0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 1
Printed in U.S.A.

Induction of the C/EBP Homologous Protein (CHOP) by Amino Acid Deprivation Requires Insulin-Like Growth Factor I, Phosphatidylinositol 3-Kinase, and Mammalian Target of Rapamycin Signaling*

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ABSTRACT

In mammalian cells, gene regulation by amino acid deprivation is poorly understood. Here, we examined the signaling pathways involved in the induction of the C/EBP homologous protein (CHOP) by amino acid starvation. CHOP is a transcription factor that heterodimerizes with other C/EBP family members and may inhibit or activate the transcription of target genes depending on their sequence-specific elements. Amino acid deficiency, when accompanied by insulin-like growth factor I signaling, results in the accumulation of CHOP messenger RNA and protein in AKR-2B and NIH-3T3 cells. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 are able to block CHOP induction in response to amino acid deprivation. Rapamycin is also able to abrogate CHOP expression, suggesting that the mammalian target of rapamycin is involved in CHOP induction by amino acid deficiency. LY294002 and rapamycin are also able to block CHOP induction by hydrogen peroxide, but do not affect expression induced by sodium arsenite or A23187. This is the first evidence that the insulin-like growth factor I/phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway is required for gene regulation by amino acid deprivation and that this pathway is involved in the induction of CHOP by both amino acid deficiency and oxidative stress by hydrogen peroxide. (*Endocrinology* **142:** 221–228, 2001)

CELLS REGULATE gene expression in response to cues from their local environment, including the availability of nutrients such as amino acids and growth factors. Amino acid deprivation provides an undue stress on cells and has adverse effects on cell growth. The *in vivo* relevance of adequate nutrient supply has been demonstrated in studies in which dietary protein restriction in young rats results in growth retardation (1). Restriction of tyrosine and phenylalanine suppresses the metastatic potential of melanoma cells *in vivo* by inducing a cell cycle arrest (2). Although nutritional control of gene expression has been well defined in prokaryotes and lower eukaryotes, less is known about the mechanisms used by multicellular organisms to respond to nutrient deprivation.

To properly adapt to the conditions of their environment, cells must be able to regulate the expression of individual genes needed to elicit responses specific to the insult. Amino acid deficiency induces a subset of messenger RNA (mRNA) products, whereas the overall rate of transcription does not change (3). Some of the genes regulated by amino acid starvation include genes involved directly in amino acid metabolism such as asparagine synthetase, the cationic amino acid transporter cat-1, and the amino acid transport system A (4–6). Other amino acid deprivation-inducible genes such as c-*myc*, c-*jun*, and insulin-like growth factor (IGF)-binding protein-1 (IGFBP-1) have been implicated in the regulation of cell growth (7, 8).

The C/EBP homologous protein (CHOP) is another gene involved in regulating cell growth and can be induced by a variety of adverse conditions, including oxidative stress, DNA damage, and amino acid deprivation (9–12). Also identified as a growth arrest and DNA damage-inducible gene (gadd153), CHOP was induced in cells that had undergone cell cycle arrest by medium depletion (10). Microinjection of CHOP into NIH-3T3 fibroblasts arrests cell in the G_1 phase of the cell cycle (13). CHOP induction by toxic stimuli such as methylmethane sulfonate and tunicamycin has also been correlated with the onset of apoptosis (14, 15). CHOP protein can influence target gene transcription by acting as a dominant negative regulator of C/EBP binding to one class of elements or by directing CHOP-C/EBP-β heterodimers to a novel DNA sequence to increase transcription in a stress-dependent manner (16, 17). Wang *et al.* (18) recently identified a set of stress-inducible, CHOP-dependent target genes, termed DOCs, that presumably contain this novel CHOP-C/EBP-binding site within their promoters. These data suggest that CHOP induction may play an important role in regulating cellular events in response to different stressful stimuli.

In cooperation with amino acid deprivation, other environmental stimuli, such as growth factors, may initiate signaling events to regulate gene expression. IGF-I is involved in preventing apoptosis induced by serum starvation or oxidative stress (19, 20). IGF-I is a potent activator of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) sig-

Received June 26, 2000.
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* This work was supported by NIH Grants CA-42572 and CA-85492. Sequencing was carried out by the DNA Sequencing Shared Resource (Grant P30-CA-68485). Data presentation was performed in part through the use of the Vanderbilt University Medical Center Cell Imaging Resource (Grants CA-68485 and DK-20593).

naling cascade, and these enzymes mediate the antiapoptotic effects of IGF-I (19, 21). PI3K transmits signals via PKB to p70S6K, which is activated by the kinase mammalian target of rapamycin (mTOR). In turn, it has been shown that PKB and p70S6K are activated by oxidative stress induced by H_2O_2 (22, 23). Therefore, the IGF-I/PI3K/mTOR pathway may be involved in signaling events that promote cell survival under adverse conditions.

