CHOP Transcription Factor Phosphorylation by Casein Kinase 2 Inhibits Transcriptional Activation

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The CAAT/enhancer binding protein homologous transcription factor CHOP, also known as GADD153, is involved in DNA damage, growth arrest, and the induction of apoptosis after endoplasmic reticulum stress and nutrient deprivation. CHOP dimerizes with and inhibits the binding of C/EBP-related transcription factors to their consensus DNA target sequences and also forms novel complexes with other transcriptional proteins (e.g., c-Jun, c-Fos). The transcriptional activation of these complexes is modified by their phosphorylation. Phosphorylation of CHOP at serine 79 and serine 81 by p38-MAP kinase enhances its transcriptional activity. Here we show that an interactive association between CHOP and casein kinase II (CK2) results in the phosphorylation of its amino-terminal transactivation domain. Mapping of the functional domains of CHOP indicates that the region in CHOP required for association with CK2 differs from that required for its phosphorylation. Th binding of CK2 to CHOP requires only the carboxyl-terminal bZip domain of CHOP, whereas phosphorylation involves residues located in the amino-terminal domain. The presence of the bZip domain, however, facilitates the phosphorylation of CHOP. Analyses of the effect of point mutations of CHOP on its transcriptional activity and the effect of specific inhibitors of CK2 lead us to conclude that CK2-mediated phosphorylation of CHOP inhibits its transcriptional activity. Our findings suggest that inhibition of the proapoptotic functions of CHOP by CK2 may be a mechanism by which CK2 prevents apoptosis and promotes cellular proliferation.

The transcription factor CHOP (GADD153), is ubiquitously expressed at low levels in proliferating cells. When cells are exposed to stressful stimuli, able to induce DNA damage and growth arrest, the expression of CHOP is remarkably increased. CHOP expression is particularly responsive to impaired protein folding in the endoplasmic reticulum (1, 2), elevated cellular levels of nitric oxide, reactive oxygen species, hypoxia, and nutrient deprivation (glucose and amino acids) (3). Cell cycle arrest and the induction of cell death by apoptosis are two responses to cellular stress in which CHOP actively participates (4–6). CHOP is a member of the C/EBP1 family of transcription factors that have a characteristic basic region involved in DNA binding and a leucine-zipper domain involved in dimerization with other transcription factors (7). Several mechanisms have been implicated in the regulation of CHOP functions as follows: 1) a dominant negative regulatory effect on gene transcription mediated by C/EBP transcription factors by inhibition of their binding to C/EBP binding sites in the promoters of genes (7); 2) transactivation of distinct downstream genes (downstream of CHOP genes) by dimerization to C/EBP factors and binding to a new set of promoter regulatory sites (1, 8, 9); and 3) regulation of the activity of other bZip transcription factors that do not belong to the C/EBP family. This is the case for the AP1 transcription factors, JunD, c-Fos, and c-Jun (10). The transcriptional activity of CHOP is also regulated by the phosphorylation of its transcriptional activation domain at the serines 79 and 81 by the stress-activated protein kinase p38-MAP kinase (11). Earlier observations indicate that the proapoptotic actions of CHOP are only partially dependent on the phosphorylation by p35-MAP kinase (6). Based in these our earlier studies and a number of additional published and unpublished contradictory observations, we considered the possibility that additional regulatory protein kinases may have an impact on the transcription functions of CHOP. Here we report evidence that CHOP activity is regulated by phosphorylations catalyzed by the ubiquitously expressed casein kinase 2 (CK2).

CK2 is a serine/threonine protein kinase required for the progression of the cell division cycle and cell viability (12). The importance of CK2 in cellular functions is evident from the observations that it phosphorylates as much as 300 known proteins (13) and is highly conserved among organisms from different kingdoms such as yeast, plants, and animals. CK2 is a heterotetramer composed of two identical regulatory β-subunits and two catalytic α-subunits. In mammalian cells, two different CK2 α-subunits encoded by two different genes substitute for one another, whereas there is only a single β-subunit. As a consequence, combinations of complexes can be formed: αβββ, αβββ, and ααββ, all of which are active kinase complexes although they may have different target specificity and mode of regulation (12). Although the expression of CK2 is considered to be constitutive, levels of CK2 expression and/or activity increase after both growth factor stimulation and in conditions that result in cellular transformation (14). In yeast and mammalian cells, CK2 activity is required at multiple transition points of the cell cycle (G1/S, G2/M) (12). Excessive activity of CK2 is associated with experimental oncogenic transformation and the development of primary and
CK2 Inhibits CHOP/GADD153 Transactivation

MATERIALS AND METHODS

Chemicals and Other Biological Reagents—DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA) and Roche Applied Science. Tissue culture media, serum, and other reagents were from Invitrogen, and the compounds DBR (5,8-dichlorobenzimidazole riboside) and purified CK2 were from Calbiochem.

