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Signals from the Stressed Endoplasmic Reticulum Induce C/EBP-Homologous Protein (CHOP/GADD153)

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The gene encoding C/EBP-homologous protein (CHOP), also known as growth arrest and DNA-damage-inducible gene 153 (*GADD153*), is activated by agents that adversely affect the function of the endoplasmic reticulum (ER). Because of the pleiotropic effects of such agents on other cellular processes, the role of ER stress in inducing *CHOP* gene expression has remained unclear. We find that cells with conditional (temperature-sensitive) defects in protein glycosylation (CHO K12 and BHK tsBN7) induce CHOP when cultured at the nonpermissive temperature. In addition, cells that are defective in initiating the ER stress response, because of overexpression of an exogenous ER chaperone, BiP/GRP78, exhibit attenuated inducibility of CHOP. Surprisingly, attenuated induction of CHOP was also noted in BiP-overexpressing cells treated with methyl methanesulfonate, an agent thought to activate *CHOP* by causing DNA damage. The roles of DNA damage and growth arrest in the induction of CHOP were therefore reexamined. Induction of growth arrest by culture to confluence or treatment with the enzymatic inhibitor *N*-(phosphonacetyl)-L-aspartate did not induce CHOP. Furthermore, both a DNA-damage-causing nucleoside analog (5-hydroxymethyl-2′-deoxyuridine) and UV light alone did not induce CHOP. These results suggest that *CHOP* is more responsive to ER stress than to growth arrest or DNA damage and indicate a potential role for CHOP in linking stress in the ER to alterations in gene expression.

CHOP (C/EBP-homologous protein; also known as GADD153) is a small nuclear protein that heterodimerizes avidly with members of the C/EBP family of transcription factors (43). The CHOP-C/EBP heterodimer is directed away from classic C/EBP sites, such as those found in the promoter regions of many genes, and binds instead a subset of C/EBP sites (49). While the target genes for CHOP-C/EBP action have not yet been identified, expression of CHOP and the formation of a dimer with C/EBP proteins significantly influence the pattern of gene expression and cellular phenotypes. For example, ectopic expression to high levels of CHOP produces growth arrest in several cell types (2, 53) and the presence of CHOP is inhibitory to adipocytic differentiation (4), a process that is dependent on the proper function of several C/EBP isoforms (31). Additional evidence for the importance of CHOP in cellular growth and differentiation comes from a molecular analysis of human sarcomas. In the vast majority of case of myxoid and round-cell liposarcoma, the *CHOP* gene is structurally rearranged (1, 25, 46), giving rise to the constitutive expression of an altered, oncogenic form of the protein (12, 42, 54).

CHOP cDNA was first identified in a screen that sought to isolate genes induced by DNA damage and was given the name *GADD153* (15). The mRNA was noted to be absent or, at most, expressed at very low levels under normal conditions. However, the alkylating agent methyl methanesulfonate (MMS) markedly induced the gene. Because MMS is capable of damaging DNA and because *GADD153* had been isolated in a screen that relied on subtractive hybridization between cDNA from cells that had been irradiated with UV light and from untreated cells, this induction was equated, at the time, with responsiveness of the gene to DNA damage. Interestingly, *GADD153* was also noted to be induced by culturing cells in nutrient-depleted media, a condition that causes growth arrest. The subset of isolated cDNAs that exhibited responsiveness to both MMS and depleted media were labeled *GADD*s (for growth arrest- and DNA damage-inducible genes [16]). Subsequent work, however, raises some doubt about the relative importance of DNA damage to the induction of the gene. UV irradiation of isolated keratinocytes was found to be associated with a very modest induction of the *GADD153* gene (17, 18), whereas a variety of metabolic insults were identified as powerful inducers (10, 11, 41, 48, 53). The role of growth arrest in the induction of CHOP has also been called into question by further analysis of the conditions used to generate that response. It was noted that the inducing activities of depleted media could be accounted for in part by the low glucose concentrations in such media (10). The induction of CHOP that occurs during adipocytic differentiation (43) can be totally abrogated if the cells are fed fresh medium at frequent intervals (4, 10). These results suggest that the association between induction of CHOP and growth-arresting conditions may have been a consequence of culture in depleted media rather than the growth-arrested state itself. This notion is supported by the observation that the nondividing cells in tissues and organs have low levels of *CHOP* mRNA.

Cellular glucose deprivation, a consequence of the depleted growth media that is known to induce CHOP, interferes with proper protein folding in the endoplasmic reticulum (ER),

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presumably by inhibiting N-linked protein glycosylation (29, 40). Other inducers of CHOP, such as tunicamycin, thapsigargin, the calcium ionophore A23187, and dithiothreitol (3, 11, 41), have all been linked directly to the abnormal function of the ER. Insults that do not primarily inhibit ER function but still induce CHOP, such as amino acid deprivation or inhibitors of energy metabolism (reference 32 and see below), may also be associated with abnormal protein synthesis and folding in the ER (9). The small soluble alkylating agent MMS may disproportionately affect ER proteins, perhaps by alkylating cysteine residues, and thereby interfere with protein folding. This body of circumstantial evidence led us to further explore the possible link between altered ER function and the induction of CHOP.

MATERIAL AND METHODS

Cell culture and treatment. All parental cell lines were obtained from the American Type Culture Collection. Chinese hamster ovary (CHO) K12 cells are temperature-sensitive mutants that have a defect in N-linked glycosylation at the nonpermissive temperature (34). BHK tsBN7 cells (38) harbor a point mutation in the DAD1 gene (37), a mammalian homolog of *Saccharomyces cerevisiae* OST2, which is a component of the oligosaccharyl-transferase complex (44). Yeast cells deficient in OST2 and tsBN7 cells exhibit a temperature-sensitive defect in N-linked glycosylation (43a, 44). Construction of the CHO cell line overexpressing hamster BiP (CHO-WT) will be described in detail elsewhere (36). Cells were cultured in Dulbecco modified Eagle medium with glucose at a high concentration (14 mM) in the presence of 10% fetal calf serum from Intergen (Denver, Colo.). To suppress CHOP induction by nutrient depletion, the growth medium was changed 4 h prior to the addition of the various CHOP inducers and at least every 2 days during routine culture of cells. MMS, dinitrophenol, and 5-hydroxymethyl-2′-deoxyuridine (HmdUrd) were purchased from Sigma (St. Louis, Mo.). Tunicamycin was purchased from Boehringer Mannheim (Indianapolis, Ind.). *N*-(Phosphonacetyl)-L-aspartate (PALA) was a gift of Jill Johnson from the Drug Synthesis Branch at the National Cancer Institute. Cells, in a thin layer of phosphate-buffered saline were irradiated with UV light from a 15-W 254-nm-wavelength bactericidal lamp bulb (Sylvania model G15-T8) at a measured dose rate of 138 $\mu W/cm^2$ (1.38 $J/s/m^2$). Proliferation assays were performed by exposing cells on coverslips to bromodeoxyuridine (BrdU; final concentration, 100 μM) for 12 h, fixing the cells with formaldehyde, staining with anti-BrdU monoclonal antibody (Amersham), and counterstaining with the karyophilic dye H33258, as described previously (45).

