TRE-dependent transcription activation by JDP2–CHOP10 association

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ABSTRACT

The c-Jun dimerization protein 2, JDP2, is a member of the activating protein 1 (AP-1) family of transcription factors. Overexpression of JDP2 has been shown to result in repression of AP-1-dependent transcription and inhibition of cellular transformation. Other studies suggested that JDP2 may function as an oncogene. Here we describe the identification of CHOP10, a member of the CCAAT enhancer binding proteins, as a protein associating with JDP2. In contrast to the inhibition of transcription by JDP2, JDP2–CHOP complex strongly enhances transcription from promoters containing TPA response elements (TRE), but not from those containing cyclic AMP response elements (CRE). The association between JDP2 and CHOP10 involves the leucine zipper motifs of both proteins, whereas, the basic domain of CHOP10 contributes to the association of the JDP2–CHOP10 complex with the DNA. DNA binding of JDP2–CHOP complex is observed both in vitro and in vivo. Finally, overexpression of JDP2 results in increased cell viability following ER stress and counteracts CHOP10 pro-apoptotic activity. JDP2 expression may determine the threshold for cell sensitivity to ER stress. This is the first report describing TRE-dependent activation of transcription by JDP2 and thus may provide an explanation for the as yet unexplored oncogenic properties of JDP2.

INTRODUCTION

The basic leucine zipper (bZIP) transcription factors form a large protein family that consists of several subgroups. They share a basic region that is responsible for specific DNA binding, and a leucine zipper domain that forms a coiled-coil structure and regulates protein oligomerization, stability and specificity (1). The activating protein 1 (AP-1) group forms one branch of the bZIP super family, and is composed of the Jun, Fos, activating transcription factor (ATF) and MAF sub-family members. The members of the AP-1 family are able to form multiple unique combinations of homo-/heterodimers that mediate the regulation of different genes. AP-1 is an immediate early transcription factor that responds to a plethora of physiological and pathological stimuli, and it has a crucial role in a variety of cellular events. The Jun and ATF proteins form stable homo- and heterodimers, while Fos proteins cannot homodimerize but can form stable heterodimers with Jun family members. The abundance of AP-1 subunits and the composition vary greatly between different cell types (2). The CCAAT enhancer binding proteins (C/EBPs), of which there are seven, are members of the bZIP family. They form dimers both with other C/EBPs and with members of other bZIP families, such as the AP-1 (1,3,4).

c-Jun dimerization protein 2 (JDP2) is a bona fide member of the AP-1 family that was isolated because of its ability to interact with c-Jun (5) and ATF2 (6). JDP2 encodes an 18-kDa protein that is able to homodimerize, as well as to form heterodimers with other AP-1 members, such as c-Jun, JunB, JunD and ATF2, and a member of the C/EBP family, C/EBPγ (7). JDP2 is a repressor of AP-1 protein family (5). The mechanism by which JDP2 represses AP-1 transcription involves competition for DNA binding, inactive heterodimer formation (5), indirect recruitment of histone deacetylase 3 (8), nucleosome assembly activity (9), inhibition of histone acetylation (9) and potential competition with JNK phosphorylation (10,11). JDP2 has been shown to play a role in the cellular differentiation of skeletal muscle (12), osteoclasts (13), adipocytes (14) and F9 cells (8).

