to determine whether individual transcription factors have unique roles in transducing the activation of specific downstream genes. Here we present an analysis of the response mediated by a specific component of the signaling pathway that is triggered by agents that perturb the function of the endoplasmic reticulum (ER)—the transcription factor GADD153/CHOP.

GADD153/CHOP (growth arrest and DNA damage/C/EBP homology protein) has been implicated in the cellular response to stress. Initially isolated as a gene that is induced rapidly by alkylating agents and UV light (Fornace *et al*., 1989), *gadd153/chop* subsequently has been found to be responsive to other forms of stress, including many conditions that are known to induce an ER stress response. The list of conditions known to trigger the ER stress response includes treatment of cells with the glycosylation inhibitor tunicamycin, agents that interfere with calcium flux across the ER membrane and reducing agents, and deprivation of nutrients such as glucose, amino acids and oxygen. All of these perturbations also induce *gadd153/chop* (Bartlett *et al*., 1992; Chen *et al*., 1992; Price and Calderwood, 1992; Carlson *et al*., 1993; Marten *et al*., 1994; Batchvarova *et al*., 1995; Halleck *et al*., 1997). Overexpression of the ER chaperone BiP/GRP78 attenuates at least part of the ER stress response (Dorner *et al*., 1992) and also attenuates the induction of *gadd153/chop* (Wang *et al*., 1996). This latter result suggests that the induction of *chop* is a direct consequence of the ER stress response. Recently, studies of cells and animals deleted for the *chop* gene have implicated the protein in programmed cell death induced by agents and conditions associated with ER stress, and suggested a possible role for the protein in cellular regeneration that follows toxic insults in organs capable of mounting such a response (Zinszner *et al*., 1998). These results indicate that cellular pathways regulated by CHOP impact on important biological processes and are therefore in need of clarification.

gadd153/chop encodes a small nuclear protein that dimerizes with members of the C/EBP family of transcription factors (Ron and Habener, 1992). The CHOP–C/EBP heterodimer does not bind classical C/EBP-binding sites, and from the perspective of such sites CHOP is inhibitory (Ron and Habener, 1992; Fawcett *et al*., 1996; Friedman, 1996). There is no evidence that CHOP homodimerizes. However, CHOP–C/EBP heterodimers are capable of binding specific DNA sequences selected from a random oligonucleotide library, and reporter genes linked to artificial CHOP–C/EBP-binding sites are activated by CHOP *in vivo* (Ubeda *et al*., 1996). Furthermore, it appears that in most physiological settings CHOP protein does not

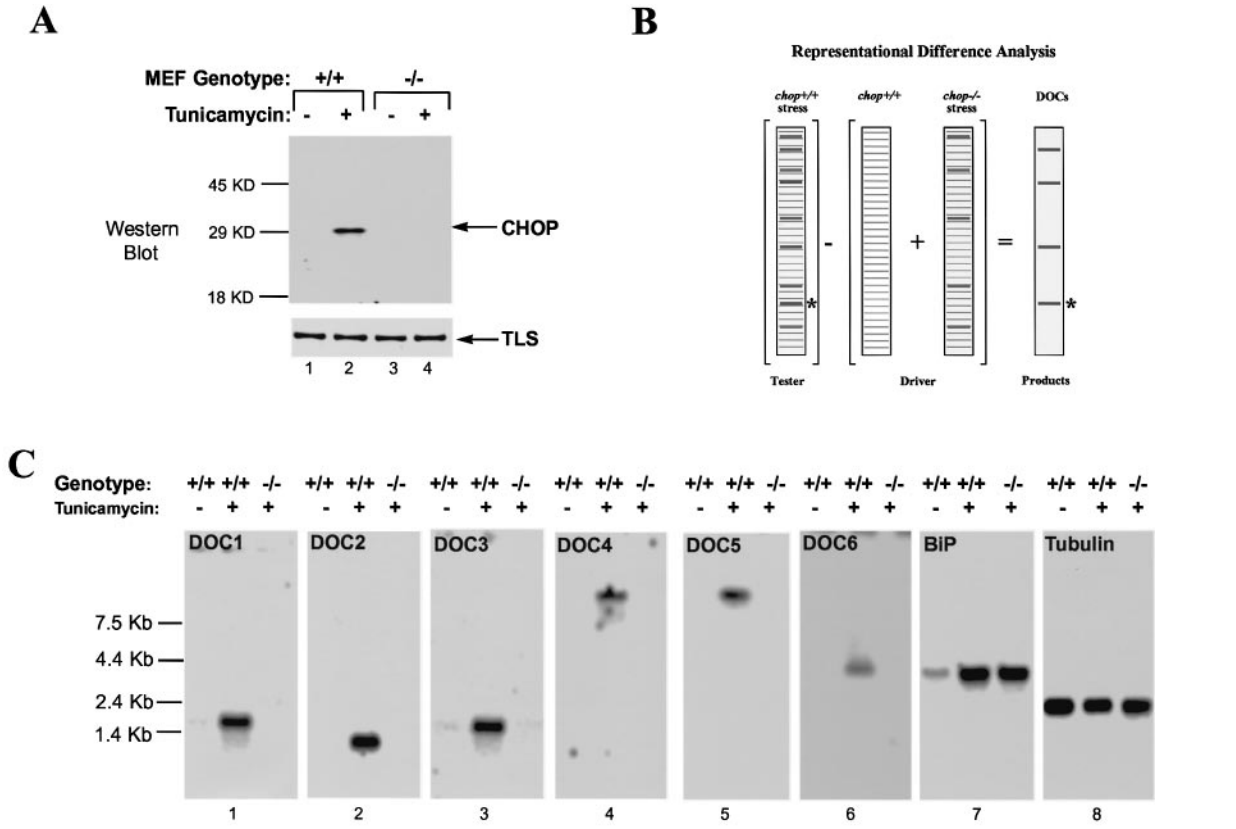


Fig. 1. Identification of ER stress-induced genes that are downstream of *chop*. (**A**) Immunoblot of CHOP protein from lysates of untreated and tunicamycin-treated (2 μg/ml, 6 h) primary MEFs that are wild-type (+/+) or nullizygous (–/–) for *chop*. The TLS immunoblot serves as an internal control for protein loading. (**B**) A cartoon depicting the strategy for isolating stress-induced genes that are downstream of *chop* (DOCs) by representational difference analysis. Tunicamycin-treated *chop*+/+ fibroblasts contain the full complement of housekeeping genes (thin lines), the non-*chop*-dependent stress-inducible genes (light thick lines) and the *chop*-dependent genes (dark thick lines). From this pool ('the tester'), representational difference analysis subtracts the non-*chop*-dependent stress-inducible genes present in tunicamycin-treated *chop*–/– cells and the housekeeping genes present in both the untreated *chop*+/+ and treated *chop*–/– pools (collectively referred to as 'the driver'). What remains as a product are the DOCs. The asterisks refers to *chop* itself that, while not a DOC, is also isolated by this scheme. (**C**) Northern blot of total cellular RNA prepared from tunicamycin-treated *chop*+/+ and *chop*–/– fibroblasts and untreated *chop*+/+ fibroblasts. Identically prepared blots were hybridized with the labeled inserts of the DOCs isolated by the RDA procedure. Tubulin serves as a control for integrity of the RNA, and BiP serves to document that an ER stress response had been induced in the *chop*–/– cells.

accumulate to levels that are sufficient to impact significantly on the total activity of the C/EBP factors present in a cell (Ubeda *et al*., 1996). Thus it seems likely that at least part of CHOP′s role in regulating gene expression is mediated by its ability to bind as a dimer with C/EBPs to certain, as yet unidentified, target genes. Other observations are also consistent with this speculation. First, CHOP protein undergoes stress-inducible phosphorylation by a p38-type MAP kinase and this phosphorylation event further enhances the transcriptional activation potential of the protein (Wang and Ron, 1996). Secondly, an altered form of CHOP, TLS-CHOP, encoded by an oncogenic fusion gene that is found in virtually all cases of human myxoid liposarcoma, requires an intact CHOP DNA-binding domain to transform cells (Zinszner *et al*., 1994). Thirdly, high level expression of CHOP will lead to cell-cycle arrest (Zhan *et al*., 1994). In this case too, the growth-suppressing properties of CHOP are dependent on the integrity of its putative DNA-binding basic region (Barone *et al*., 1994).