Cell Lines and Tissue Culture—NIH-3T3 fibroblasts and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 100 microunits/ml penicillin G and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator in 5% CO2. Cell cultures were passaged by trypsinization and subculture every 5 days.

Plasmid DNA Constructs—Deletion mutants of CHOP were created by PCR and inserted into the plasmid pGST-KG in-frame with the GST-coding sequence. Transformed Escherichia coli were induced with isopropyl-1-thio-β-D-galactopyranoside to produce the corresponding proteins. Point mutations in the CHOP-coding sequence were generated by site-directed mutagenesis through the use of the kit QuikChange (Stratagene). A Gal4-CHOP expression plasmid was constructed by inserting in-frame the CHOP-coding sequence into the pM plasmid (Stratagene). A Gal4-CHOP expression plasmid was constructed by inserting in-frame the CHOP-coding sequence into the pM plasmid (Stratagene). A Gal4-CHOP expression plasmid was constructed by inserting in-frame the CHOP-coding sequence into the pM plasmid (Stratagene). A Gal4-CHOP expression plasmid was constructed by inserting in-frame the CHOP-coding sequence into the pM plasmid (Stratagene).

GST Pull-down and Kinase Assay—Cells or tissues were homogenized in lysis buffer A containing 20 mM Tris-HCl, pH 7.2, 2 mM MgCl2, 0.5% Nonidet P-40, 150 mM NaCl, and 1 mM dithiothreitol as previously described. The following protease and phosphatase inhibitors were also added: phenylmethylsulfonyl fluoride (1 mM), leupeptin (2 μg/ml), aprotinin (1 μg/ml), NaF (5 mM), and NaVO4 (1 mM). Protein extracts (200 μg) were incubated with 5 μg of the GST fusion proteins such as CHOP, wild type and the indicated mutants for 1 h at 4°C. The incubation buffer contained 20 mM sodium phosphate, pH 7.3, 150 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin (2 μg/ml), and aprotinin (1 μg/ml). The samples were washed three times with the same buffer and used for kinase activity determination after two additional washes in kinase buffer. The in vitro kinase reaction was performed at 30°C for 30 min in kinase buffer (50 mM HEPES, pH 7.0, 10 mM MgCl2, 5 mM MnCl2, 1 mM dithiothreitol containing 1 μCi of [γ-32P]ATP (specific activity 1,000 Ci/mmol) and 1 μM of substrate peptide). After 30 min of incubation, 4 μl of a phosphatase buffer was added to stop the reaction, and samples were loaded in a 10% SDS-PAGE after boiling for 5 min. Gels were dried and exposed for autoradiography.

Casein kinase 2-specific activity was determined by the use of a specific peptide substrate and reagents provided in the CK2 assay activity kit (Upstate Biotechnology, Lake Placid, NY).

Transfection Experiments and Luciferase Assays—Transfection experiments were performed in HeLa cells by using the GenePorter system (Gene Therapy System, San Diego, CA) using 2 μg of the luciferase reporter and 0.5 μg of the expression vector. Cells were harvested 48 h after transfection, and luciferase activity was determined using a commercially available luciferase assay kit from Promega (Madison, WI). All of the transfections were performed in duplicate at least in three different occasions. Statistical significance was determined using the Student’s t test.

RESULTS

CHOP Is Phosphorylated by a Ubiquitously Distributed Protein Kinase in Nuclear and Cytoplasmic Extracts—We reported earlier that the proapoptotic actions of CHOP are regulated by protein phosphorylation (6). However, phosphorylation by p38 MAP kinase is only partially responsive for the apoptotic effect of CHOP, suggesting that other mechanisms including additional protein kinases may also participate in the regulation of the function of CHOP. To identify additional protein kinases involved in the regulation of CHOP, we investigated the presence of protein kinases with the capacity to phosphorylate CHOP in both nuclear and cytoplasmic preparations (Fig. 1A) (see “Materials and Methods”). In this protocol, protein kinases that associate with and phosphorylate CHOP were isolated by GST-CHOP fusion protein pull-down experiments followed by assay of the kinase activity on CHOP in the presence of radioactive [γ-32P]ATP (Fig. 1, B and C). A GST control protein without CHOP sequences and a deletion mutant of CHOP lacking its amino-terminal transactivation domain (GST-CHOP(36–162)) were included (Fig. 1, B and C). Recombinant proteins were incubated in the presence of nuclear and cytoplasmic extracts prepared from the cell lines HeLa and NIH-3T3. Phosphorylation of CHOP was found in the presence of both nuclear and cytoplasmic extracts (Fig. 1D). No phosphorylation of either the GST protein or the CHOP amino-terminal deletion mutant (lacking residues 1–36) was observed (Fig. 1A).