Here, we investigate the induction of CHOP by amino acid deprivation to identify coordinating signals that regulate gene expression in response to stressful stimuli. Our findings demonstrate that CHOP induction under nutritional starvation requires both the deprivation of amino acids and the activation of the IGF-I/PI3K/mTOR signaling cascade. Inhibitors of this pathway block the amino acid-dependent induction of CHOP as well as the expression of the downstream target genes, DOCs. We have also observed that PI3K and mTOR activities are required for CHOP induction by H_2O_2, but are not required for induction of CHOP by $NaAsO_2$ or A23187.

Materials and Methods

Cell culture and treatment conditions

AKR-2B and NIH-3T3 mouse fibroblasts were maintained in minimal Eagle's medium (MEM) containing 10% FBS in a 5% CO_2 environment. To deprive cells of amino acids, subconfluent cultures were washed twice in PBS and incubated in the appropriate amino acid-deficient medium containing 10% FBS. AKR-2B cells were incubated in a MEM-based medium lacking cysteine, isoleucine, leucine, and tryptophan (Life Technologies, Inc., Gaithersburg, MD). Although this medium lacks four amino acids, we have observed that CHOP is induced by the individual depletion of leucine, methionine, or tryptophan (data not shown). NIH-3T3 cells were incubated in RPMI medium lacking cysteine and methionine. For growth factor studies, epidermal growth factor (EGF) or IGF-I was added to serum-free amino acid-deficient medium, whereas IGFBP-3 and IGFBP-3:IGF-I experiments were performed in the presence of 10% FBS. IGF-I, IGFBP-3, and IGFBP-3:IGF-I were provided by Desmond Mascarenhas (Celtrix Pharmaceuticals, Inc., San Jose, CA).

For inhibitor studies, stock solutions of wortmannin (500 μM), LY294002 (10 mM), rapamycin (20 μM), FK506 (20 μM), SB203580 (20 mM; Alexis Biochemicals, San Diego, CA), or dimethylsulfoxide vehicle were diluted into the medium before placement on the cells. Hydrogen peroxide (H_2O_2) and sodium arsenite ($NaAsO_2$) were diluted in PBS to a final concentration of 200 μM and placed on the cells for 30 min. These treatments were then replaced with MEM and 10% FBS with or without the indicated inhibitors. A23187 (Sigma, St. Louis, MO) was used at a final concentration of 1 μg/ml in MEM and 10% FBS. Unless otherwise indicated, cells were harvested after 18 h of incubation.

Cell lysis, immunoblotting, and kinase assay

Cells were lysed in extraction buffer containing 50 mM HEPES, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.1% TritonX-100, 0.1% β-mercaptoethanol, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 nM microcystin, 10 μg/ml trypsin inhibitor, 0.5 μg/ml leupeptin, and 0.2 mM phenylmethylsulfonylfluoride. Extracts were sonicated and centrifuged for 10 min. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Inc., Richmond, CA). For immunoblotting, samples were boiled in Laemmli sample buffer, and 20 μg total protein were resolved on 10% or 12% SDS-containing polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, blocked with 5% BSA, and probed with antibodies directed against CHOP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-tubulin (Sigma), or p70S6K (Santa Cruz Biotechnology, Inc.). Blots were probed with the appropriate peroxidase-conjugated (CHOP and β-tubulin; Amersham Pharmacia Biotech) or alkaline phosphatase-conjugated (p70S6K) secondary antibody and developed with enhanced chemiluminescence (Amersham Pharmacia Biotech) or nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, respectively. p70S6K kinase assay was performed as previously described (24).

RNA extraction, Northern blotting, and RT-PCR

Total RNA was isolated using the single step method of Chomczynski and Sacchi (25). For Northern analysis, 30 μg total RNA were separated on a formaldehyde agarose gel and transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech). Full-length CHOP complementary DNA (cDNA; provided by Dr. David Ron, Boston, MA) was labeled with [α-^{32}P]ATP using a random primer labeling kit (Roche). Prehybridization and hybridization were carried out at 42 degree in 50% formamide, 5 × SSC (standard saline citrate), 1× Denhardt's solution, 0.1% SDS, 150 μg/ml sonicated salmon sperm DNA, and 50 μg/ml polyadenylase. Hybridized signals were detected using PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA). To control for loading, blots were reprobed with 18S cDNA.

To analyze the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CHOP, carbonic anhydrase VI (CA-VI), and advillin by PCR, 1 μg total RNA was primed with oligo(deoxythymidine) to synthesize cDNA by reverse transcriptase. The samples were diluted 5-fold, and 5% of the volume was used for subsequent PCR. Primers and conditions for PCR were modified from those described by Wang *et al.* (18) and are as follows: GAPDH primer 1, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; primer 2, 5′-CATGAGGCCATGAGGTCCACCAC-3′; CHOP primer 1, 5′-GCAGTCATGGCAGCTGAGTCCCTGCCTTTC-3′; primer 2, 5′-CAGACAGGAGGTGATGCCCACTGTTCATGC-3′; CA-VI primer 1, 5′-AGTGCTGGGCTTAGTTTAGAGCTTTCC-3′; primer 2, 5′-AGATCGATCGATACTGTGTGTCCGTTG-3′; advillin primer 1, 5′-ATCCACGGGAACGACAAATCCAAC-3′; and primer 2, 5′-AGGATGTGTGACCAGGTACTCCTG-3′. GAPDH and CHOP PCRs were performed in the same reaction using PCR buffer II (Perkin-Elmer Corp., Norwalk, CT) supplemented with 4 mM $MgCl_2$ at 95 C for 1 min and 70 C for 3 min for 24 cycles. CA-VI and advillin PCR were performed in PCR buffer II with 2 mM $MgCl_2$ at 94 C for 1 min, 60 C for 1 min, and 72 C for 1 min for 27 cycles.