Based on these findings, we hypothesized that CHOP may mediate the induction of cellular genes in the context of the ER stress response. We predicted that such genes would not be induced in cells that lack *gadd153/chop* and that their activation by ER stress would also depend on the presence of a suitable dimerization partner for CHOP. The identification of such downstream genes should shed light on the role of *gadd153/chop* gene induction and CHOP protein post-translational modification in transducing stress signals.

Results

Identification of genes whose induction by ER stress is chop dependent

Genes that are *chop* dependent should not be inducible in cells that are nullizygous for *chop*. Having produced murine lines that carry a deletion allele of *chop* (Zinszner *et al*., 1998), we procured mouse embryonic fibroblasts (MEFs) from embryonic day 14.5 siblings produced by mating *chop*+/– animals. Western blotting confirmed that tunicamycin treatment of *chop*+/+ MEFs leads to the appearance of large amounts of CHOP protein, but identically treated *chop*–/– MEFs exhibited no accumulation of anti-CHOP-reactive protein (Figure 1A). Similar results were obtained with a panel of monoclonal and polyclonal antibodies that span the length of the protein, indicating that the mutant *chop* allele does not lead to the production

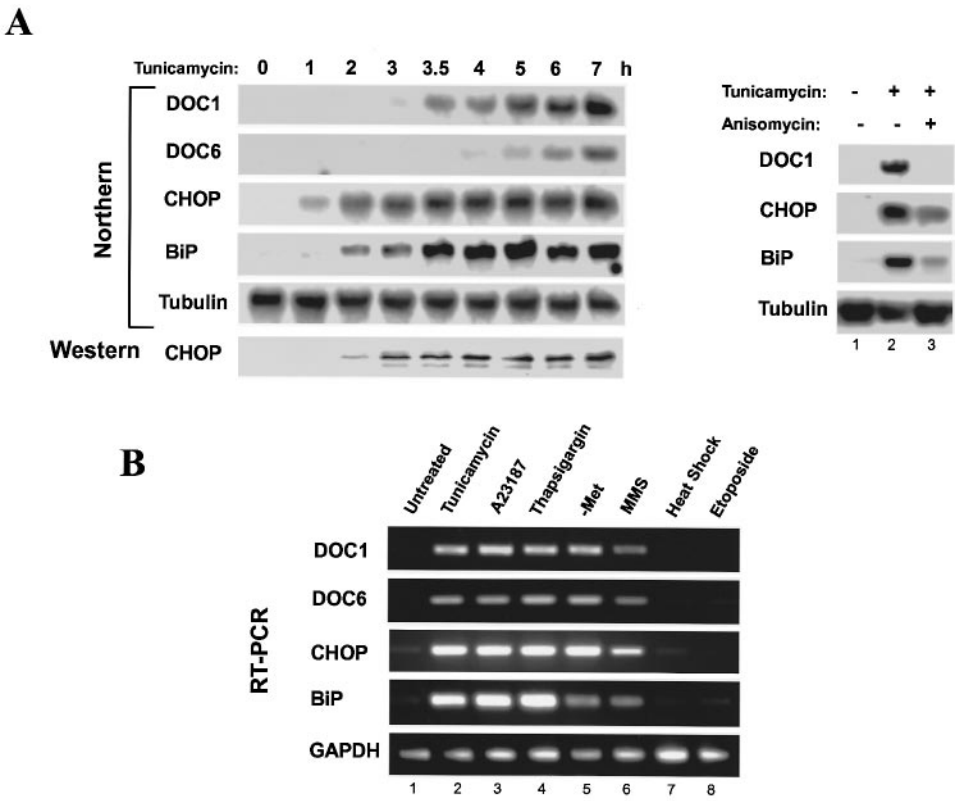


Fig. 2. DOC induction is rapid, dependent on new protein synthesis and proceeds in response to multiple agents that trigger an ER stress response. (**A**) Northern blot of NIH 3T3 cells treated with tunicamycin (2 μg/ml) for the indicated period of time. Note that DOC1 and DOC6 induction follows closely the accumulation of CHOP mRNA and protein (left panel). The same cells were treated with tunicamycin with or without anisomycin (a protein synthesis inhibitor, 10 μg/ml) and RNA was isolated for Northern blot 6 h later. Note that CHOP induction is not blocked by anisomycin but DOC1 induction is (right panel). (**B**) NIH 3T3 cells were treated with agents that induce an ER stress response [tunicamycin, 2 μg/ml; the Ca^{+2} ionophore A23187, 2 μM; thapsigargin, 2 μM; all for 10 h; methyl methanesulfonate (MMS, 100 μg/ml), for 6 h or methionine-deficient media (–Met), 16 h] or agents that do not induce ER stress (heat shock at 45°C for 3 h; or etoposide 100 μM for 12 h) and RNA was isolated. Levels of DOC1, DOC6, CHOP and BiP mRNAs were estimated by RT–PCR using specific primers. The GAPDH signal serves as an internal control for integrity of the mRNA.

of any CHOP protein. To isolate genes that are dependent on CHOP for their induction by ER stress, we performed a representational difference analysis (Lisitsyn *et al*., 1993; Hubank and Schatz, 1994) of cDNA produced from wild-type cells treated with tunicamycin compared with pooled cDNA from *chop–/–* cells that had been treated identically (Figure 1B). The cDNA from the treated *chop–/–* cells contains, in theory, all the constitutive and stress-inducible genes except those that are dependent on CHOP. cDNA from the untreated wild-type cells was also added to the subtracting pool. This was done to minimize the chance that subtle differences in the non-stress-dependent gene expression program between the MEFs with different *chop* genotypes might create false positives in the representational difference analysis (RDA).

Three cycles of RDA yielded several distinct bands on a 2% agarose gel. Six of these were subcloned and used as targets in Southern blots that compared the intensity of the hybridization signal obtained with labeled cDNA prepared from tunicamycin-treated *chop+/+* and *chop–/–* cells. Most of the subcloned inserts reacted more strongly with labeled cDNA from *chop+/+* than from *chop–/–* cells (not shown). These inserts were labeled individually and used as probes on Northern blots prepared from tunicamycin-treated and untreated *chop+/+* RNA and treated *chop–/–* RNA. All six mRNAs that were strongly inducible by tunicamycin in wild-type cells were completely non-inducible in *chop–/–* cells. Probing the Northern blot with tubulin indicated that the RNA was intact. Comparable levels of BiP mRNA in both cell types indicate that the *chop–/–* cells respond vigorously to ER stress (Figure 3C). The genes that satisfied these criteria were named provisionally DOCs (downstream of chop). *DOC2* proved to be *chop* itself, a predictable result in this screen. *DOC1* and *3*, and *DOC4* and *5* proved to be different fragments of the same cDNAs, respectively. Three different genes were therefore isolated by this screen (*DOC1*, *DOC4* and *DOC6*). Their identity will be discussed below.