Northern (RNA) blotting, Western blotting (immunoblotting), and immune precipitation to detect protein levels. Cell lysis, RNA and protein preparation, and gel electrophoresis were performed as previously described (43). In Western blots, CHOP was detected with the murine anti-CHOP monoclonal antibody 9C8 which recognizes an epitope common to human and mouse CHOP (4). To detect the hamster protein in lysates of CHO cells, the previously described rabbit anti-CHOP polyclonal serum was used at a dilution of 1:10,000 (43). p53 was detected with the monoclonal antibody DO-1 (purchased from Santa Cruz Biotechnology, Santa Cruz, Calif.), and heat shock protein 70 (Hsp70) was detected with a monoclonal anti-Hsp70 antibody from Stressgen. CREB and TLS were detected as previously described (12, 50). The murine *CHOP* cDNA, hamster *BiP/GRP78* cDNA, and murine β-tubulin cDNA were used as probes to hybridize to Northern blots of total cellular RNA. Metabolic labeling of cells and immunoprecipitation of BiP and associated proteins with a rat monoclonal antibody and GRP94 with a rabbit polyclonal antiserum were performed as previously described (20). The control antibody used in Fig. 3C is a rat monoclonal anti-mouse μ chain antibody (Zymed Inc.).

In-gel kinase assays to detect the activity of the stress-activated protein kinases (SAPKs) SAPK1α and SAPK1β were performed as previously described (21, 23). Briefly, whole-cell extracts from untreated plates or plates 1 h after UV irradiation were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a gel that contained the bacterially expressed SAPK substrate (GST-Jun 2-233). Following removal of the SDS and renaturation of the proteins, the gel was soaked in kinase buffer with [γ-^{32}P]ATP for 1 h, the proteins in the gel were fixed, and unincorporated ATP was removed by extensive washing of the gel, which was then dried and exposed to autoradiography. Activated SAPK1α and SAPK1β register as ^{32}P-labeled bands of 46 and 55 kDa, respectively.

Analysis of DNA content of cells treated with MMS and HmdUrd. Subconfluent cultures of V79 hamster cells (8) were either treated with 100 μg of MMS per ml for 1 h, after which the inducer was removed, and then cultured in MMS-free medium for an additional 16 h, or continuously cultured in 1 μM HmdUrd for 16 h. The cells were trypsinized, fixed in paraformaldehyde, permeabilized with Triton X-100, digested with RNase A, and stained with propidium iodine. The intensity of the fluorescent signal was measured at the single-cell level by fluorescence-activated flow cytometry analysis with a Becton Dickinson FACScan flow cytometer.

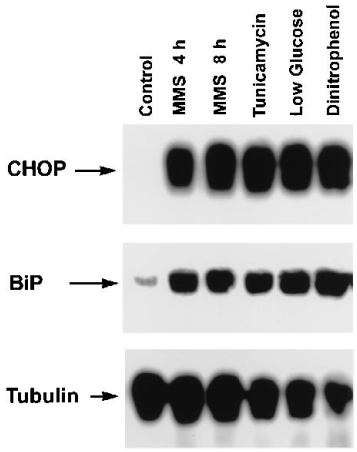

FIG. 1. Conditions that induce CHOP also activate an ER stress pathway. NIH 3T3 cells were treated with the alkylating agent MMS (100 μg/ml for 4 h or 50 μg/ml for 8 h), the glycosylation inhibitor tunicamycin (25 μg/ml for 4 h), low-concentration glucose medium (2 mM, overnight), and the inhibitor of oxidative phosphorylation dinitrophenol (100 μM, overnight). Shown is an autoradiogram of a Northern blot of total cellular RNA hybridized to the labeled *CHOP*, *BiP/GRP78*, and β-tubulin cDNAs. CHOP induction correlates with induction of the ER protein chaperone BiP.

RESULTS

Defective function of the the ER triggers the induction of CHOP. The Northern blot shown in Fig. 1 confirms that CHOP is induced in cultured cells by physiological stress. The mode of action of these inducers of CHOP is also consistent with the possibility that they lead to the formation of malfolded proteins and trigger stress in the ER. The latter possibility is supported by the induction of the ER stress-induced protein BiP/GRP78 under the same circumstances.

Because the inducers of CHOP are rather pleiotropic in their effects, it was decided to examine a more defined system in which stress can be localized to a discrete cellular compartment. The CHO cell line K12 and the BHK cell line tsBN7 exhibit temperature-sensitive defects in N-linked glycosylation of nascent proteins (34, 37; see also reference 44). The resulting accumulation of unglycosylated, malfolded proteins at the nonpermissive temperature leads to increased synthesis of the ER stress-induced protein BiP (28) (Fig. 2). Culturing the parental CHO and BHK cells at nonpermissive temperatures does not affect the glycosylation of ER proteins and only modestly up-regulates the synthesis of BiP. When K12 and tsBN7 cells were cultured at a nonpermissive temperature, CHOP was markedly induced, whereas the parental cells at the same temperature did not express CHOP (Fig. 2, left panels). Hsp70 was induced in both K12 and parental cells at 42°C, demonstrating that both cell types were responding to the stressful effects of heat. In the BHK cells cultured at the lower temperature of 39.5°C, Hsp70 was not induced. The decrease in the CHOP level at the 6-h time point in K12 cells at the nonpermissive temperature is a reproducible finding. In the tsBN7 cells, a similar drop in the CHOP level is observed between the 6- and 12-h time points (data not shown).