JDP2 is most closely related to ATF3, a member of the ATF/CREB (CRE-binding protein) subfamily of the AP-1 group (15). ATF3 is a stress inducible gene: as its mRNA level greatly increases upon exposure of cells to stress signals (15). ATF3 has been reported to activate transcription as a heterodimer with c-Jun, whereas it represses...
transcription as a homodimer (16). ATF3 dimerizes with CHOP10/GADD153 (referred as CHOP10 for simplicity), a member of the C/EBP family, to form a nonfunctional complex (17). JDP2 and ATF3 exhibit 61% overall homology. While the bZIP domains of these two proteins show 90% homology, outside the bZIP domain JDP2 and ATF3 display very low similarity. Both JDP2 and ATF3 bind to TRE as well as to CRE DNA elements in vitro. It has recently been shown in our laboratory (18) that transgenic mice with temporal cardiac expression of JDP2 exhibit a similar phenotype to ATF3 transgenic mice (19). This suggests that these two proteins have a similar role in the heart (18,20). Despite the high degree of similarity in this aspect, there appear to be considerable differences between JDP2 and ATF3 with regard to their regulation. JDP2 is ubiquitously expressed and its level remains unchanged following various stimuli (10). Nevertheless, in a number of processes, such as differentiation of skeletal muscle cells (12), osteoclasts (13) and ultraviolet irradiation (21), a modest increase in JDP2 expression is observed. JDP2 undergoes phosphorylation by c-Jun N-terminal kinase (JNK) and p38 kinase on threonine 148, but the precise role of this phosphorylation in terms of its biological function is currently unknown (10,11). Different studies suggest that JDP2 has a dual role in malignant transformation. On the one hand, it is well established that JDP2 efficiently counteracts AP-1 transcription (5), and thus may interfere with the oncogenic properties of c-Jun. JDP2 has been found to inhibit cell transformation induced by Ras in vitro and in xenografts injected into SCID mice (22). On the other hand, JDP2 has been identified as a candidate oncogene in a high-throughput screen based on viral insertional mutagenesis in wild-type mice (23). It has also been associated to potentiate multicentric lymphogenesis in collaboration with either a loss of function mutation of p27 (24) or with the overexpression of c-Myc and Runx2 oncogenes (25).

In the present study, we identified CHOP10 as a novel protein partner for JDP2. We have shown for the first time that the repression of TRE-containing promoters by JDP2 may be switched to strong transcription activation by the association of JDP2 with CHOP10. The leucine zipper domain of CHOP10 is necessary for its association with JDP2, and the basic region of CHOP10 plays a role in mediating the binding of the complex to the TRE DNA sequences. JDP2 counteracts the pro-apoptotic activity of CHOP10 and thus provides increased cell resistance to endoplasmic reticulum (ER) stress. The recruitment of CHOP10 to TRE containing promoters resulting in the potentiation of transcription, may provide a molecular basis for the role of JDP2 as a tumor promoter.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Thapsigargin was purchased from Sigma–Aldrich Ltd. (cat# T9033) and was dissolved in DMSO to generate a 1 mM stock solution. Antibodies against JDP2 were used as described (5). Polyclonal antibodies directed against: CHOP10, c-Jun and THTR1 were purchased from Santa Cruz. Monoclonal antibodies against: hemagglutinin (HA) (12CA5) from Babco Inc, CHOP10 (9C8) from Abcam Inc., α-tubulin from Sigma–Aldrich Ltd.

**Plasmids**

The expression plasmids encoding wild-type CHOP10 and the CHOPΔLZ mutant were kindly donated by Prof. David Ron (Skirball Institute, NY, USA) (26). CHOPΔBasic was generated using a site-directed mutagenesis kit (Stratagene Inc.), using appropriate oligonucleotides to generate a deletion of the entire basic domain sequences encoding amino acids 101–122. The deletion was confirmed by DNA sequencing.

The expression plasmid pcDNA 3.1 (Invitrogen Inc.) was used to express His-JDP2. JDP2 fused to HA (HA-JDP2) was expressed using the pCEFL expression plasmid, which was kindly donated by Dr SJ Gutkind (NIH, Bethesda, MD, USA). CyCD1-Luciferase reporter plasmid (27) was kindly donated by Dr Richard G. Pestell (Thomas Jefferson University, Philadelphia).

Tethered dimers were cloned into a His tag-containing pcDNAc expression plasmid (Invitrogen Inc.) in the following manner. A BamHI–EcoRI double-stranded oligonucleotide linker encoding a polypeptide linker (5’-GGA TCC GGG GGA TCA GGC GGA GGT GGA GGT TCC GGT GGC GGT GAA TTC-3’) was introduced into a BamHI–EcoRI linearized pcDNAc-His vector. PCR fragments encoding for JDP2 and CHOP were generated using appropriate specific oligonucleotides. A 5′ BamHI site was generated for JDP2, together with a BglII site at the 3′ end that included the complete coding region but avoided the JDP2 stop codon. In addition, a CHOP10 DNA fragment was designed to harbor an in-frame EcoRI site at the 5′-end, and an XhoI site was designed at the 3′ end of the complete coding sequence.