Results

To understand the signaling pathways involved in amino acid-dependent gene regulation, we investigated the induction of CHOP by amino acid deprivation. We first examined the kinetics of CHOP induction in response to amino acid deprivation in the mouse fibroblast cell lines AKR-2B and NIH-3T3. As the results of the experiments in these two cell lines were identical, only results for one of the cell lines are shown in any given figure. AKR-2B cells were incubated in amino acid-deficient medium (plus 10% FBS) for the indicated times. As shown in Fig. 1A, amino acid deprivation induced *CHOP* mRNA beginning at 9 h and was sustained through 24 h of incubation. *CHOP* levels peaked at 12 h, with an 8-fold increase when normalized to the 18S ribosomal RNA loading control. In parallel with *CHOP* mRNA expression, CHOP protein levels also increased from 9–24 h in amino acid-deficient medium, whereas the levels of β-tubulin remain unchanged (Fig. 1B). Therefore, the lack of amino acids resulted in accumulation of CHOP mRNA and protein levels in AKR-2B and NIH-3T3 cells.

We next examined the biochemical pathways necessary for CHOP expression in response to the depletion of amino acids. As we performed the original experiments in the presence of serum, we could not rule out the possibility that a component of the serum was contributing to the induction of CHOP. Therefore, to isolate the effects specific for the loss of amino acids, we incubated the AKR-2B or NIH-3T3 cells in medium lacking both amino acids and serum. However, in

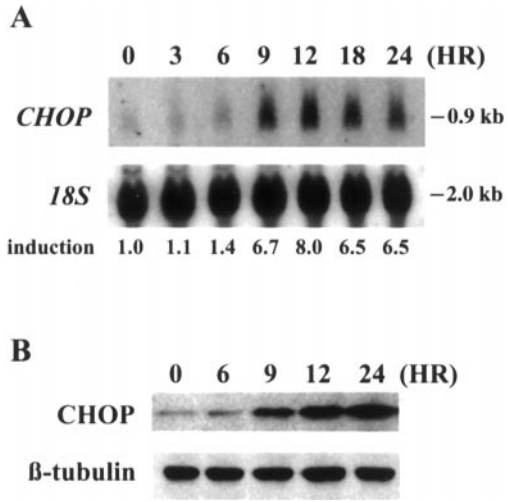

FIG. 1. Amino acid deprivation induces CHOP mRNA and protein. AKR-2B cells were incubated in amino acid-deficient medium with 10% FBS, and extracts were harvested at the indicated time points. A, Total RNA was extracted, and a Northern blot was prepared using a ^{32}P-labeled CHOP cDNA probe. The blot was rehybridized with an 18S probe to normalize for loading. Fold induction over 0 h is indicated. B, Whole cell protein lysates were prepared and probed with CHOP- and β-tubulin-specific antibodies.

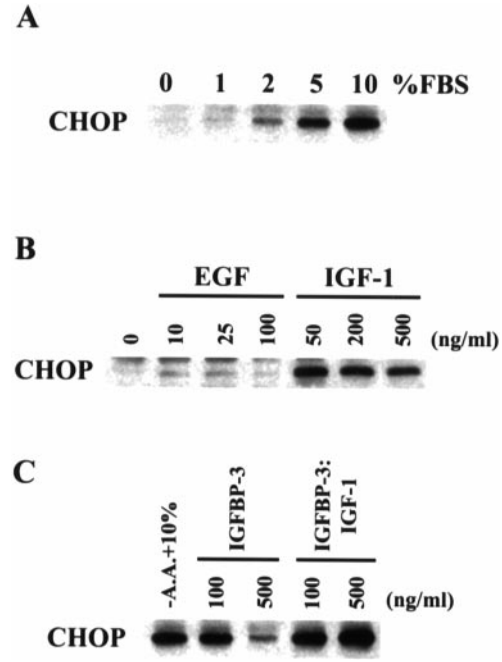

FIG. 2. CHOP induction by amino acid deprivation requires IGF-I. Cells were treated in the following medium conditions, protein was harvested after 18 h, and CHOP immunoblots were performed. A, AKR-2B cells were incubated in amino acid-deficient medium with increasing concentrations of serum (%FBS). B, AKR-2B cells were incubated in amino acid-deficient medium supplemented with the indicated doses of EGF or IGF-I. C, AKR-2B cells were incubated in amino acid-deficient medium plus serum (−A.A.+10%) containing either IGFBP-3 or a preformed complex of IGFBP-3 and IGF-I (IGFBP-3:IGF-I).