To further explore the relationship between the induction of CHOP and the cellular response to the accumulation of malfolded proteins in the ER, CHO cells that overexpress the ER chaperone BiP (CHO-WT) were used. These cells exhibit a drastically attenuated ER stress response when treated with agents that disrupt protein folding in the ER (14). CHO and CHO-WT cells were treated with either tunicamycin, known to

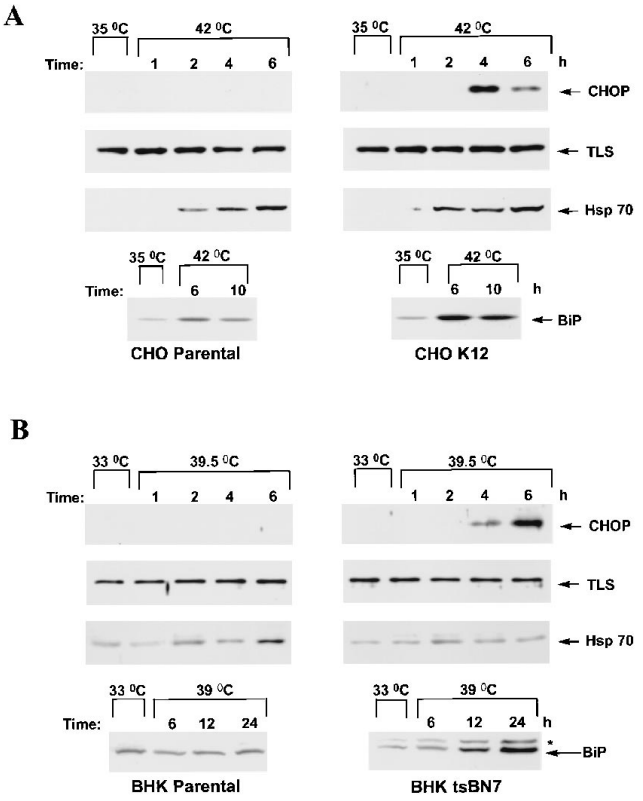

FIG. 2. CHOP is induced in cells defective in N-linked glycosylation. (A) CHO K12 cells, which have a temperature-sensitive defect in glycosylation, were cultured at a permissive temperature, 35°C, or switched to the nonpermissive temperature, 42°C, for the indicated periods of time. Induction of CHOP, estimated by Western blotting of whole-cell extracts (upper panel), was noted only in the defective K12 cells but not in the normal CHO parental cells. Identical blots were reacted with antisera to the RNA-binding protein TLS (an internal control) and antisera to Hsp70, an indicator of stress in the cytoplasmic compartment. The lowest panel shows enhanced synthesis of the ER stress-induced protein BiP in K12 cells following the temperature shift as revealed by autoradiography of immune precipitated proteins from cells metabolically labeled with [^{35}S]methionine. (B) Experiment identical to that described for panel A performed with parental (wild-type) BHK cells and the tsBN7 mutant cell line. BiP was detected by immunoblotting and migrates as a doublet in tsBN7 cells (asterisk). Hsp70 is not induced in these cells at 39.5°C.

block N-linked glycosylation, or the alkylating agent MMS. Both are potent inducers of CHOP (Fig. 1). The parental CHO cells responded by up-regulating the synthesis of BiP, GRP94, and CHOP (Fig. 3A and B). However, the CHO-WT cells minimally up-regulated BiP and GRP94 in response to tunicamycin and exhibited a markedly attenuated induction of CHOP. Tunicamycin was effective in blocking N-linked glycosylation in both cell types as evidenced by the increased mobility of GRP94, a protein that normally bears a single N-linked glycan (Fig. 3A, lower panel). Similarly, although MMS treatment strongly induced CHOP in the CHO line, it had a reduced ability to induce the protein in the BiP-overexpressing cells (Fig. 3B). Overexpression of BiP is thought to inhibit the development of an ER stress signal. This leads to attenuated induction of the ER chaperones in response to agents that perturb ER function. Thus, these data argue that CHOP is induced in response to cellular stress signals originating in the ER. Furthermore, they suggest that MMS may induce CHOP synthesis via an ER stress signal rather than exclusively through DNA damage.

BiP associates with unfolded or malfolded proteins. Consequently, agents that affect folding of ER proteins cause an increased association of proteins with BiP (5, 13). To determine if MMS affects proteins in the ER, we examined the pattern of BiP-associated proteins in untreated and MMS- and tunicamycin-treated cells. As expected, inhibition of N-linked glycosylation by tunicamycin resulted in an increase in the level of BiP-associated proteins and an increase in BiP biosynthesis (Fig. 3C). Importantly, incubation of cells with the alkylating agent MMS also resulted in the coprecipitation of several additional proteins with BiP. These proteins were specifically associated with BiP, as they were not detected with an irrelevant antibody (Fig. 3C). In agreement with the Northern blot data (Fig. 1), an increase in BiP biosynthesis was also observed. The most likely explanation for this is a direct effect of MMS on protein folding in the ER. However, an indirect mechanism cannot be excluded. For example, the induction by MMS treatment of a subset of ER proteins that associate with BiP even when normally folded would give a similar result. Regardless of the mechanism, this result indicates that in addition to its well-characterized effect on DNA, MMS alters proteins in the ER and leads to an enhanced association of a subset of proteins with BiP.

Growth arrest does not uniformly induce CHOP. K12 cells have been shown to undergo G_1- and S-phase cell cycle arrest at the nonpermissive temperature (35). We reasoned that if the induction of CHOP is secondary to the inducible defect in cell growth, CHOP should also be activated by other conditions that drive cells to growth arrest. 3T3 L1 cells and primary human foreskin fibroblasts (nonimmortalized primary cells) were allowed to reach confluence and remained confluent without an increase in cell number and exhibited a marked inhibition of BrdU incorporation (Fig. 4C). As seen in Fig. 4A (lanes 2, 5, and 6), the growth-arrested state is not sufficient to induce CHOP. The confluent cells remained responsive to MMS, however, excluding the possibility that prolonged culture under these conditions attenuated the ability of the cells to activate *CHOP*.

Confluence represents a relatively physiological inducer of growth arrest, mimicking the behavior of cells in nongrowing tissues. The relationship between growth arrest and CHOP was also tested in a more stressful system. PALA, a metabolic inhibitor of the trifunctional enzyme CAD, is a potent inducer of growth arrest in nontransformed cells (30, 39) (Fig. 4C). Its mechanisms of action are thought to be depletion of nucleotides and interference with DNA synthesis (47). The induction of growth arrest by this compound requires the activation of a signaling pathway that involves the tumor suppressor p53 and hence is observed in nontransformed cells that have intact p53, such as primary human foreskin fibroblasts (30, 52). Addition of PALA to the medium of growing subconfluent cells caused them to assume a flat morphology and inhibited incorporation of BrdU. But PALA, in doses as high as 120 μM that clearly arrest cell growth, did not induce CHOP (Fig. 4B and D). The increase in the level of p53 (Fig. 4B), which may be due to cell cycle changes induced by PALA, serves as an indication that the cells responded to the agent. Thus, growth arrest is not uniformly associated with the induction of CHOP.