Agents that activate the ER stress response rapidly induce DOC expression

chop mRNA induction proceeds in the absence of new protein synthesis—high dose anisomycin is an inducer of CHOP. However, the induction of DOCs is dependent on new protein synthesis (Figure 2A, right panel). This result is consistent with a requirement for CHOP protein synthesis and accumulation for the induction of DOCs. The attenuated induction of BiP mRNA in anisomycin-treated cells is probably due to the lack of something other than CHOP because BiP induction is the same in *chop+/+* and *chop–/–* cells (Figure 1C, panel 7). The

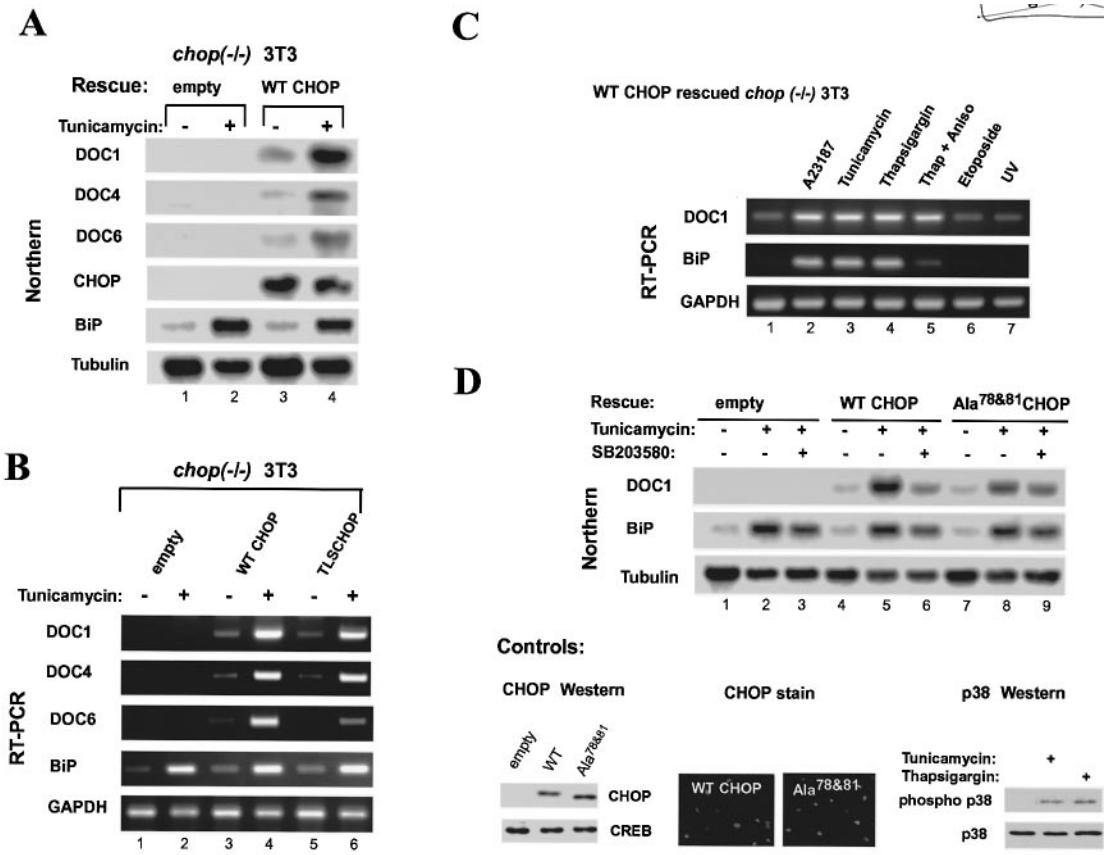


Fig. 3. Rescue of *chop–/–* cells. (**A**) Northern blot of RNA from untreated and tunicamycin-treated (2 μg/ml, 6 h) pools of *chop–/–* 3T3 cells that had been transduced with a wild-type CHOP-expressing retrovirus or an 'empty' retrovirus. Note that DOC expression is only partially rescued by CHOP (lane 3) but is fully restored by a combination of CHOP expression and tunicamycin treatment (lane 4). (**B**) RT–PCR analysis of DOC expression in untreated and tunicamycin-treated (2 μg/ml, 6 h) *chop–/–* cells expressing TLS-CHOP, CHOP and empty virus, as a control. (**C**) RT–PCR analysis of RNA from *chop–/–* 3T3 cells rescued by the CHOP-expressing retrovirus. Note that three different agents that induce ER stress can provide the necessary synergistic signal to fully activate DOC1 (lanes 2–4). Cells in lane 5 were pre-treated with anisomycin (10 μg/ml) for 30 min before and continuously for 6 h after the administration of tunicamycin. (**D**) *chop–/–* 3T3 cells were rescued with wild-type or the $Ala^{78\&81}$ mutant CHOP-expressing retrovirus (Wang and Ron, 1996). The upper panel is a Northern blot from the cells treated with tunicamycin (2 μg/ml, 6 h) in the absence and presence of the p38-type MAP kinase inhibitor SB203580 (10 μM). The control panels below are of a CHOP Western blot (left), immunocytochemical detection of CHOP in the rescued cells (middle) and Western blot detection of p38 MAP kinase activation in 3T3 cells treated by tunicamycin and thapsigargin (right).

temporal relationship between CHOP induction and the induction of DOC1 and DOC6 was examined in NIH 3T3 cells. These cells were chosen because they are easier to grow and because their ER stress response is more vigorous than that of MEFs. DOC mRNA is detectable after as little as 3–4 h of tunicamycin treatment, which follows shortly on the heels of the induction of CHOP protein, which is first detectable by 2 h (Figure 2A, left panel). The kinetics of the response are consistent with *DOC1* and *DOC6* being direct target genes of CHOP, a result supported by the identification of a functional CHOP-binding site in the DOC1 promoter (J.Sok, unpublished observations).

All perturbations known to trigger the ER stress response will lead to the accumulation of CHOP protein (see Introduction). To test if the DOCs share this property with CHOP, NIH 3T3 cells were subjected to multiple treatments known to induce the ER stress response, and the induction of DOC mRNA was estimated by an RT–PCR assay. Tunicamycin, the calcium ionophore A23187, thapsigargin, methionine-depleted media and the alkylating agent methyl methanesulfonate (MMS) all induced DOC1, DOC6, CHOP and BiP. The stress of heat shock or short-term treatment with the genotoxic topoisomerase inhibitor, etoposide, does not induce CHOP or BiP and therefore does not induce the DOCs (Figure 2C). DOC4 and DOC5 were excluded from this analysis because of their high basal level of expression in NIH 3T3 cells. It seems likely, however, that this high basal level is dependent on CHOP as it is not seen in immortal 3T3 cells derived from *chop–/–* MEFs (see Figure 3A). We conclude that multiple inducers of the ER stress response will activate *DOC1* and *DOC6* gene expression.

Rescue of chop–/– cells by exogenously expressed CHOP reveals the need for a second signal in DOC induction

The criterion used to identify the DOCs—non-inducibility by tunicamycin in *chop–/–* MEFs—leaves open the possibility that these genes are not truly dependent on *chop* expression at the time of stress, but rather that *chop* may have been required at one time to effect the development of a cellular phenotype that is necessary for DOC induction by stress. To address this issue, immortal 3T3 *chop–/–* cells were transduced with a retrovirus expressing epitope-tagged CHOP. Constitutive expression of CHOP led to