the absence of serum, amino acid deprivation was unable to induce CHOP expression (Fig. 2A). The addition of increasing amounts of serum (5–10%) was able to restore CHOP induction in the absence of amino acids. Therefore, there appeared to be at least two requirements for CHOP induction, the absence of amino acids and the presence of a component found in the serum. To identify the serum factor, we incubated cells in amino acid-free medium supplemented with two growth factors present in serum, EGF and IGF-I. At the doses tested, IGF-I restored CHOP induction by amino acid deprivation, but EGF did not (Fig. 2B), indicating that IGF-I can substitute for the serum requirement. At the indicated concentrations, EGF activated extracellular regulated protein kinase 1/2 (ERK1/2), confirming that the EGF signaling pathway was intact in these cells (data not shown). To verify that IGF-I was the serum factor required for CHOP induction, we inhibited IGF-I signaling with IGFBP-3. IGFBP-3 is able to complex with IGF-I and inhibits the binding of IGF-I to its receptor (26). When added in the presence of serum, IGFBP-3 sequestered the IGF-I present in the serum and blocked CHOP induction by amino acid deprivation in the AKR-2B cells (Fig. 2C). However, a preformed complex of IGFBP-3 and IGF-I (IGFBP-3:IGF-I), which is unable to bind and inhibit serum-supplied IGF-I, did not block CHOP induction. Taken together, these data suggest that IGF-I is the serum component required for the induction of CHOP in response to amino acid deficiency.

As we had established that IGF-I is necessary for CHOP induction by amino acid deficiency, we wanted to identify the downstream signaling molecules activated by IGF-I. IGF-I signaling weakly activates the ERK pathway, but potently stimulates the PI3K signaling cascade (27). Under the treatment conditions tested here, we noted that IGF-I did not stimulate ERK1/2, and the MEK-1 inhibitor PD98059 did not significantly inhibit CHOP induction by amino acid deprivation (data not shown). Likewise, activation of ERK1/2 by EGF is not sufficient to restore CHOP expression in the absence of serum (see above). These data indicate that the ERK pathway is not an essential component in the expression of CHOP by amino acid starvation. To determine whether the requirement for IGF-I in CHOP induction was dependent upon the activation of PI3K, AKR-2B or NIH-3T3 cells were incubated in amino acid-deficient medium containing serum with the addition of the PI3K inhibitors, wortmannin and LY294002. Both of the PI3K inhibitors inhibited the induction of CHOP in a dose-dependent manner, with wortmannin producing maximal inhibition at 100 nM and LY294002 producing maximal inhibition at 5 μM (Fig. 3). Neither of the inhibitors altered the expression of β-tubulin, indicating that their effect on CHOP was not due to inhibition of general protein synthesis. Wortmannin was also able to block CHOP induction in the presence of IGF-I (data not shown). These data indicate that PI3K activity is necessary for CHOP expression in conjunction with amino acid depletion.

We next wanted to determine whether other components of the PI3K signaling cascade were important for the accumulation of CHOP by amino acid deprivation. PI3K has been shown to indirectly activate mTOR, which, in turn, stimulates p70S6K activity. The macrolide rapamycin, in complex with its receptor FKBP12, inhibits mTOR and abolishes p70S6K activity. Although the levels of β-tubulin remained unchanged, the induction of CHOP by amino acid depletion was blocked by rapamycin in a dose-dependent manner at

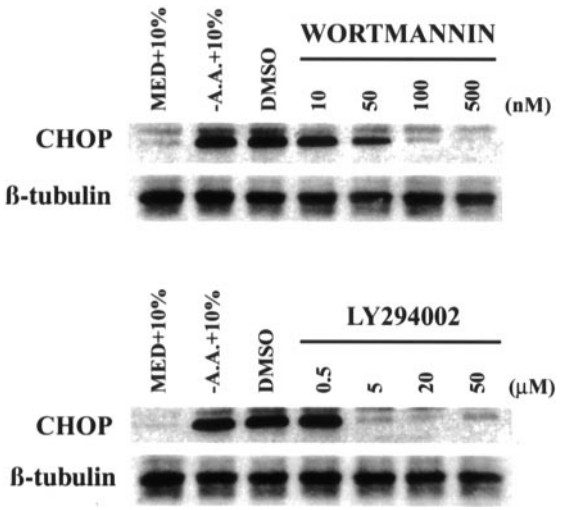

FIG. 3. PI3K inhibitors block CHOP induction by amino acid deprivation. AKR-2B cells were incubated in amino acid-deficient medium plus serum (−A.A.+10%) including DMSO vehicle or the indicated doses of wortmannin (*upper panel*) or LY294002 (*lower panel*) for 18 h. Cell lysates were analyzed for CHOP and β-tubulin protein expression as described in *Materials and Methods*.