DNA damage does not uniformly induce CHOP. The ability of BiP overexpression to attenuate CHOP induction by MMS (Fig. 3B) suggests the possibility that the effects of MMS are mediated through the development of stress in the ER and not, as has been thought, solely through the induction of DNA damage (16). Because of the potential for MMS to alkylate both proteins and DNA, a compound was sought that could mimic the effects of MMS on DNA, without modifying proteins. HmdUrd is incorporated into DNAs of cells and leads to

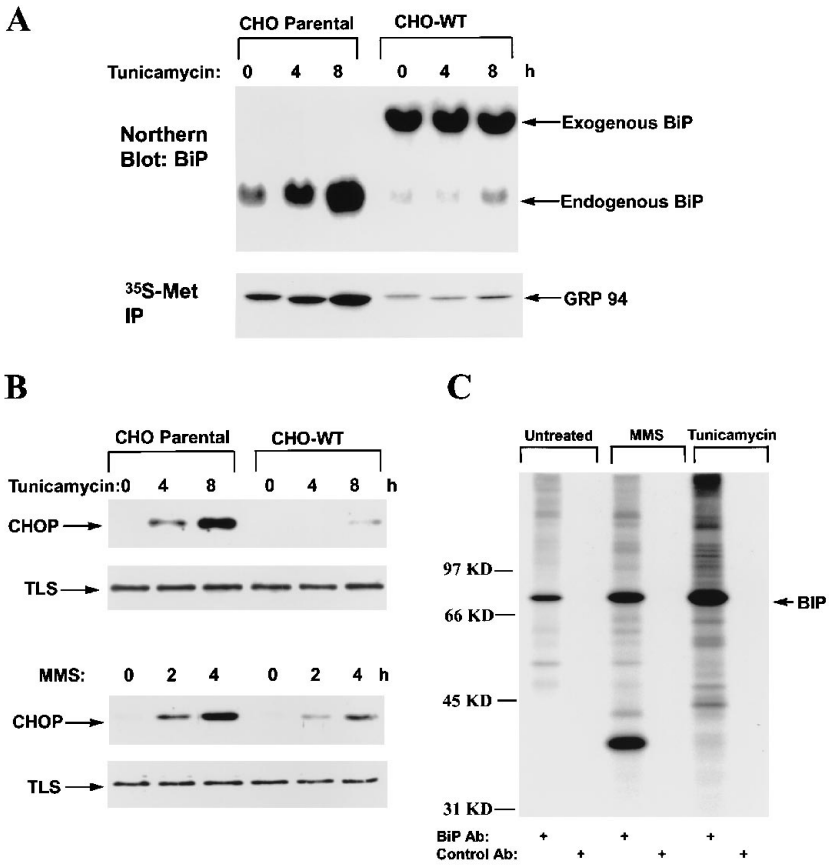

FIG. 3. Overexpression of the ER chaperone BiP attenuates the induction of CHOP. (A) Characterization of the BiP-overexpressing cells, CHO-WT. The upper panel is a Northern blot of whole-cell RNA hybridized with the hamster *BiP* cDNA. The positions of the endogenous *BiP* mRNA and the larger exogenous form are indicated to the right of the autoradiogram. The lower panel shows metabolically labeled immune precipitated (IP) GRP94. Note the induction of endogenous BiP and GRP94 in response to tunicamycin (10 μg/ml) in the parental CHO cells and the attenuated induction in the cells overexpressing BiP. (B) Attenuated induction of CHOP in response to tunicamycin and MMS in the BiP-overexpressing cells. A Western blot of CHOP and TLS (the internal control) in parental and CHO-WT cells following treatment with tunicamycin (10 μg/ml) or MMS (100 μg/ml) is shown. (C) MMS treatment causes ER proteins to associate with BiP. [^{35}S]methionine-labeled proteins were immunoprecipitated with a monoclonal antibody (Ab) to BiP (or an unrelated antibody) from detergent lysates of cells treated for 2.5 h with MMS (100 μg/ml), tunicamycin (25 μg/ml), or untreated cells. BiP and its associated proteins are revealed by autoradiography of the SDS-polyacrylamide gel of the coimmunoprecipitating proteins. Molecular mass markers are noted at the left.

the creation of an apurinic site (8) similar to the one remaining after excision repair of bases methylated by MMS (27). Either because of reduced incorporation of the agent into DNA (7) or because of failure to excise the modified base once incorporation has occurred (6), not all cells are responsive to the DNA-damaging effects of HmdUrd. We chose therefore to examine a clone of Chinese hamster lung fibroblasts (V79) that is known to be responsive to HmdUrd (8). In these cells, HmdUrd in doses of up to 4 μM failed to induce CHOP, whereas MMS was fully active (Fig. 5A). Both compounds led to an induction of G_2 cell cycle arrest, as revealed by FACS analysis of cellular DNA content (Fig. 5B). This indicates that HmdUrd was active in this experiment. We also note that HmdUrd did not lead to induction of BiP, whereas MMS did (Fig. 5C), further strengthening the correlation between ER stress and the induction of CHOP.

To analyze further the relationship between DNA damage and the induction of CHOP, a physical agent that induces DNA damage by a mechanism different from that of MMS was used. UV light leads to the formation of pyrimidine dimers and induces growth arrest by activating a p53-dependent pathway (22, 24). *CHOP* expression in response to UV light was examined in several cellular systems. In primary cultures of human foreskin fibroblasts that have wild-type p53 protein, the protein is induced by UV light, serving as an indicator that DNA damage has occurred (Fig. 6A, lower panel), but CHOP is not induced (upper panel). Since in HeLa and 3T3 cells changes in p53 levels are a poor reflection of the response to UV light, we relied on the ability of UV light to activate members of the stress-activated protein kinase family as an indication of the cellular response to the agent. UV light led to a dramatic increase in levels of SAPK1α and SAPK1β enzymatic activity, but not to induction of CHOP (Fig. 6B). Both MMS and UV light induce the SAPKs, indicating that their activation is not sufficient to induce CHOP.