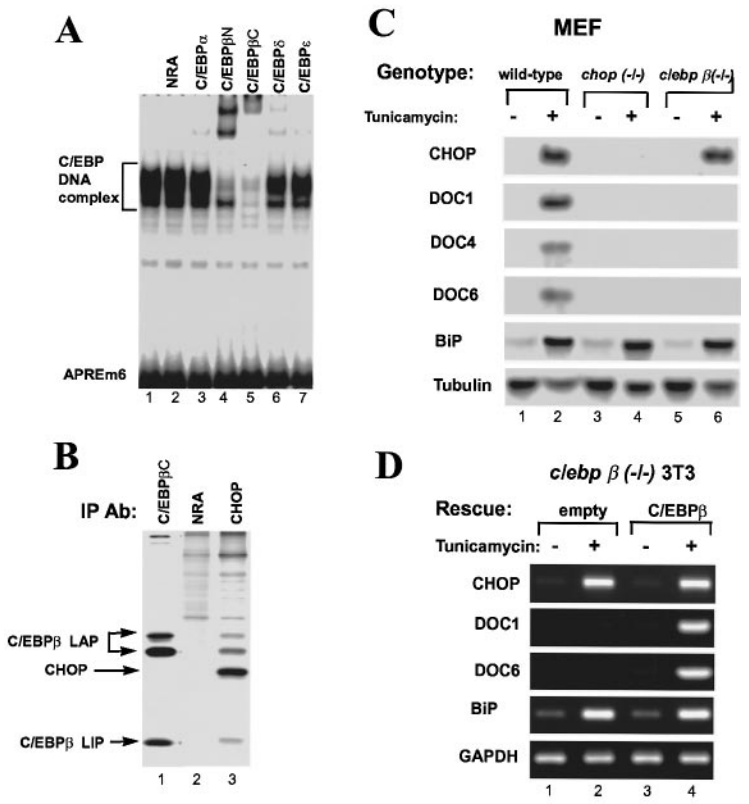


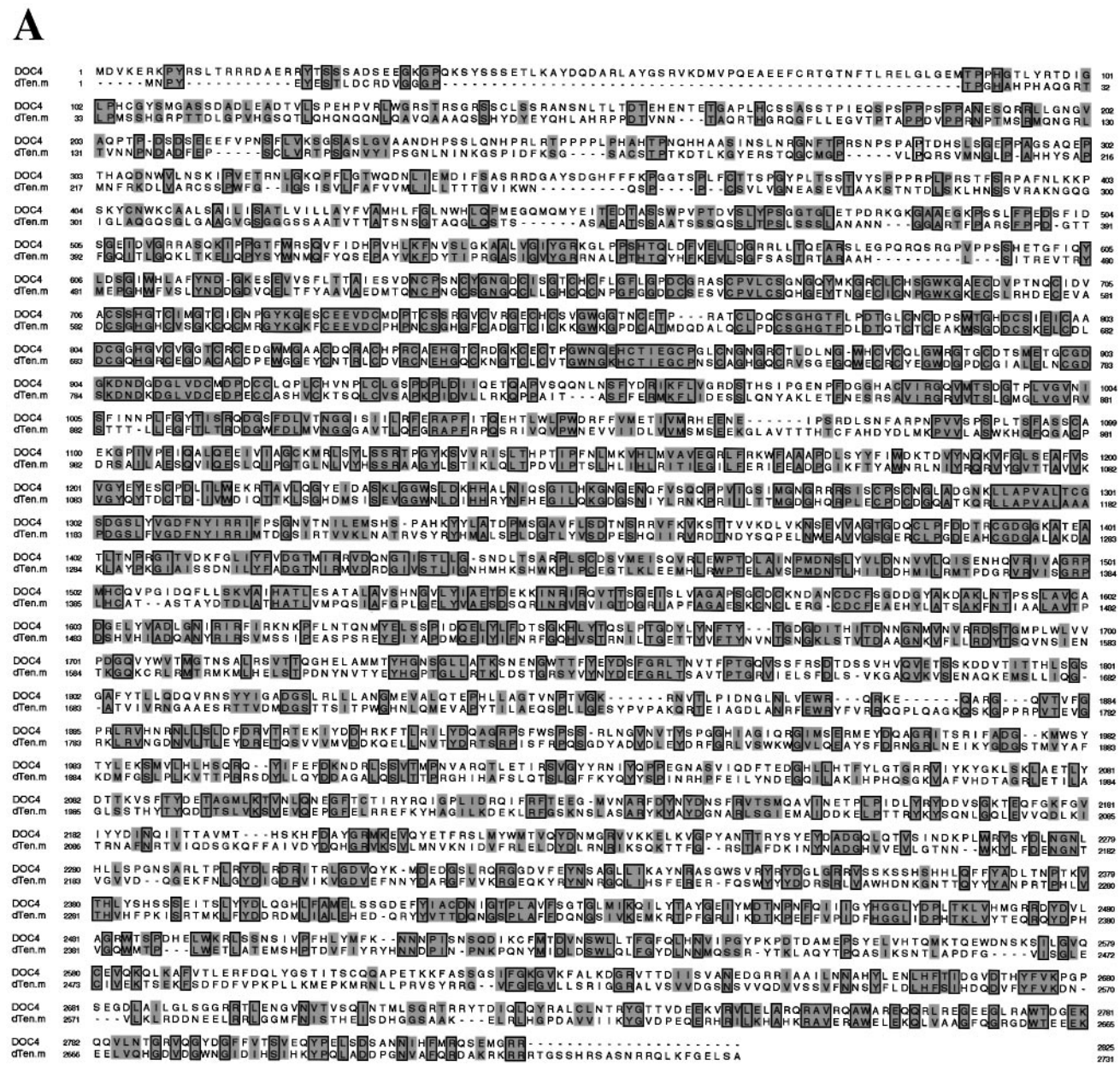
Fig. 4. C/EBPβ is required for DOC expression. (**A**) Gel-shift of a ^{32}P-labeled C/EBP-binding site-containing double-stranded oligonucleotide. Antibodies to specific C/EBP family members were added to the fibroblast nuclear extract before the binding reaction. C/EBPβN and C/EBPβC refer to antisera directed against the N- and C-termini of the protein respectively; NRA is a non-related antibody (rabbit anti-CREB polyclonal antibody). The position of the C/EBP–DNA complex is indicated. (**B**) Proteins in tunicamycin-treated fibroblasts were metabolically labeled with [^{35}S]methionine and immunoprecipitated with antibodies to C/EBPβ and CHOP (9C8). The C/EBPβ immunoprecipitation (lane 1) was carried out after boiling the lysate in 1% SDS to dissociate non-covalently bound proteins and reveal the position of the major C/EBPβ isoforms referred to as LAP and LIP and indicated by the arrows to the left of the autoradiogram (Descombes and Schibler, 1991). The NRA (the 9E10 monoclonal antibody) and CHOP immunoprecipitations (lanes 2 and 3) were carried out under conditions that retain CHOP-associated proteins in a complex. (**C**) Northern blot of RNA from tunicamycin-treated and untreated wild-type, *chop–/–* and *c/ebp*β*–/–* fibroblasts. (**D**) RT–PCR analysis of RNA from tunicamycin-treated and untreated *c/ebp*β*–/–* 3T3 cells that had been rescued by transduction with the LAP form of C/EBPβ.

the induction of DOC expression when cells were stressed (Figure 3A). TLS-CHOP, an oncogenic version of CHOP that contains an intact CHOP DNA-binding and dimerization domain, was also found to be capable of rescuing DOC expression in *chop–/–* cells (Figure 3B). The two proteins thus appear to have overlapping sets of target genes. These results also strongly suggest that the DOCs are dependent on CHOP expression at the time of stress. In addition, these rescue experiments exclude the possibility that variation at loci other than *chop* account for the differences observed in the inducibility of the DOCs between the different pools of MEFs.

Interestingly, tunicamycin treatment of the rescued *chop–/–* cells was required for full expression of the DOCs, though this treatment had no effect on the CHOP expression level in these cells (Figure 3A, last lane). The super-induction of DOCs by tunicamycin in CHOP-expressing *chop–/–* cells is shared by other agents that induce an ER stress response, but not by the heterologous stress of etoposide or UV light (Figure 3C). Furthermore, super-induction of DOCs in response to stress in CHOP-expressing *chop–/–* cells was no longer dependent on new protein synthesis (Figure 3C, lane 5), indicating that the propagation of this second signal can proceed by modification of pre-formed factors.