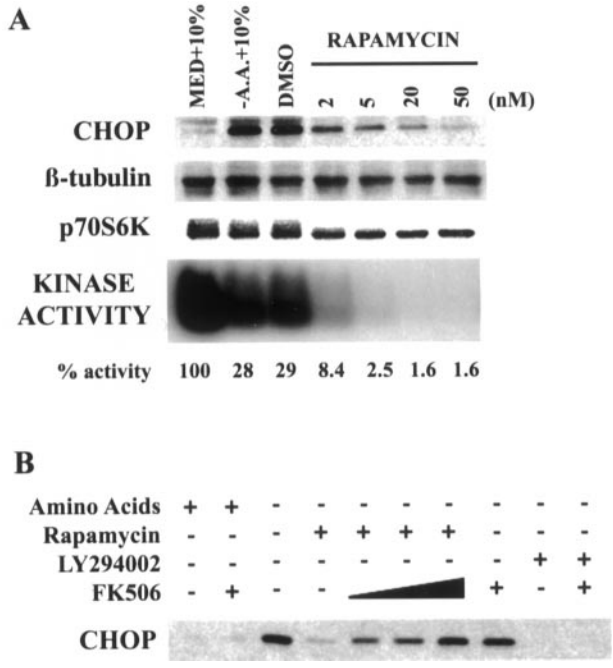

FIG. 4. Rapamycin inhibits the induction of CHOP by amino acid deprivation. A, AKR-2B cells were incubated in amino acid-deficient medium (−A.A.+10%) with the DMSO vehicle or the indicated dose of rapamycin for 18 h. Total protein was extracted, and equal amounts of protein were analyzed as described in *Materials and Methods*. *Upper panel*, CHOP and β-tubulin immunoblots; *middle panel*, p70S6K immunoblot; *lower panel*, p70S6K kinase assay. The percentage of the control kinase activity is indicated. B, NIH-3T3 cells were incubated in serum-containing medium either containing (+) or lacking (−) amino acids with the addition (+) of rapamycin (20 nM), LY294002 (10 μM), or FK506 (500 nM) in the indicated combinations. ▲, Increasing concentrations of FK506 of 20, 100, and 500 nM. After 18 h, protein lysates were analyzed for CHOP protein expression.

concentrations that completely inhibited the activity of p70S6K (Fig. 4A). This inhibition of p70S6K was demonstrated by Western analysis in which the mobility shift of bands into a single migrating species is indicative of the loss of phosphorylated forms of the kinase (Fig. 4A, *middle panel*). The changes in mobility of p70S6K correlated with the activity of the kinase, as assessed directly by kinase assay (Fig. 4A, *lower panel*). The molecule FK506 competes with rapamycin for binding to FKBP12, and excess FK506 blocks the ability of rapamycin to inhibit mTOR. In the presence of rapamycin, increasing concentrations of FK506 restored the induction of CHOP in response to amino acid deficiency (Fig. 4B). FK506 did not induce CHOP and was unable to reverse the inhibition by LY294002, confirming that the effect seen with FK506 was due to the competition with rapamycin for FKBP12. Rapamycin specifically inhibited mTOR and blocked CHOP expression, suggesting that mTOR is necessary for CHOP induction by amino acid deprivation. The inhibition of CHOP induction by PI3K and mTOR inhibitors was observed in the fibroblast cell lines described above as well as in HeLa cells and normal murine mammary gland cells (data not shown). These latter two cell lines are both epithelial in origin, indicating that the responses we observed were not cell type specific.

CHOP expression is also induced by diverse stimuli, including the oxidizing agent H_2O_2, the genotoxic compound $NaAsO_2$, and the calcium ionophore A23187 (9, 28). To determine whether the involvement of the PI3K/mTOR pathway was universally required for the induction of CHOP by these stressful agents, or whether it was specific to amino acid deprivation, we treated AKR-2B cells with H_2O_2, $NaAsO_2$, or A23187 in the presence or absence of LY294002 or rapamycin. Similar to the results attained under amino acid-deprived conditions, the induction of CHOP by H_2O_2 was inhibited by both LY294002 and rapamycin (Fig. 5), suggesting that the PI3K/mTOR pathway is also involved in H_2O_2 signaling. H_2O_2 has been shown to activate p70S6K, a downstream target of mTOR, in a PI3K-dependent manner (23). Therefore, both amino acid depletion and H_2O_2 treatment incorporate the PI3K/mTOR pathway to induce CHOP. However, LY294002 or rapamycin did not inhibit CHOP induction by either $NaAsO_2$ or A23187. Thus, these two agents do not require the PI3K/mTOR pathway and most likely use other signaling cascades to induce CHOP expression. Although all of these stressful conditions result in the accumulation of CHOP protein, they incorporate distinct pathways to achieve that end.