CK2 Associates with and Phosphorylates CHOP—To identify the protein kinase that associates with and phosphorylates CHOP, we initially examined the amino acid sequence of the CHOP amino-terminal domain (1–36) for consensus phosphorylation sites. Two putative sequence motifs that fit the consensus, (S/T)XX(D/E), were identified as characteristic of those phosphorylated by CK2 (Fig. 2A). We then determined that purified CK2 binds and phosphorylates CHOP but not the GST control or the NIH3T3-terminal truncated mutant (GST-CHOP(36–168)) proteins (Fig. 2B). To identify the specific sites in the NIH3T3-terminal amino acid sequence of CHOP that are phosphorylated by CK2, we generated a CHOP mutant (MUT2A) in which the serine residues (amino acids 14–15 and 30–31) in the sequence 1–35 were substituted by alanines (Fig. 2, C and D). The serine to alanine substituted site CHOP mutant was not phosphorylated by CK2, indicating that serines 14–15 and 30–31 of CHOP are the CK2 phosphoacceptor sites (Fig. 2E).

Distinct Domains of CHOP Bind and Are Phosphorylated by CK2—The binding sites and phosphorylation sites of protein kinases on their substrate proteins are often separated by some distance in the amino acid sequence. We examined the possibility that the association of CK2 with CHOP may occur on a domain that contains neither the amino-terminal domain nor the DNA binding region (GST-CHOPΔDBR) result in a decrease of precipitated activity to background levels. However, the CHOP mutant containing only the basic region and leucine-zipper domains (GST-CHOP bezip) showed a high capacity to precipitate CK2, even higher.
than that of the wild type GST-CHOP. These experiments indicate that the association of CHOP with CK2 is mediated by the bZip domain of CHOP, whereas the phosphorylation of CHOP occurs at a more distant site within the amino-terminal domain. We further delineated the CK2 phosphorylation activity on CHOP by directly incubating CK2 with equal amounts of...
the mutant substrates and performing the kinase assay without previous pull-down precipitation. The results of this experiment show a decreased phosphorylation acting on the mutants CHOP-LZ and CHOP-ΔBR (Fig. 3D), indicating that both the leucine-zipper domain and the basic region of CHOP are not only required for association with CK2 but these regions also...
enhance the kinase activity of CK2 on the phosphorylation of the amino-terminal domain of CHOP.

CK2-mediated Phosphorylation of CHOP Results in Downregulation of Transcriptional Activity—To determine the role of CK2-mediated phosphorylation on the activity of CHOP, we conducted an experiment that minimizes the complexity of CHOP and CK2 interactions. A luciferase reporter-promoter construct under the control of three Gal4 binding elements was used (Fig. 4A). Expression constructs in which the CHOP or the mutant CHOP-MUT2A cannot be phosphorylated by CK2 (Fig. 4B) were cotransfected with the reporter gene in HeLa cells. We found that serine to alanine substitutions in the CK2 phosphorylation sites of CHOP results in an augmented transcriptional activation. This finding suggests the existence of an inhibitory effect of CK2 phosphorylation on CHOP transactivation (Fig. 4C). To corroborate these findings, we performed additional experiments in which the activity of CK2 is modified. Overexpression experiments were uninformative because
constitutive CK2 expression and activity are high in all of the cells tested so that the transfection of a CK2 expression vector fails to induce a significant change of activity. Therefore, we carried out experiments in which the activity of CK2 could be decreased by using the compound DRB, a specific inhibitor of CK2. In these experiments, we found that the reporter activity of both the control and the CHOP mutants that are insensitive to CK2 were decreased by DRB and that the activity of CHOP was increased (Fig. 4D). The negative effect of DRB probably represents the inhibition of the already known positive effect of CK2 on the basal transcription machinery. The enhanced activity of CHOP in response to DRB is better appreciated when the effect of DRB is normalized to the effect of DRB on the control empty expression vector (Fig. 4E). We suggest that an inhibitory effect of CK2 on the transcriptional activation function of CHOP may contribute to the antiapoptotic and proproliferative roles of CK2 in mammalian cells (Fig. 5).