It has been found previously that CHOP protein undergoes stress-inducible phosphorylation during the ER stress response and that this phosphorylation event maps to two serine residues, 78 and 81. Phosphorylation of these residues leads to enhanced transcriptional activation of a reporter gene by the CHOP activation domain, and mutation of the two residues to alanine diminishes this stress-inducible enhancement in transactivation (Wang and Ron, 1996). To determine if the super-induction of DOC1 by the ER stress response is dependent on this inducible phosphorylation event, the ability of wild-type and Ala$^{78\&81}$ mutant CHOP to rescue DOC1 expression was compared in *chop–/–* cells. Western blot and immunochemistry showed that similar levels of expression of the two proteins had been attained in the rescued population of *chop–/–* cells (Figure 3D, lower left and middle panels). Expression of either protein resulted in a significant basal and tunicamycin-inducible expression of DOC1. The level of tunicamycin-induced expression of DOC1 in the cells rescued by CHOP Ala$^{78\ \&\ 81}$ was reproducibly 1.5- to 3-fold lower than that observed in cells rescued with wild-type CHOP (the experiment was repeated in three independent rescued pools of *chop–/–* cells, with identical results). Furthermore, ER stress leads to activating phosphorylation of p38 MAP kinase on Tyr82 (detected

by a phosphopeptide-specific antibody, Figure 3D, lower right panel). Significantly, the p38 MAP kinase inhibitor SB203580, a compound previously found to inhibit the stress-inducible phosphorylation of CHOP (Wang and Ron, 1996), attenuated the tunicamycin-induced expression of DOC1 in cells rescued with wild-type CHOP but had much less of an effect on the cells rescued by CHOP Ala$^{78\ \&\ 81}$ (Figure 3D, compare lanes 5 and 6 with 8 and 9). These experiments suggest that phosphorylation of CHOP on Ser78 and Ser81 plays a role in mediating the second signal that links the ER stress response to DOC1 induction (the first signal being the induction of *chop* itself), but that there are other components of the second signal that are not dependent on CHOP phosphorylation.



The requirement for c/ebpβ in DOC induction suggests an active role for CHOP–C/EBP heterodimers

The induction of DOCs during the ER stress response could reflect either a positive role for CHOP in activating their promoter (or the promoter of an upstream mediator gene) or, in theory, a negative role for CHOP that acts by relieving a tonic inhibitory influence exerted by CHOP's dimerization partner. In fibroblasts, the predominant C/EBP protein is C/EBPβ. This is revealed by the fact that most of the activity in fibroblast nuclear extract that can bind to a classical C/EBP site is supershifted by antibodies to C/EBPβ, but not by antibodies to other C/EBP isoforms (Figure 4A). C/EBPβ also accounts for most of the material that is co-immunoprecipitated by anti-CHOP antibodies from such cells (Figure 4B, see also Barone *et al*., 1994; Zinszner *et al*., 1994). Recalling that CHOP does not form homodimers readily (Ubeda *et al*., 1996), one would predict that if DOC induction proceeded via a positive effect of CHOP, cells that have no C/EBPβ would have a significantly attenuated DOC response to ER stress. If, on the other hand, DOC expression were due to CHOP-mediated relief of repression, cells lacking C/EBPβ would have wild-type or

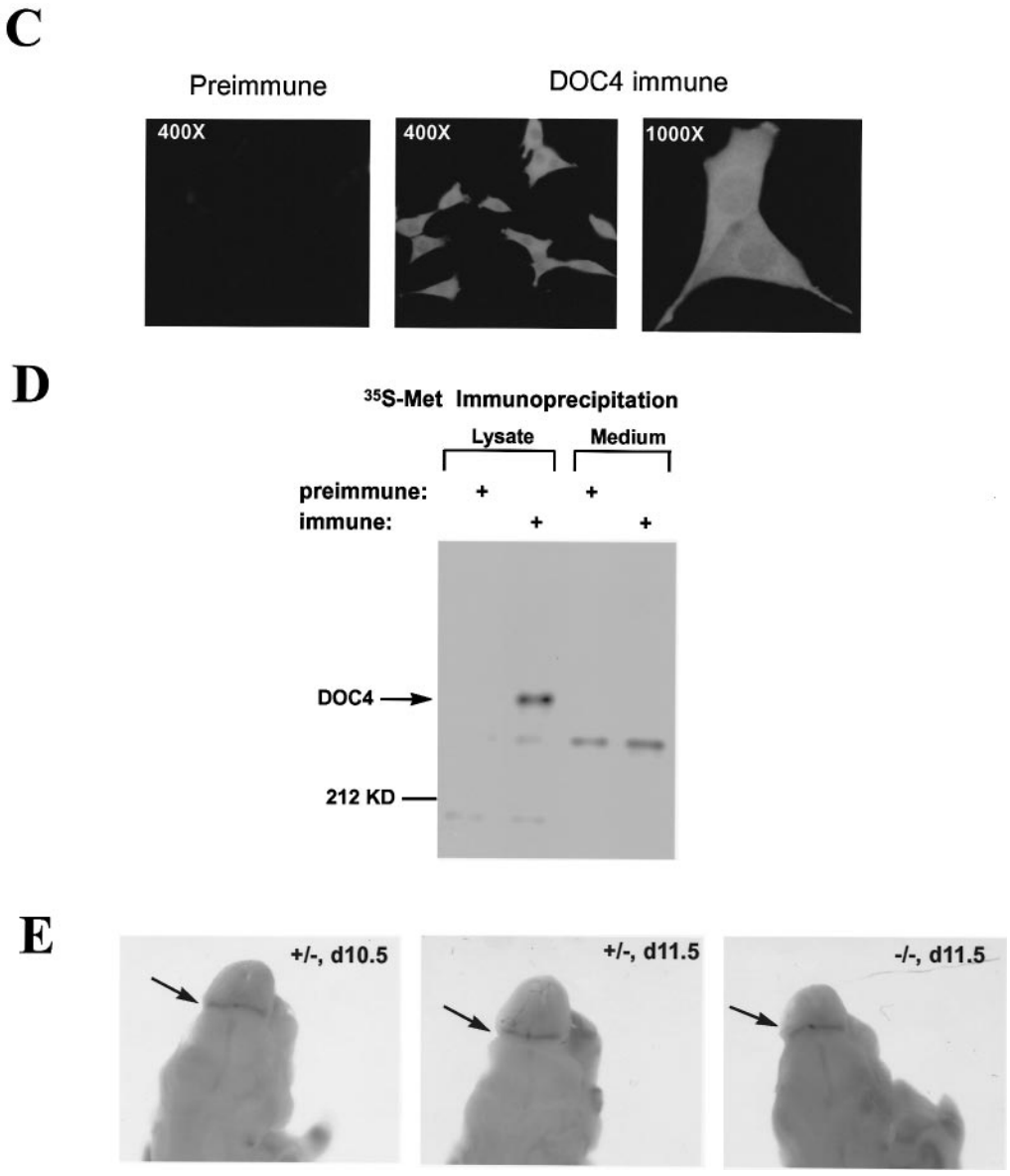


Fig. 5. (**A**) Alignment of the peptide sequence of DOC4 with *Drosophila* Tenm/Odz. Identical residues are boxed and similar residues are shaded. The nucleotide sequence of the cDNA has been deposited in GenBank under accession No. AF059485. (**B**) A bar diagram of the domain structure of the DOC4 mRNA and encoded protein. Non-coding regions are denoted by the thin horizontal lines and the coding region by the thick bar. Both the hydrophobic stretch at amino acid residues 411–433 (TM) and the potential furin cleavage site at residue 591 (Furin) are indicated. The eight EGF repeats, residues 633–893, are lightly stippled, and the conserved cysteine-rich domain (CC), residues 894–1009, is darkly stippled. The position of the DOC4 and DOC5 inserts is indicated by the horizontal bars. (**C**) Surface immunostaining of non-permeabilized NIH 3T3 cells with DOC4 antiserum and pre-immune serum. (**D**) Immunoprecipitation of metabolically labeled DOC4 from methionine-starved NIH 3T3 whole cell lysates and culture conditioned medium. The positions of the 212 kDa marker and the DOC4 signal are indicated to the left of the autoradiogram. (**E**) DOC4 *in situ* histohybridization of whole-mount mouse embryos with the indicated *chop* genotypes. The position of the sharp band of DOC4 staining at the midbrain–hindbrain junction is indicated by the arrows.