Under all of the stressful conditions tested, the presence of functional CHOP protein has been able to induce expression of its downstream target genes, DOCs (18). In $CHOP^{-/-}$ fibroblasts, tunicamycin is unable to induce the expression of DOCs, but introduction of wild-type CHOP into these cells restores DOC expression through the presence of a functional CHOP-C/EBP-binding site in the promoter. To determine that inhibiting CHOP expression would disrupt downstream signaling events, we examined the induction of two of these target genes. DOC1 encodes a stress-inducible form of CA-VI, and DOC6 has been identified as the gelsolin homologue advillin (18, 29). As we noted previously, accumulation of *CHOP* mRNA by amino acid starvation requires serum and is inhibited by LY294002 and rapamycin (Fig. 6).

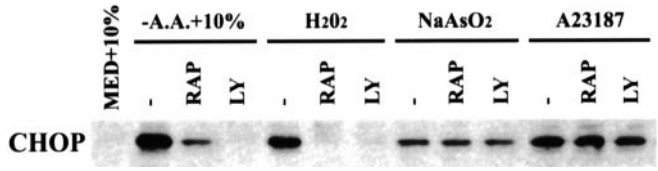

FIG. 5. Rapamycin and LY294002 do not universally inhibit the induction of CHOP. AKR-2B cells were treated with amino acid-deficient medium (−A.A.+10%), H_2O_2; 200 μM), $NaAsO_2$ (200 μM), or A23187 (1 μg/ml) in the presence or absence of rapamycin (RAP; 20 nM) or LY294002 (LY; 10 μM). Cells were harvested at 18 h, and CHOP protein levels were detected by immunoblotting.

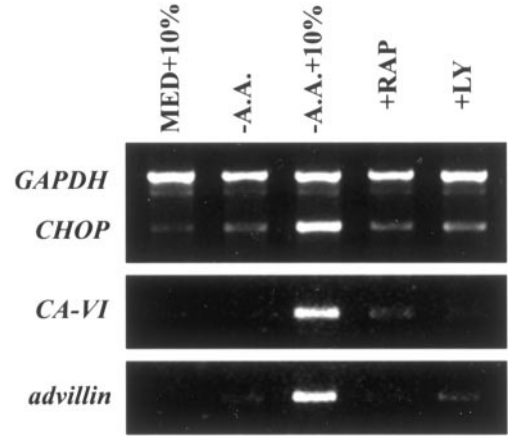

FIG. 6. Amino acid deprivation induces downstream targets of CHOP. NIH-3T3 cells were incubated in complete medium (MED+10%), amino acid-deficient medium (−A.A.), or amino acid-deficient medium plus serum (−A.A.+10%). Where indicated, rapamycin (RAP; 20 nM) or LY294002 (LY; 10 μM) was added to −A.A.+10%. RNA was extracted, and RT-PCR was performed as described in *Materials and Methods*.

The levels of the housekeeping gene GAPDH were not changed by the treatments and were used as an internal loading control. In accordance with published results (18), amino acid depletion induced *CA-VI* and *advillin* mRNA in NIH-3T3 cells in a CHOP-dependent manner. Because of their dependence on CHOP for expression, *CA-VI* and *advillin* were not induced in the presence of rapamycin or LY294002 where *CHOP* expression was inhibited. These data indicate that the CHOP protein induced by amino acid deprivation increased the expression of CHOP-dependent genes.

Previously, Wang and Ron (30) have shown that the p38 mitogen-activated protein kinase (p38MAPK) phosphorylates CHOP, and this event is required for CHOP to activate transcription and inhibit adipogenesis. Reconstitution of $CHOP^{-/-}$ fibroblasts with a mutant of CHOP lacking the p38 consensus phosphorylation sites partially restores *CA-VI* expression in response to tunicamycin, but *CA-VI* levels are reduced compared with those in $CHOP^{-/-}$ fibroblasts reconstituted with wild-type CHOP. Also, the p38MAPK inhibitor SB203580 is able to partially inhibit the induction of *CA-VI* by tunicamycin (18). These data suggest that stress-induced expression of *CA-VI* may require posttranslation modification of CHOP by p38MAPK. To determine that the induction of *CA-VI* by amino acid deprivation also requires this regulation, we incubated NIH-3T3 cells in amino acid-deficient medium with increasing concentrations of SB203580. As shown in Fig. 7, inclusion of the p38MAPK inhibitor was able to block *CA-VI* expression by amino acid deprivation, with partial inhibition occurring at 10–20 μM and full inhibition at 100 μM. However, SB203580 did not affect the expression levels of *CHOP* or the internal loading control *GAPDH*, indicating that the inhibitor is affecting the ability of CHOP to modulate transcription without influencing CHOP expression. These results are similar to those seen with tunicamycin and suggest a common mechanism by which p38MAPK activity is able to modulate CHOP transcriptional activity.

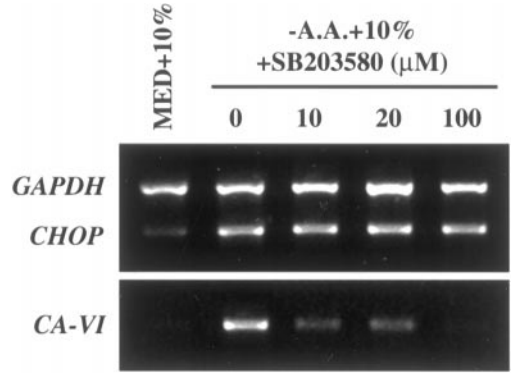

FIG. 7. Induction of CA-VI expression by amino acid deprivation is inhibited by SB203580. NIH-3T3 cells were incubated in amino acid-deficient medium (−A.A.+10%) with the indicated doses of SB203580. After 18 h, RNA was extracted, and RT-PCR was performed as detailed in *Materials and Methods*.