These results differ from those of other experiments in which *CHOP* or *GADD153* has been found to be modestly responsive to UV light (15–18). Close examination of the published reports revealed that CHOP induction by UV light was associated with high baseline levels of the mRNA in the untreated cells (reference 15, Fig. 2A therein, and reference 18, Fig. 6 therein; contrast these figures with reference 16, Fig. 1 and the untreated lane therein, and Fig. 5 here). This finding suggests that culture conditions of the cells in those specific experiments may have favored the induction of CHOP by mechanisms independent of (or possibly even synergistic with) UV light. To

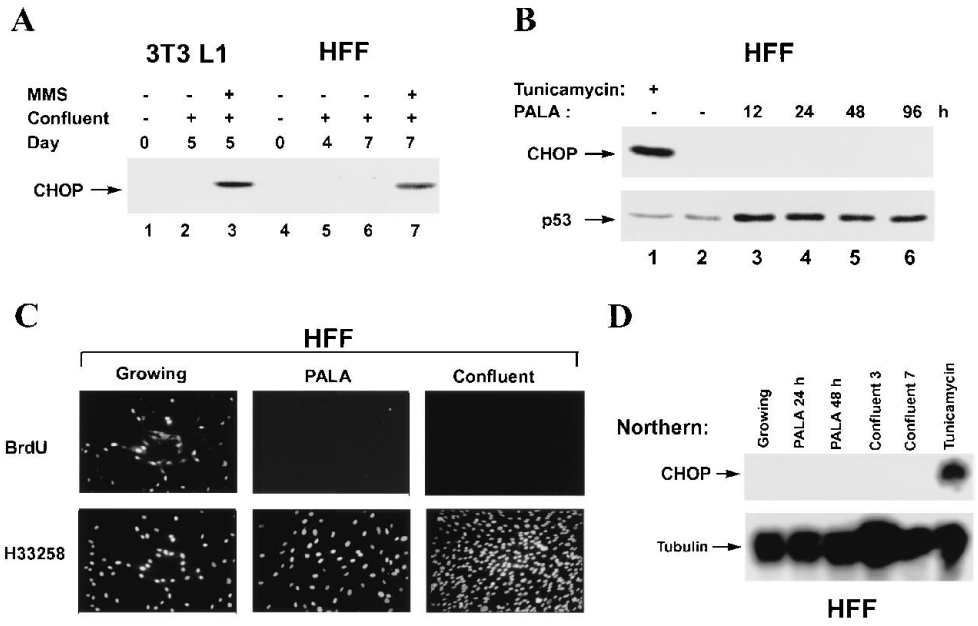

FIG. 4. Growth arrest does not uniformly induce CHOP. (A) 3T3 L1 cells and human foreskin fibroblast cells (HFF) were kept at confluence for the indicated time periods to reach contact inhibition and growth arrest. Whole-cell lysates were prepared and probed for the presence of CHOP by Western blot analysis. Induction of CHOP by MMS controls for the inducibility of CHOP under these culture conditions (lanes 3 and 7) is shown. (B) The enzymatic inhibitor PALA, which interferes with nucleotide metabolism and induces growth arrest, does not induce CHOP. Human foreskin fibroblasts were cultured in the presence of PALA (120 μM). At the indicated time points whole-cell lysates were analyzed for CHOP and p53 content by Western blotting (upper and lower panels). (C) Incorporation of BrdU as revealed by staining of subconfluent (growing) human foreskin fibroblasts, subconfluent cells exposed to PALA (120 μM for 24 h), and cells maintained at confluence for 3 days. The lower panel shows the nuclei of the same cells stained with the karyophilic dye H33258. (D) Northern blot analysis of CHOP and β-tubulin performed with cells in parallel with the experiment shown in panel C.

examine this possibility directly and thereby to try to reproduce the published results, we exposed CHO cells that had been cultured overnight in a low concentration of glucose to UV light. As expected, when compared with cells cultured in standard medium, the cells in low-concentration glucose had elevated basal levels of CHOP. Interestingly, when they were irradiated with UV light, CHOP was further induced (Fig. 6C). This induction of CHOP by UV light was also associated with a superinduction of BiP, suggesting that UV light is capable of inducing CHOP by a mechanism that is synergistic with the ER stress pathway.

DISCUSSION

This study suggests a key role for signals elicited by the stressed ER in the induction of CHOP. Correlative pharmacological data indicate that the inducers of CHOP also interfere with proper protein folding in the ER and result in the induction of BiP (3, 10, 11, 41). More direct evidence is provided by the induction of CHOP in cells with a conditional defect in N-linked glycosylation of ER proteins. The fact that this defect is specific for proteins entering the ER supports the conclusion that a stress signal emanating from that site is a major inducer of CHOP. The relatively short latency period between the induction of the ER defect by switching to culture at a nonpermissive temperature and the appearance of CHOP protein suggests that *CHOP* is responding to an early ER stress signal.

The primary molecular defect in K12 is not known. The possibility that these cells harbor a temperature-sensitive mutation in a different gene that is responsible for CHOP induction, though formally possible, is rendered unlikely by our observation of CHOP induction in a different, independently derived hamster cell line, BHK tsBN7, with a temperature-sensitive mutation in a known component of the protein glycosylation apparatus. The experiments with the two cell lines do not permit us to distinguish between a situation in which *CHOP* responds directly to an ER signal and one in which *CHOP* responds to a perturbation in a downstream cellular process that is itself dependent on the integrity of ER function. Examples of such a downstream cellular process might include essential transport functions or signaling processes that require proper expression of cell surface or secreted molecules.

The experiments in cells overexpressing BiP, however, strongly support a model in which *CHOP* responds directly to an ER stress signal. Overexpression of exogenous BiP inhibits the induction of genes encoding the glucose-regulated proteins in response to agents that affect protein folding in the ER (14) (Fig. 3A). This occurs by attenuation of the ER stress signal, not by abrogation of the folding defect or its downstream consequences that persist unabated (14). Therefore, the ability of BiP overexpression to attenuate CHOP inducibility means that *CHOP* is not responding to a distant consequence of ER malfunction but rather to a proximal stress signal generated in the ER.

The surprising observation that BiP overexpression attenuates the induction of CHOP in response to MMS led us to examine both the effects of MMS on ER proteins and the role of DNA damage in CHOP induction. We find that treatments that cause DNA damage (such as UV light and HmdUrd) do not induce CHOP and do not induce an ER stress signal. Furthermore, treating cells with MMS has a profound effect on proteins in the ER compartment, which is reflected in an enhanced association of proteins with BiP and an induction of BiP and other GRPs. These data, taken together with the attenuated induction of CHOP in response to MMS in BiP-