Fig. 6. Alignment of the peptide sequence of DOC6 with that of mouse villin and gelsolin. Identical residues are shaded. Note the similar location of the DXXD motif (underlined) in DOC6 (residues 370–373) and gelsolin (residues 349–352). The nucleotide sequence of the cDNA has been deposited in GenBank under accession No. AF059486.

enhanced DOC inducibility. MEFs produced from *c/ebp*β*–/–* mice were compared for DOC inducibility by tunicamycin with wild-type and *chop–/–* MEFs. Cells lacking C/EBPβ, like their *chop–/–* counterparts, were deficient in DOC inducibility (Figure 4C). Introducing C/EBPβ back into these *c/ebp*β*–/–* cells rescued the defect in DOC expression (Figure 4D). This result is most consistent with CHOP–C/EBPβ dimers playing an active role in DOC induction.

Identity of the DOCs

Sequence analysis revealed DOC1 and DOC3 to be fragments of a mouse homolog of human and sheep carbonic anhydrase VI (CA-VI). The CA-VI gene encodes a secreted form of an enzyme that catalyzes the reversible hydration of CO_2 and has been known to be expressed at high levels in the salivary gland (Fernley *et al*., 1989). The significance of its expression in response to stress in cells is not known at present. It is tempting to speculate, however, that DOC1 may play a role in controlling hydrogen ion concentration in the extracellular environment in response to stress.

DOC4 and DOC5 are two fragments of the same large mRNA (~14 kb) that encodes a novel protein of 2825 amino acids. The protein is highly similar throughout its length to the product of a *Drosophila* gene known as *Tenm*/*Odz* (Figure 5A, 31% identity, 50% similarity). The *Drosophila* protein has been found in both secreted and membrane-bound forms and is expressed in the developing fly embryo at several compartment boundaries (Baumgartner *et al*., 1994; Levine *et al*., 1994, 1997). Examination of the full-length mammalian cDNA reveals it to encode a protein with a single internal hydrophobic stretch (residues 411–433) followed by a domain with a series of eight epidermal growth factor (EGF) repeats (residues 633–893), a unique cysteine-rich domain (residues 894–1009) and a long C-terminus conserved between invertebrates and mammals (Figure 5B). The presence of EGF repeats suggests that the C-terminus is exposed to the extracellular environment. This prediction is borne out experimentally by the observation that antibodies directed against the C-terminal portion of DOC4 stain non-permeabilized DOC4-expressing cells (Figure 5C). Metabolic labeling followed by immunoprecipitation indicates that the protein is not present in soluble form in the media. Instead, it remains in the cell-associated pellet (Figure 5D), suggesting that it has either a type II transmembrane topology, or that it is secreted and associates with the membrane or matrix in an insoluble complex.

A database search reveals that the 632 N-terminal amino acids of the human homolog of DOC4 (>90% identity) are found as part of a novel secreted protein referred to as γ-heregulin (Schaefer *et al*., 1997). In the latter entity, the C-terminus of DOC4 is replaced by the immunoglobulin and EGF domains of human heregulin-1. Presently, it is not known if γ-heregulin is the product of a single locus that would harbor both the known human *HRG1* gene and the new *DOC4* gene, or if it represents a translocation product between the two genes that is unique to the breast cancer line (or tumor cell line) in

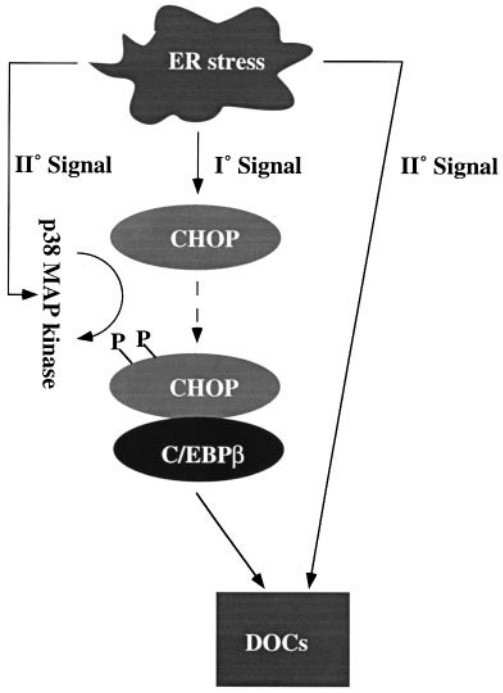


Fig. 7. A cartoon depicting components of the signaling pathway hypothesized to play a role in DOC expression in response to ER stress. ER stress leads to the induction of the *chop* gene (the I° signal). A second signal consists of the post-translational modification of CHOP protein by a p38-type MAP kinase on Ser78 and Ser81, enhancing CHOP transactivation potential (II° signal, left arrow). An additional component of this second signal is propagated independently of the phosphorylation of CHOP on these two residues and is depicted here as a path that converges on DOC in parallel with the CHOP-mediated signal (II° signal, right arrow). It remains possible, however, that CHOP is also modified by mechanisms distinct from the previously described p38-type MAP kinase phosphorylation at Ser78 and Ser81. In such a case, both II° signal arrows should be seen as converging on CHOP.

which γ-heregulin was identified. To the extent that the processing signals that specify a secreted fate to γ-heregulin (these include the hydrophobic stretch at residues 411–433 and the furin-type cleavage site at amino acid 591) are also present in DOC4, it is likely that the latter is also a secreted protein whose processed N-terminus is identical to that of γ-heregulin and begins at Gly591 (Schaefer *et al*., 1997).

The expression pattern of DOC4 in developing mouse embryos has features that suggest conservation of function with Tenm/Odz. The mRNA is found to be localized to a fine stripe at the midbrain–hindbrain junction of day 9.5–11.5 mouse embryos (Figure 5E). This 'organizing center' in the mouse has functional similarity to the posterior portion of the body segments in the fly embryo and the morphogenetic furrow in the fly eye imaginal discs—areas in which developmental boundaries are being established (Joyner, 1996). In the fly, this process is associated with and dependent on expression of Tenm/Odz (Baumgartner *et al*., 1994; Levine *et al*., 1994, 1997). *chop* appears to play no role in the developmental expression of DOC4, as *chop–/–* mouse embryos are indistinguishable from their wild-type counterparts in terms of DOC4 expression (Figure 5E). The results further indicate that whatever role DOC4 may have in development, it is 'recycled' as a stress-responsive and CHOP-dependent component in the adaptation of cells to stress.

DOC6 encodes a previously undescribed protein of 819 amino acids that is most similar to the actin filament-binding and severing proteins villin (60% identity) and gelsolin (48% identity). Figure 6 shows an alignment of DOC6 with the murine proteins. Of particular interest is the occurrence of a similarly situated DXXD motif in DOC6 (residues 370–373) and in gelsolin (residues 349–352). In the latter protein, this motif is implicated in a cleavage reaction mediated by caspases and occurring during programmed cell death. The N-terminal fragment of gelsolin, generated by this cleavage event, mediates changes in the actin cytoskeleton during apoptosis (Kothakota *et al*., 1997). The conservation of the potential caspase cleavage site in DOC6 suggests that it may mediate some of the changes in the cytoskeleton observed during programmed cell death induced by ER stress.