Discussion

We have shown that amino acid deprivation induces CHOP in the mouse fibroblast cell lines AKR-2B and NIH-3T3. Although depletion of amino acids was able to induce CHOP, expression also required signaling events originating from a serum factor, which we have identified as IGF-I. This is the first instance reported where gene regulation by amino acid deprivation requires the presence of a specific growth factor. Previously, Gazzola *et al.* (6) showed that under starvation conditions the increased activity of amino acid transport by system A requires serum in human fibroblasts. Together, these data indicate that cells are able to regulate gene expression by coordinating signals from amino acid availability and from serum growth factors.

In contrast to the results presented here, Marten *et al.* (3) showed that in the hepatoma cell line H4-II-E, induction of CHOP by amino acid deprivation occurred in the absence of serum. This apparent discrepancy could be due in part to the method of deprivation and to the cell types used. In the previous study hepatoma cells were first grown to confluence, then placed in serum-free medium for 24 h before being incubated in amino acid-free medium. These pretreatments may have placed additional stresses on the cells that could have contributed to the induction of CHOP by independent signaling cascades. In the current study subconfluent cultures of nontransformed fibroblasts were directly treated with amino acid-deficient medium such that this was the only stress placed on the cells. Alternatively, the hepatoma

cells used in the previous study may have accumulated mutations that allow these cells to bypass the requirement for the IGF-I cascade. For example, many tumor cells up-regulate the expression of eIF4E whose activity is positively regulated by mTOR signaling in normal cells (31).

As IGF-I is a potent stimulator of PI3K activity, we investigated the potential role of PI3K in CHOP induction. We have shown that PI3K is necessary for CHOP induction by amino acid depletion, because the PI3K inhibitors wortmannin and LY294002 completely blocked CHOP expression. Downstream effectors of PI3K are also involved in CHOP induction. The mTOR inhibitor rapamycin blocked the expression of CHOP by amino acid deprivation. Therefore, we have defined a signaling cascade leading from an external growth factor through the activation of its downstream mediators that can influence gene expression. This is the first report of the involvement of the IGF-I/PI3K/mTOR signaling cascade in the induction of CHOP, and activation of this cascade is a novel element of CHOP induction by either amino acid deprivation or H_2O_2 (see below).

Previously, it has been demonstrated that amino acid deprivation decreases *IGF-I* mRNA levels *in vitro* and *in vivo*. However, we have shown that the accumulation of CHOP by starvation requires IGF-I (8). This paradox may be explained by the fact that IGF-I levels are diminished only 30–50% by amino acid deprivation, and the remaining levels of IGF-I may surpass a threshold required for the induction of CHOP in specific tissues. Similarly, most evidence suggests that mTOR activity is inhibited by the lack of amino acids (32), but we have shown here that mTOR activity is required for CHOP expression by amino acid deprivation. This introduces a contradiction where mTOR activity is required in the absence of amino acids to induce a cellular event. However, in amino acid-deficient medium, p70S6K activity was inhibited by 70% compared with the activity seen in complete medium (Fig. 4A, *lower panel*). Therefore, in this situation, amino acid deprivation reduced, but did not fully abolish, the activity of mTOR. In accordance with our results, Shigemitsu *et al.* (33) and Patti *et al.* (34) showed that insulin is still able to activate p70S6K in the absence of amino acids. The residual p70S6K kinase activity seen under starvation conditions is apparently sufficient to induce CHOP.

Additionally, inhibition of mTOR by rapamycin completely blocks p70S6K activity, which is generally associated with controlling translation by phosphorylating the ribosomal S6 protein that is assembled into the translation machinery (35). Another target of the kinase mTOR also involved in translation is the eukaryotic initiation factor 4E binding protein (eIF4E-BP), also known as PHAS-1 (36). In nonstimulated cells, eIF4E-BP binds to and inhibits eIF4E from binding the methyl-guanine cap of mRNAs, thereby inhibiting translation initiation. Phosphorylation of eIF4E-BP by mTOR releases eIF4E, which then binds to the mRNA cap in complex with other initiation factors important for translation. The activity of mTOR does not directly influence the translation of CHOP, but, instead, is required for accumulation of CHOP mRNA. The protein synthesis inhibitor cycloheximide is also able to block CHOP mRNA levels (data not shown) (12). Together, these data suggest that synthesis of some protein, possibly a transcription factor, is required for induction of CHOP mRNA. This model is similar to that observed in yeast where amino acid starvation is able to preferentially initiate translation of the transcription factor GCN4, which is required to activate transcription of a number of genes involved in amino acid metabolism (37). To date, no mammalian homologue of GCN4 has been identified, but other molecules in the general control pathway, such as GCN2, GCN1, and GCN20, have mammalian counterparts (38–40), suggesting conservation of this pathway in higher eukaryotes. Under amino acid-deficient conditions, mTOR activity may be necessary to coordinate assembly of the translation initiation complex at the mRNA of a transcription factor that regulates CHOP expression. It has also been shown that mTOR is able to directly phosphorylate STAT3, and this phosphorylation may be necessary for the full activation of STAT3 (41). It is possible that the increase in CHOP expression by amino acid deprivation may require the PI3K/mTOR pathway to activate translation or phosphorylation of a transcription factor. These possibilities are currently being investigated.