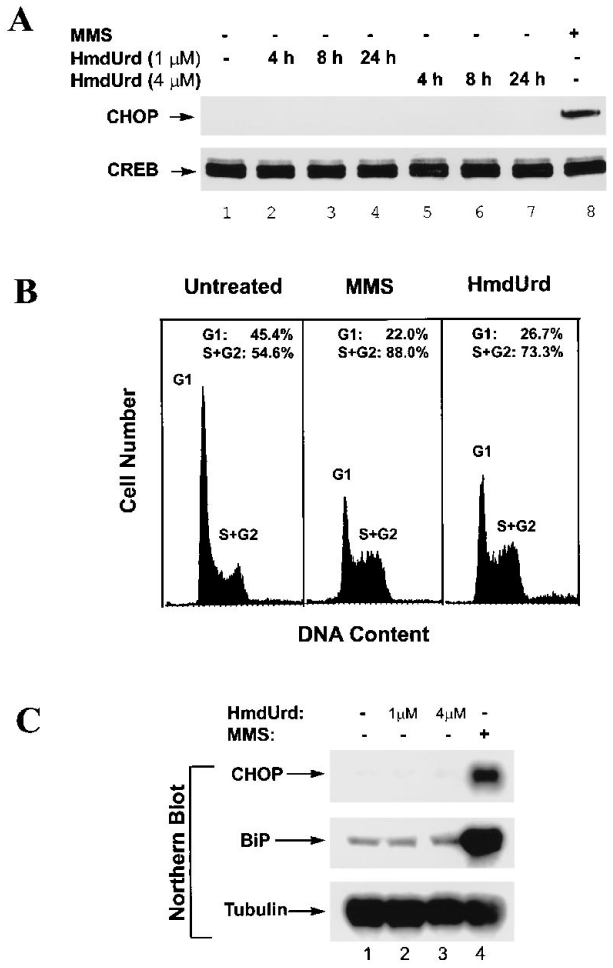

FIG. 5. Treatment of cells with HmdUrd, a compound that mimics the effect of MMS on DNA but that does not modify other macromolecules and does not induce CHOP. (A) V79 hamster cells were treated with HmdUrd for the indicated periods of time. Whole-cell lysates were evaluated for CHOP content by Western blot analysis with a rabbit polyclonal serum to CHOP (upper panel). Induction of CHOP by MMS (lane 8) serves as a control for the inducibility of CHOP in these cells. The same blot was probed with an antiserum to CREB to serve as an internal control. (B) Histograms of the distribution of DNA contents of untreated cells and cells treated with MMS (100 μg/ml for 1 h, 16 h before analysis) and HmdUrd (1 μM, continuous treatment for 16 h). Both compounds induce similar accumulations of cells in the G_2 and S phases of the cell cycle. (C) Northern blot of RNA from V79 cells either treated continuously with HmdUrd (16 h at the indicated dose) or pulsed for 1 h with MMS (100 μg/ml, and harvested 4 h later) and hybridized with CHOP, BiP, and β-tubulin probes.

overexpressing cells, lead us to conclude that CHOP induction occurs primarily in response to unfolded proteins in the ER and not, as previously suggested, through a DNA-damage-mediated pathway or through growth arrest.

How can one reconcile our finding that two well-established DNA-damaging stimuli, UV light and HmdUrd, do not induce CHOP with previous work suggesting that *CHOP* is a *GADD* gene? The results of the original experiments showing that CHOP is inducible by UV irradiation must be interpreted in light of the observation that *CHOP* mRNA levels were unphysiologically elevated in the untreated cells in those experiments. We were able to reproduce these observations by treating glucose-starved cells with UV light. We interpret the observed synergism of UV light with low-concentration glucose in the induction of CHOP to indicate that in such a setting UV light could be working synergistically with an ER stress signal. The superinduction of BiP in response to UV light in the glucose-starved cells and its absence in the cells growing in rich medium (Fig. 6C, compare the BiP signals in lanes 1 and 2 with those in lanes 3 and 4) lends further support to this interpretation.

What might be the physiological significance of the induction of CHOP by an ER stress signal? In adipogenic cell lines, culture in low-concentration glucose, a physiological inducer of CHOP known to cause ER stress, markedly inhibits the differentiation process (4, 10). Forced expression of *CHOP* can fully account for this inhibition of differentiation, which is dependent on the ability of CHOP to antagonize the activities of C/EBP proteins at several levels (4). Taken together with the findings presented here, this supports a model, originally alluded to by Carlson et al. (10), in which CHOP is an effector of the response of cells to ER overload. In the adipogenic lineage, this translates to inhibiting differentiation, a process known to be associated with increased levels of protein secretion (26). To the extent that C/EBP transcription factors positively regulate the expression of secreted proteins in a variety of organs (33), induction of CHOP may broadly mitigate ER stress by attenuating the expression of genes that encode secreted proteins. The induction of *GRP* genes is a late event compared with the induction of CHOP (Fig. 3) and, in contrast to the induction of CHOP, is dependent on new protein synthesis (reference 41 and our unpublished observations). It is possible therefore that CHOP participates in the induction of the GRPs. Our recent finding that CHOP has a stress-regulated positive transcriptional activation function (49, 51) fits well with such a speculation.

In addition to its effects on differentiated gene expression, *CHOP* may also play a role in regulating cell growth. Forced expression of *CHOP* to a high level can, under some conditions, lead to cell cycle arrest (2, 53). It is thus possible that CHOP participates in a signaling pathway that modifies cellular proliferation in response to stress in the ER. The need to modify growth in response to stress is shared by organisms from bacteria to mammals and presumably reflects the important role this process has in adaptation to a hostile environment (19). One attractive hypothesis is that the cell has distinct pathways for transducing growth-arrest signals in response to specific forms of stress. K12 cells undergo cell cycle arrest at the nonpermissive temperature, suggesting a direct link between ER stress and cell cycle control (35). We speculate that CHOP occupies a niche in a pathway linking the ER to this response, whereas p53, for example, serves a functionally similar role in response to various forms of DNA damage. A naturally occurring altered form of *CHOP* that is incapable of eliciting growth arrest (2) has been identified as the myxoid liposarcoma oncogene (12, 54). This suggests that derangement in the pathway linking ER stress to growth arrest may predispose cells to the development of neoplastic transformation.