Discussion

Before this study, it was not known whether CHOP, other than serving as an inhibitor of C/EBPs, had a role in regulating gene expression. The experiments described above establish the protein as a stress-inducible transcription factor that activates a novel set of target genes in response to stress. CHOP′s ability to activate these target genes is dependent on the presence of a C/EBP dimerization partner, suggesting that activation takes place by the binding of a CHOP–C/EBP heterodimer, as predicted by *in vitro* site selection experiments (Ubeda *et al*., 1996). The identification of such a CHOP–C/EBP-binding site in the promoter of one of the target genes, *DOC1*, lends further support to this concept (J.Sok, unpublished observations). Based on the results presented here, we propose that an early stressful event in the ER leads to the induction of *chop* gene expression. Subsequently, a second signal that is mediated in part by the phosphorylation of two adjacent serines on CHOP (Ser78 and Ser81) leads to further augmentation of the transcriptional activation of DOCs. This hypothetical chain of events is depicted in cartoon form in Figure 7. It appears, however, that part of this second signal can also proceed via path(s) that are not dependent on phosphorylation of CHOP at these two known residues. This conclusion is based on two observations. First, a mutant form of CHOP, that has alanines substituting for Ser78 and Ser81 still retains significant ability to activate DOC1 in response to ER stress (compare lanes 7 and 8 in Figure 3C). Secondly, SB203580, an inhibitor of p38 MAP kinase that is known to block the stress-induced phosphorylation of CHOP at Ser78 and Ser81 (Wang and Ron, 1996), attenuates but does not abolish the induction of DOCs. The additional component of the second signal also appears to be dependent on phosphorylation events, as it is sensitive to the broad spectrum kinase inhibitors H7 and genistein (data not shown), but at this point in time we have no further clues as to its nature.

The presence of two components in this CHOP-dependent signaling pathway has interesting parallels with the propagation of stress signals via the c-JUN constituent of the AP-1 complex. There too a synergism is found between transcriptional activation of c-*jun* and post-

transcriptional activation of JUN protein through its phosphorylation by a MAP kinase (reviewed in Karin and Hunter, 1995). In the latter system, it appears that the transcriptional component is less important than the rapid post-transcriptional one, whereas in the CHOP-dependent pathway, described here, transcriptional induction of *chop* is a requirement. Perhaps the fact that the AP-1 complex evolved to respond to physical agents that immediately modify pre-existing macromolecules (e.g. UV light) imposed a requirement that new protein synthesis should not play such an important role in the propagation of that signal. ER stress, on the other hand, is thought to develop predominantly in the context of interference with synthesis and folding of new proteins. Therefore, pathways responsive to that perturbation can be endowed with greater latency and hence may be dependent on synthesis of mediators such as CHOP.

Cells modify their gene expression pattern in response to altered function of the ER. This response is best characterized in yeast where it has been referred to as the unfolded protein response (UPR) and consists of the transcriptional activation of a set of genes encoding ER-resident proteins. The UPR, therefore, represents a positive feedback loop by which unfolded proteins in the ER trigger the enhanced expression of components of the machinery that affects proper folding in that environment (Shamu *et al*., 1994). In yeast, the signaling pathway linking events in the ER to changes in gene expression has been worked out in great detail. However, much less is known about the response of mammalian cells to ER stress. At least part of the response appears to be shared with yeast in so much as ER stress triggers the induction of a homologous set of ER-resident proteins in both phyla (Lee, 1992). However, the diversity of the response may be greater in mammalian cells. For example, NF-κB activity is induced by ER stress in mammalian cells (Pahl and Baeuerle, 1995, 1997). Because of the pleotrophic nature of NF-κB, this finding suggests that perturbations in the ER may activate a broad stress response rather than one limited to genes encoding ER-resident proteins. The description here of a CHOP-dependent ER stress pathway that activates a set of genes that are clearly not ER-resident proteins lends further support to this concept. Cells lacking *chop* are not globally deficient in the ER stress response; BiP, for example, is normally induced in these cells (Zinszner *et al*., 1998; Figure 1C, panel 6). The ER stress response of mammalian cells, therefore, has at least one bifurcation at the level of CHOP and contains a novel branch that is dependent on CHOP.

ER stress modifies the proliferation and survival of cells (Carlberg and Larsson, 1993; Larsson *et al*., 1993; Nakashima *et al*., 1993; Pérez-Sala and Mollinedo, 1995; Carlberg *et al*., 1996; Chang and Korolev, 1996). It is encountered under physiological conditions such as renal ischemia (Kuznetov *et al*., 1996) in which it is associated with CHOP induction and cell death (Zinszner *et al*., 1998; H.Zinszner, unpublished observations). CHOP has an important role in programmed cell death and may also play a role in renal tubular regeneration in response to ER stress (Zinszner *et al*., 1998). DOC6 is a close homolog of gelsolin. The latter has been found to undergo cleavage by caspase 3 during programmed cell death, and the N-terminal fragment generated by this cleavage causes the collapse of the actin cytoskeleton, a feature of apoptosis (Kothakota *et al*., 1997). DOC6 shares a potential, DXXD caspase cleavage site with gelsolin. It is tempting, therefore, to speculate that an N-terminal cleavage fragment of DOC6 may have similar effects on the actin cytoskeleton in cells exposed to ER stress.

DOC4 is a mammalian homolog of an intriguing *Drosophila* protein, Tenm/Odz. Though there is some disagreement on the topology of Tenm/Odz (Baumgartner *et al*., 1994; Levine *et al*., 1994), all the experimental data are in agreement that the protein has potent signaling functions. In the early fly embryo, Tenm/Odz is expressed by the posterior cells of the odd-pair segment. In its absence, the adjacent even-pair segment fails to develop, giving rise to a pair-rule defect (Baumgartner *et al*., 1994; Levine *et al*., 1994). This suggests that Tenm/Odz affects the fate of cells adjacent to those that produce the protein, a result consistent with evidence that in *Drosophila* Schneider S2 cells the protein is secreted (Baumgartner *et al*., 1994). A secreted fate for DOC4 is suggested by the fact that the N-terminus of the protein is identical to that of a novel secreted form of the paracrine hormone heregulin, known as γ-heregulin (Schaefer *et al*., 1997). DOC4 shares extensive sequence identity with Tenm/Odz, and its pattern of expression in mouse embryos is also consistent with a role in signaling at compartment boundaries (Figure 5E). Our experiments indicate that the DOC4 protein remains in close association with the cells that make it (Figure 5D). We therefore propose a model whereby expression of DOC4 by stressed cells serves to propagate a signal to adjacent cells. We further speculate that this signal may be important to the phenotypic alteration in cell fate that accompanies the regeneration process that occurs when tissues are subjected to circumstances associated with ER stress. According to this speculation, products of some genes downstream of CHOP, such as DOC6, have cell-autonomous effects that contribute to the development of programmed cell death, whereas others, like DOC4, have signaling properties that effect the process of regeneration.

Materials and methods

Cell culture and treatment

chop–/– mice were generated by homologous recombination in embryonic stem cells. In the targeted allele, the *neo* gene replaces all but the last 34 amino acids of CHOP. A detailed description of the targeting strategy and phenotype observed in mice has been described elsewhere (Zinszner *et al*., 1998). *c/ebp*β*–/–* mice have been described previously (Screpanti *et al*., 1995). MEFs with wild-type or mutant genotypes were generated from day 14.5 mouse embryos (Joyner, 1993). *chop–/–* or *c/ebp*β*–/–* 3T3 fibroblasts were produced from the MEFs by serial passage as described (Todaro and Green, 1963), and were studied here between passages 20 and 25. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal calf serum from Intergen (Denver, CO). MMS, tunicamycin, thapsigargin, calcium ionophore A23187 and etoposide were purchased from Sigma (St Louis, MO). The concentration and time of treatment are indicated in the figure legends. UV irradiation (1.38 mJ/cm^2) was performed as previously described (Wang *et al*., 1996).

cDNA synthesis, representational difference analysis and full-length cDNA cloning

All procedures were performed essentially as described by Hubank and Schatz (1994). *chop+/+* and *chop–/–* MEFs at ~75% confluence were treated with tunicamycin (2 μg/ml, 10 h). Poly(A)$^+$ RNA was prepared. Double-stranded cDNA synthesis was based on Stratagene's ZAP-cDNA synthesis kit with the modification that the incorporation of 5-methyl-

dCTP into first-strand synthesis was omitted. The cDNAs were restricted with *Dpn*II, ligated into R-linkers, amplified with R-24 primer, and restricted again to generate a tester pool consisting of cDNA from tunicamycin-treated *chop*+/+ cells and a driver pool consisting of cDNA from untreated *chop*+/+ and treated *chop*–/– MEFs. In the first round of subtractive hybridization, a tester:driver ratio of 1:100 was used, in the second, 1:800 and in the third, 1: 40 000. After three cycles of subtraction and amplification, the *Dpn*II-digested differential products were fractionated on a 2% NuSieve agarose gel and individual identifiable bands were subcloned into the *Bam*HI site of pGEM3 vector. The cloned cDNA fragments were then used for Northern blot analysis and cDNA cloning. To isolate full-length clones of the DOCs, both oligo(dT)- and random-primed cDNA libraries made from tunicamycin-treated (12 h, 2 μg/ml) NIH 3T3 cell RNA were constructed in λ-Zap phage vector (Stratagene). Overlapping clones were assembled to 'walk' along the cDNA. The entire coding region was sequenced and the position of the predicted initiator methionine was assigned based on the presence of in-frame 5′ upstream stop codons. The nucleotide sequences have been deposited in DDBJ/EMBL/GenBank under the following accession numbers DOC 4, AF059485; DOC 6, AF059486.