**DISCUSSION**

Here we describe the association of CHOP with the ubiquitously expressed protein kinase CK2. As a result of this association, CHOP is phosphorylated by CK2 in its transcriptional activation domain, resulting in an impairment of its transcriptional transactivational activity. This effect of CK2 on CHOP is consistent with the opposing roles that CHOP and CK2 exert on the control of cell proliferation and the induction of apoptosis. CHOP exerts proapoptotic role under conditions of cellular stress induced by oxidative stress, nutrient deprivation, and unfolded protein response and also endoplasmic reticulum stress (6, 19). However, CK2 is known to have an antiapoptotic role that is achieved by regulating the function of several other proteins involved in the induction of apoptosis (16–18, 20, 21).

For example, in type II cells, the induction of apoptosis by the Fas ligand requires a positive amplification loop exerted by the BH3 only protein Bid (17). Cleavage and translocation of Bid to the mitochondria are required for this amplification, and CK2 phosphorylation of Bid at a site close to the site cleaved by caspase-8 blocks its cleavage (17), thereby interrupting the apoptotic signaling that results in prevention of cell death. Inhibition of the proteolytic activity of caspase-8 by CK2 phosphorylation was also reported for the protein substrates Max (18), connexin-45.6 (20), and the hematopoietic-specific factor constitutive CK2 expression and activity are high in all of the cells tested so that the transfection of a CK2 expression vector fails to induce a significant change of activity. Therefore, we carried out experiments in which the activity of CK2 could be decreased by using the compound DRB, a specific inhibitor of CK2. In these experiments, we found that the reporter activity of both the control and the CHOP mutants that are insensitive to CK2 were decreased by DRB and that the activity of CHOP was increased (Fig. 4D). The negative effect of DRB probably represents the inhibition of the already known positive effect of CK2 on the basal transcription machinery. The enhanced activity of CHOP in response to DRB is better appreciated when the effect of DRB is normalized to the effect of DRB on the control empty expression vector (Fig. 4E). We suggest that an inhibitory effect of CK2 on the transcriptional activation function of CHOP may contribute to the antiapoptotic and proproliferative roles of CK2 in mammalian cells (Fig. 5).

**FIG. 4.** Phosphorylation of CHOP by CK2 results in inhibition of its transcriptional activation. Map of the luciferase reporter (A) and expression vectors (B) used in this experiment is shown. C, luciferase activity 48 h after transfection into HeLa cells. D, effect of the CK2 inhibitor DRB (50 μM) on the transactivation potential of CHOP and the mutant CHOP-MUT2A. E, normalization of the effect of DRB.
HS1 (21). The inhibition of apoptosis appears to be an important function of CK2. In fact, CK2 also inhibits apoptosis by a completely different mechanism that involves inhibition of caspases by the activation of the antiapoptotic protein ARC. In this instance, CK2 phosphorylation is required for ARC to exert its inhibitory effect on caspase-8 activity (16).

Our findings identify CHOP as a target for CK2 and define a novel mechanism by which CK2 is able to inhibit apoptotic cell death. The association of CK2 with CHOP may also have implications for the regulatory activities of CK2 in general. It was reported previously that the α-subunit of CK2 binds to a promoter region of the gene coding for the β-subunit of CK2 and activates its transcription (22). It is possible that a complex of CK2 and CHOP may bind specific DNA regulatory elements and regulate gene expression. We are currently investigating this possibility. Because CHOP and CK2 exert antagonistic effects on cell cycle progression, we anticipate that the interactions of CK2 and CHOP that we observe may play a role in the molecular mechanism responsible for such functions. However, little information regarding the mechanisms by which CHOP and CK2 influence cell proliferation is available. It is possible that the negative effect of CK2 on CHOP transactivation may have evolved to silence antiapoptotic and antiproliferative actions of any residual expression of CHOP in proliferating cells. An alternative or an additional possibility is that CK2 phosphorylation of CHOP may down-regulate CHOP actions in cells that have experienced stress stimulation and therefore express high levels of CHOP. The mechanism may be designed to bypass the stress-induced cell cycle checkpoint. At least one previous report supports this idea. Yeast cells arrested in G₂/M because of the induction of DNA damage will eventually continue through the cell cycle, even if they are unable to repair the DNA damage (23). This process of overriding a cell cycle checkpoint, also called adaptation, requires the actions of CDC5 (polo-like kinase) and the yeast CKB2 gene, which encodes a subunit of the yeast CK2 (23). This process of adaptation is not unique to DNA damage but also occurs for the spindle checkpoint (24) and the cell morphology checkpoint (25). We suggest that the phosphorylation of CHOP by CK2 may also be an important factor contributing to checkpoint adaptation or overriding mechanisms in mammalian cells.

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