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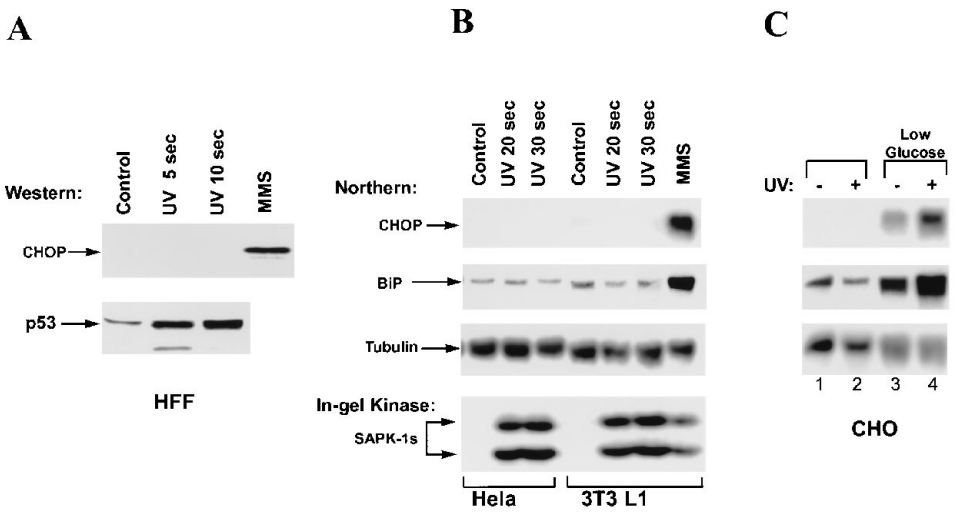

FIG. 6. UV irradiation, a potent DNA-damaging agent, does not by itself induce CHOP. (A) Human foreskin fibroblasts were irradiated with UV light (at 1.38 J/s/m^2 for the indicated periods) or treated with MMS (100 μg/ml) and harvested for CHOP immunoblot analysis 4 h later (upper panel). Induction of p53 (immunoblot analysis, lower panel) indicates that a genotoxic stress signal was registered by the cells. (B) Doses of UV light sufficient to activate the stress-activated protein kinase cascade do not induce CHOP in HeLa and 3T3 L1 cells. Shown is a Northern blot probed sequentially with CHOP, BiP, and β-tubulin; conditions of treatment and the cell harvesting procedure were the same as described for panel A. Induction of the SAPK1s, an indication that a UV light-induced stress signal has been registered, is revealed by an in-gel kinase assay with GST-Jun 2-233 as a substrate (lowest panel). (C) UV light is capable of activating *CHOP* in synergism with an ER stress signal. CHO cells cultured in standard medium or in medium lacking glucose (2 mM, for 16 h) were irradiated with UV light (27 J/m^2 as described for panels A and B). Four hours later RNA was prepared for Northern blot analysis and hybridized to CHOP, BiP, and β-tubulin probes.

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REFERENCES

1. **Åman, P., D. Ron, N. Mandahl, T. Fioretos, S. Heim, K. Arhenden, H. Willén, A. Rydholm, and F. Mitelman.** 1992. Rearrangement of the transcription factor gene *CHOP* in myxoid liposarcomas with t(12;16)(q13;p11). Genes Chromosomes Cancer **5:**271–277.
2. **Barone, M. V., A. Y. Crozat, A. Tabaee, L. Philipson, and D. Ron.** 1994. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, differ in their ability to induce G1/S arrest. Genes Dev. **8:**453–464.
3. **Bartlett, J., J. Luethy, S. Carlson, S. Sollott, and N. Holbrook.** 1992. Calcium ionophore A23187 induces expression of the growth arrest and DNA damage-inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. J. Biol. Chem. **267:**20465–20470.
4. **Batchvarova, N., X.-Z. Wang, and D. Ron.** 1995. Inhibition of adipogenesis by the stress-induced protein CHOP (GADD153). EMBO J. **14:**4654–4661.
5. **Bole, D., L. Hendershot, and J. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with the nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102:**1558–1566.
6. **Boorstein, R. J., L.-N. Chiu, and G. W. Teebor.** 1992. A mammalian cell line deficient in activity of the DNA repair enzyme 5-hydroxymethyluracil-DNA glycosylase is resistant to the toxic effects of the thymidine analog 5-hydroxymethyl -2′-deoxyuridine. Mol. Cell. Biol. **12:**5536–5540.
7. **Boorstein, R., J. Haldar, G. Poirier, and D. Putnam.** 1995. DNA base excision repair of 5-hydroxymethyl-2-deoxyuridine stimulates poly(ADP-ribose) synthesis in Chinese hamster cells. Carcinogenesis (Oxford) **16:**1173–1179.
8. **Boorstein, R., and G. Teebor.** 1989. Effects of 5-hydroxymethyluracil and 3-aminobenzamide on the repair and toxicity of 5-hydroxymethyl-2′-deoxyuridine in mammalian cells. Cancer Res. **49:**1509–1514.
9. **Braakman, I., J. Helenius, and A. Helenius.** 1992. Role of ATP and disulphide bonds during protein folding in the endoplasmic reticulum. Nature (London) **356:**260–262.
10. **Carlson, S. G., T. W. Fawcett, J. D. Bartlett, M. Bernier, and N. J. Holbrook.** 1993. Regulation of the C/EBP-related gene *gadd153* by glucose deprivation. Mol. Cell. Biol. **13:**4736–4744.
11. **Chen, Q., K. Yu, N. J. Holbrook, and J. L. Stevens.** 1992. Activation of the growth arrest and DNA damage-inducible gene gadd153 by nephrotoxic cysteine conjugates and dithiothreitol. J. Biol. Chem. **267:**8207–8212.
12. **Crozat, A. Y., P. Åman, N. Mandahl, and D. Ron.** 1993. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma with t(12;16)(q13;p11). Nature (London) **363:**640–644.
13. **Dorner, A., D. Bole, and R. Kaufman.** 1987. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. J. Cell Biol. **105:**2665–2674.
14. **Dorner, A., L. Wasley, and R. Kaufman.** 1992. Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. EMBO J. **11:**1563–1571.
15. **Fornace, A., I. Alamo, and M. Hollander.** 1988. DNA damage-inducible transcripts in mammalian cells. Proc. Natl. Acad. Sci. USA **85:**8800–8804.
16. **Fornace, A. J., D. W. Neibert, M. C. Hollander, J. D. Luethy, M. Papathanasiou, J. Fragoli, and N. J. Holbrook.** 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol. Cell. Biol. **9:**4196–4203.
17. **Garmyn, M., M. Yaar, N. Boileau, C. Backendorf, and B. Gilchrest.** 1992. Effect of aging and habitual sun exposure on the genetic response of cultured human keratinocytes to solar-simulated irradiation. J. Invest. Dermatol. **99:**743–748.
18. **Gujuluva, C., J.-H. Baek, K.-H. Shin, H. Cherrick, and N.-O. Park.** 1994. Effect of UV-irradiation on cell cycle, viability and the expression of p53, gadd153 and gadd45 genes in normal and HPV-immortalized oral keratinocytes. Oncogene **9:**1819–1827.
19. **Hartwell, L.** 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell **71:**543–546.
20. **Hendershot, L. M., J. Ting, and A. S. Lee.** 1988. Identity of the immunoglobulin heavy-chain-binding protein with the 78,000-dalton glucose-regulated protein and the role of posttranslational modifications in its binding function. Mol. Cell. Biol. **8:**4250–4256.
21. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
22. **Hupp, T. R., D. W. Meek, C. A. Midgley, and D. P. Lane.** 1992. Regulation of the specific DNA binding function of p53. Cell **71:**875–886.
23. **Kamashita, I., and H. Fujisawa.** 1989. A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. Anal. Biochem. **183:**139–143.
24. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig.** 1991. Participation of p53 in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
25. **Knight, J. C., P. J. Renwick, P. Dal Cin, H. Van Den Berghe, and C. D. M. Fletcher.** 1995. Translocation t(12;16)(q13;p11) in myxoid liposarcomas and round cell liposarcoma: molecular and cytogentic analysis. Cancer Res. **55:**24–27.
26. **Kuri-Harcuch, W., C. Argüello, and M. Marsch-Moreno.** 1994. Extracellular matrix production by mouse 3T3-F442A cells during adipocytic differentiation in culture. Differentiation **28:**173–178.
27. **Laval, J., J. Pierre, and F. Laval.** 1981. Release of 7-methylguanine residues from alkylated DNA by extracts of micrococcus luteus and Escherichia coli. Proc. Natl. Acad. Sci. USA **78:**852–855.
28. **Lee, A.** 1981. The accumulation of three specific proteins related to glucose-