RNA isolation Northern blot and RT–PCR analysis

Total RNA for cDNA synthesis was prepared by GTC lysis and CsCl ultracentrifugation. Poly(A)$^+$ RNA was isolated by passes over an oligo(dT) cellulose column (Molecular Research Center, Inc.). For Northern blots, total RNA (20 μg/lane) was fractionated on a formaldehyde agarose gel and transferred onto HyBond-N nylon membrane (Amersham). The murine CHOP, murine BiP, murine α-tubulin and isolated individual DOC cDNA fragments were labeled with ^{32}P by a random primer labeling technique and hybridized at 65°C in a buffer containing 7% SDS, 1 mM EDTA and 0.5 M sodium phosphate pH 7.4.

To analyze the expression of DOCs by RT–PCR, 1 μg of total RNA from each sample was primed with oligo(dT) to synthesize first-strand cDNA by reverse transcriptase. After reverse transcription, the reaction mixture was diluted to a volume of 100 μl in TE; 5% of the reaction mixture was used for PCR.

The primers and conditions for PCR analysis were: CHOP primer 1, GCAGCCATGGCAGCTGAGTCCCTGCCTTCC; primer 2, CAGACTCGAGGTGATGCCCACTGTTCATGC; BiP primer 1, GAAAGGATGGTTAATGATGCTGAG; primer 2: GTCTTCAATGTCCGCATCCTG; GAPDH primer 1, ACATCAAGAAGGTGGTGAAGCAGG; primer 2, CTCTTTCCTCTTGTGCTCTTGCTGG; DOC1 primer 1, AGTGCTGGGCTTAGTTTAGAGCTTTCC; primer 2, AGATCGATCGATACTGTGTGTCCGTTG; DOC4 primer 1, AGTACAAGATGGATGAGGATGGCTC; primer 2, TGGCTGCTCTTGCTGGATACTC; DOC 6 primer 1, ATCTACGGGAACGACAAATCCAAC; primer 2, AGGATGTGTGACCAGGTACTCCTG. CHOP PCR was performed in a buffer containing 67 mM Tris–HCl pH 8.8, 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 33 mg/ml bovine serum albumin (BSA), 200 μM dNTP at 95°C (1 min), 70°C (3 min), 24 cycles. PCR analyses of GAPDH (22 cycles), BiP (22 cycles), DOC1 (30 cycles) and DOC6 (33 cycles) were done in the 'Expand™ Long Template PCR Buffer 2' (Boehringer Mannheim) at 94°C (1 min), 60°C (1 min), 72°C (1 min). DOC4 (30 cycles) and DOC5 (26 cycles) PCR analyses were done in the aforementioned buffer 2 with the addition of $MgCl_2$ to a final concentration of 3 mM and dNTP to 500 mM at 94°C (1 min), 60°C (1 min), 72°C (1 min).

Western blot, immunocytochemistry, in situ histohybridization, immunoprecipitation and gel shifts

CHOP immunoblotting was performed using the murine anti-CHOP monoclonal antibody 9C8, as previously described (Batchvarova *et al*., 1995). The rabbit anti-CREB, and anti-TLS polyclonal antisera used as internal controls have also been described previously (Waeber and Habener, 1991; Zinszner *et al*., 1994). Rabbit anti-CHOP polyclonal antibody was used in immunocytochemistry to visualize the CHOP staining (Ron and Habener, 1992). For immunoprecipitation analysis of CHOP-associated proteins, tunicamycin-treated cells (2 μg/ml) were metabolically labeled with [^{35}S]methionine (500 μCi/ml) for 3 h, lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaC1, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol (DTT)]. The lysate was pre-cleared by Sepharose Cl-4B beads and immunoprecipitated with the murine 9C8 monoclonal antibody against CHOP. The anti-myc 9E10 monoclonal antibody was used as a negative control (Evan *et al*., 1985). To immunoprecipitate C/EBPβ without associated proteins (Figure 4B, lane 1), the labeled cells were first lysed in a disrupting buffer (1% SDS, 10 mM Tris pH 7.5, 1 mM EDTA), boiled for 5 min to dissociate protein complexes and then diluted into RIPA and immunoprecipitated with rabbit anti-C/EBP C-terminal antibody (Santa Cruz). Gel shift assays of C/EBP proteins were performed exactly as described using the C/EBP site from the angiotensinogen promoter, APRE M6 as a probe (Brasier *et al*., 1990). Rabbit anti-C/EBPα or anti-C/EBPβ N-terminal polyclonal antibodies were described previously (Ron *et al*., 1992). Rabbit anti-C/EBPδ and anti-C/EBPε polyclonal antibodies were purchased from Santa Cruz. Antiserum to active p38 MAP kinase was from New England Biolabs.

Rabbit antiserum to DOC4 was developed to a soluble 6× His-tagged peptide encoded by an *Nco*I fragment (nucleotides 7791–8237) from the 3′ end of the cDNA. The antisera was used at a dilution of 1:200 for immunochemistry on paraformaldehyde-fixed, non-permeabilized NIH 3T3 cells grown on coverslips. The pre-immune serum served as a negative control. Immunoprecipitation of DOC4 was performed from RIPA lysates of [^{32}S]methionine + cysteine-labeled NIH 3T3 cells that had been cultured for 12 h in methionine minus media (methionine depletion is known to induce CHOP gene expression, see Figure 2C, lane 5). In parallel, conditioned media from the labeled cells were collected and immunoprecipitated.

In situ histohybridization of whole-mount fixed mouse embryos was performed exactly as described (Wilkinson, 1992). The digoxigenin-labeled antisense cRNAs corresponding to the cloned DOC4 and DOC5 fragments were used as probes. Hybridization was performed at 65°C in 50% formamide-containing buffer and was followed by extensive digestion with RNase A to degrade unhybridized probe. Staining was revealed by an alkaline phosphatase-coupled anti-digoxigenin sheep antibody and a 'BM purple' chromogenic substrate (both from Boehringer Mannheim).

Retroviral production and infection

Wild-type (*Bam*HI–*Xho*I) or Ala$^{78\&81}$ (*Bam*HI–*Sal*I)-substituted 9E10-tagged CHOP (Wang and Ron, 1996) or TLS-CHOP (*Bam*HI/*Mun*I) cDNA (Zinszner *et al*., 1994) were ligated into pBabe-puromycin retroviral vector (Morgenstern and Land, 1990) digested with *Bam*HI–*Sal*I or *Bam*HI–*Eco*RI. The LAP form of human C/EBPβ cDNA was cloned into pBabe-puromycin as a *Bam*HI–*Sal*I fragment. Replication-defective retroviral particles carrying a vesicular stomatitis virus G-envelope protein pseudotype were produced as previously described (Landau and Littman, 1992). Cells were transduced with the retrovirus and selected with puromycin (1 μg/ml) for stable CHOP-expressing cell lines. TLS-CHOP could not be stably expressed in *chop*–/– 3T3 cells; therefore, these cells were analyzed 48 h after viral transduction when 30–50% of the cells were expressing the protein.

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