Many studies have examined the pathways that are responsible for CHOP expression induced by various stressful stimuli, and it appears that different agents may induce CHOP by activating distinct signaling cascades. Although originally identified as a gene that was induced by both growth arrest and DNA damage, induction of CHOP by growth arrest under medium-deprived conditions occurs independently of induction by DNA-damaging signals (42). Deletion analysis of the CHOP promoter indicates that CHOP regulation by the DNA-damaging agent ultraviolet-C involves response elements different from those involved in the regulation by H_2O_2 and $NaAsO_2$ (9). Although these initial studies indicated that H_2O_2 and $NaAsO_2$ might regulate CHOP expression via common promoter elements, our results demonstrate that H_2O_2 and $NaAsO_2$ instead may use different signaling pathways to induce CHOP. However, these signaling pathways may lie upstream of and independently activate a common set of transcription factors that converge on specific promoter elements. Jousse *et al.* (43) showed that amino acid deprivation regulates CHOP expression by a different mechanism than agents such as tunicamycin, which induce CHOP by activating the unfolded protein response. The unfolded protein response involves the endoplasmic reticulum resident protein Ire1p that splices and activates the novel transcription factor Hac1 (44). These data indicate that multiple stimuli induce CHOP by activating diverse signaling events. However, the common induction of CHOP by these agents may signal cells to arrest under stressful conditions to repair cellular damage. If the insult is too great for the cells to recover expediently, CHOP induction may then signal the cells to undergo apoptosis.

Induction of CHOP by multiple stimuli also coordinates the expression of CHOP target genes such as *CA-VI* and *advillin*. Expression of these downstream genes suggests that CHOP is functional under amino acid-deprived conditions and may have a physiological role in eliciting responses to amino acid deficiency such as cell cycle arrest (2). Although the known target genes of CHOP do not have an apparent role in cell cycle arrest or amino acid metabolism, these genes

most likely represent only a subset of the genes regulated by CHOP.

In addition to the regulation of CHOP activity by increasing protein abundance, CHOP activity is regulated by phosphorylation. The posttranslational activation of CHOP by p38MAPK may be a common element of CHOP induction and activation by stressful agents. Induction of *CA-VI* by tunicamycin and amino acid deprivation is partially inhibited by the p38MAPK inhibitor SB203580 (18) (data not shown). Many adverse stimuli, such as $NaAsO_2$, induce CHOP as well as activate p38MAPK (45). p38MAPK is associated with both cell cycle arrest and apoptosis (46, 47), and CHOP has been implicated in both of these processes (10, 13–15). These data suggest that under stressful conditions, CHOP induction and p38MAPK activity may be coordinated to regulate the expression of specific target genes.

Recent research has allowed us to begin to understand the pathways by which amino acid deprivation signals change during gene expression in mammalian cells. It has been suggested that starvation of amino acids leads to the accumulation of uncharged transfer RNA (tRNA), and this uncharged tRNA is responsible for altering gene expression by altering the rate of transcription or translation (48, 49). A probable correlation between accumulation of uncharged tRNA and CHOP induction has been shown using CHO cells carrying a temperature-sensitive mutation in leucyl-tRNA synthetase. Under permissive temperature, these cells express low levels of *CHOP* mRNA, but *CHOP* levels are greatly induced when these cells are grown at the restrictive temperature (43). Although we have not determined the mechanisms by which the depletion of individual amino acids from the medium stimulates gene expression, we have identified a growth factor-stimulated pathway that coordinates the induction of an amino acid-regulated gene. Although we have shown that the induction of CHOP by amino acid deficiency requires the IGF-I/PI3K/mTOR pathway, amino acid regulation of other inducible genes may not involve this signaling cascade. In fact, the increased activity of system A amino acid transport in response to starvation requires the activation of ERK1/2. This response is not inhibited by the addition of wortmannin or rapamycin, indicating that the PI3K/mTOR pathway may not be a universal component of regulation by amino acid deprivation (50). By carefully dissecting the biochemical pathways that lead to CHOP induction, we can elucidate how the depletion of specific nutrients, such as amino acids, regulates gene expression and other biological responses in multicellular organisms.

Acknowledgments

The authors thank D. Ron for providing CHOP cDNA, Dr. S. Pearsall and Dr. M. McDonnell for critical reading of the manuscript, and M. Aakre for technical assistance.

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