regulated proteins in a temperature-sensitive hamster mutant cell line K12. J. Cell. Physiol. **106:**119–125.
29. **Lee, A. S.** 1987. Coordinate regulation of a set of genes by glucose and calcium ionophores in mammalian cells. Trends Biochem. Sci. **12:**20–23.
30. **Livingston, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty.** 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell **70:**923–935.
31. **MacDougal, O., and M. Lane.** 1995. Transcriptional regulation of gene expression during adipocytic differentiation. Annu. Rev. Biochem. **64:**345–373.
32. **Marten, N. W., E. J. Burke, J. M. Hayden, and D. S. Straus.** 1994. Effect of amino acid limitation on the expression of 19 genes in rat hepatoma cells. FASEB J. **8:**538–544.
33. **McKnight, S.** 1992. CCAAT/enhancer binding protein. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Melero, J.** 1981. Identification of the glucose/glycosylation-regulated proteins as those which accumulate in the temperature-sensitive cell line K12. J. Cell. Physiol. **109:**59–67.
35. **Melero, J., and V. Fincham.** 1978. Enhancement of the synthesis of specific cellular polypeptides in a temperature-sensitive Chinese hamster cell line (K12) defective for entry into S phase. J. Cell. Physiol. **95:**295–306.
36. **Morris, J., A. Dorner, L. Hendershot, and R. Kaufaman.** BiP ATPase activity is required to protect cells from ER stress and for the secretion of selective proteins. Submitted for publication.
37. **Nakashima, T., T. Sekiguchi, A. Kuraoka, K. Fukushima, Y. Shibata, S. Komiyama, and T. Nishimoto.** 1993. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell. Biol. **13:**6367–6374.
38. **Nishimoto, T., and C. Basilico.** 1978. Analysis of a method for selecting temperature sensitive mutants of BHK cells. Somatic Cell Genet. **4:**323–340.
39. **Otto, E., S. McCord, and T. Tlsty.** 1989. Increased incidence of CAD gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. J. Biol. Chem. **264:**3390–3396.
40. **Poussegur, J., R. P. C. Shiu, and I. Pastan.** 1977. Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. Cell **11:**941–947.
41. **Price, B., and S. Calderwood.** 1992. Gadd 45 and Gadd 153 messenger RNA levels are increased during hypoxia and after exposure of cells to agents which elevate the levels of glucose-regulated proteins. Cancer Res. **52:**3814–3817.
42. **Rabbitts, T. H., A. Forster, R. Larson, and P. Nathan.** 1993. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. Nat. Genet. **4:**175–180.
43. **Ron, D., and J. F. Habener.** 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant negative inhibitor of gene transcription. Genes Dev. **6:**439–453.
43a. **Sanjay, A., and G. Kreibich.** Unpublished observations.
44. **Silberstein, S., P. Collins, D. Kelleher, and R. Gilmore.** 1995. The essential OST2 gene encodes the 16-kD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. J. Cell Biol. **131:**371–383.
45. **Sorrentino, V., R. Pepperkok, R. L. Davis, W. Ansorge, and L. Philipson.** 1990. Cell proliferation inhibited by *MyoD1* independently of myogenic differentiation. Nature (London) **345:**813–815.
46. **Sreekantiaiah, S., C. P. Karakousis, S. P. L. Leong, and A. A. Sandberg.** 1992. Cytogenetic findings in liposarcoma correlate with histopathologic subtypes. Cancer **69:**2484–2495.
47. **Swyryd, E., S. Seaver, and G. Stark.** 1974. N-(phosphonacetyl) L-aspartate, a potent transition state analog inhibitor of aspartate trans-carbamylase, blocks proliferation of mammalian cells in culture. J. Biol. Chem. **249:**6945–6950.
48. **Sylvester, S. L., C. M. J. ap Rhys, J. D. Leuthy-Martindale, and N. J. Holbrook.** 1994. Induction of *GADD153* a CCAAT/enhancer-binding protein-related gene during the acute-phase response in rats. J. Biol. Chem. **269:**20119–20125.
49. **Ubeda, M., X.-Z. Wang, H. Zinszner, I. Wu, J. Habener, and D. Ron.** 1996. Stress-induced binding of transcription factor CHOP to a novel DNA-control element. Mol. Cell. Biol. **16:**1479–1489.
50. **Waeber, G., and J. F. Habener.** 1991. Nuclear translocation and DNA recognition signals colocalized within the bZIP domain of cyclic adenosine 3′-5′-monophosphate response element-binding protein CREB. Mol. Endocrinol. **5:**1431–1438.
51. **Wang, X.-Z., and D. Ron.** 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP-kinase. Science, 272:1347–1349.
52. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by HPV oncoproteins. Genes Dev. **8:**666–677.
53. **Zhan, Q., K. A. Lord, I. Alamo, Jr., M. C. Hollander, F. Carrier, D. Ron, K. W. Kohn, B. Hoffman, D. A. Liebermann, and A. J. Fornace, Jr.** 1994. The *gadd* and *MyD* genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. Mol. Cell. Biol. **14:**2361–2371.
54. **Zinszner, H., R. Albalat, and D. Ron.** 1994. A novel effector domain from the RNA-binding proteins TLS or EWS is required for oncogenic transformation by CHOP. Genes Dev. **8:**2513–2526.