**Ras recruitment system and yeast manipulations**

Conventional yeast transfection and manipulations were performed as previously described (28). The interaction of CHOP10 with JDP2-LZ and ATF3 was examined by mating. Cdc25-2a, and z haploid yeast strains were transfected with the pMyr (uracil selectable marker) and Bait (leucine selectable marker) expression plasmids. Transformants were selected and grown on glucose-containing plates that lacked either uracil or leucine. Following mating, colonies were replica plated on to YPD plates overnight at 24°C. Following mating, colonies were replica plated on to plates that lacked uracil and leucine to test for diploid transformants and incubated at 24°C. Subsequently, colonies were replica plated on to galactose plates that lacked methionine to induce the expression of the prey and bait proteins. Plates were incubated at 36°C to test for Ras-Bait membrane translocation and complementation of the Cdc25-2 temperature-sensitive phenotype.

**Cell culture and transfections**

Mouse fibroblast cells (NIH3T3) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.5 g/ml d-glucose containing 10% fetal.
calf serum and 1% penicillin and streptomycin, grown in a 5% CO₂ incubator. Stable NIH3T3 cell lines were generated by retroviral infection using a pBabe vehicle, pBabe-JDP2 and pBabe-CHOP10 expression plasmids as previously described (22). Following infection, colonies were selected using 2 μg/ml puromycin.

For the luciferase reporter assays, cells were grown in 60-mm dishes (2.5 × 10⁵ cells/dish) and were transfected the following day using the X-TremeGENE Q2 transfection reagent (Roche Inc.). For the co-immunoprecipitation assay, NIH3T3 cells were transfected with the jetPEI transfection reagent (Polyplus transfection Inc.), following the manufacturer’s protocol; 7 μg of DNA and 14 μl of reagent were used for each 100-mm plate.

SV40 large T antigen immortalized mouse embryo fibroblasts (MEF) corresponding to parental and mCys protocol; 7° (Polyplus transfection Inc.), following the manufacturer’s protocol; 7 μg of DNA and 14 μl of reagent were used for each 100-mm plate.

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SV40 large T antigen immortalized mouse embryo fibroblasts (MEF) corresponding to parental and CHOP10^-/- cell lines were kindly provided by Prof. David Ron (Skirball Institute, NY, USA) (29). These cells were used for retroviral infection as described above to produce stable cell lines expressing JDP2, CHOP10 and JDP2-CHOP chimera.

**Reporter assays**

NIH3T3 cells were co-transfected with expression plasmids and reporter plasmids, using the X-TremeGENE Q2 transfection reagent (Roche) and following the manufacturer’s protocol; 3 μg of DNA and 7.5 μl of reagent were used for each 60-mm plate. Twenty-four hours after transfection, cells were harvested and tested for luciferase activity using the Luciferase Assay System (Promega Corp.) as described in the manufacturer’s protocol.

**Western blot**

Western blot analysis was performed in 5% nonfat dry milk (Carnation); washes were performed with PBS. Polyclonal antibodies against JDP2 were used at a dilution of 1:500; polyclonal antibodies against CHOP10; and c-Jun were used at a 1: 200 dilution. Monoclonal anti-α-tubulin was used at a 1:5000 dilution. Secondary HRP-conjugated antibodies directed against rabbit and mouse antigens were purchased from Sigma–Aldrich Inc.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were performed using ChIP assay kit (Upstate Millipore) according to the manufacturer’s instructions. In principle, NIH stable cell lines were transfected with the indicated combination of reporter plasmid and expression vector. Twenty-four hours following transfection cells were cross-linked with formaldehyde lysed and sonicated on ice for eight cycles of 5 s each with 30 s intervals. This resulted in DNA fragmentation to an average length of 500–1000 bp. Immunoprecipitation was performed with either nonrelevant serum (anti-THTR1 SC#27656) or anti-CHOP10 polyclonal antibodies (SC#575) both were purchased from Santa Cruz. Precipitated protein–DNA complexes were extensively washed according to the manufacturer’s instructions. Cross-link was reversed by addition NaCl to final concentration 200 mM for 4 h at 65°C. DNA was phenol–chlorophorm extracted and ethanol precipitated. PCR was performed with the following oligonucleotides corresponding to a 200-bp DNA fragment adjacent to the Jun TRE element located at the −79 position of the human e-Jun reporter plasmid. Top-primer 5'-TCATGTCTGGATCCAGCTTC bottom-primer 5’- GCAGTTCGGACTATACTGCC. PCR reaction was performed with 23 cycles and 63°C annealing temperature.

Oligonucleotides corresponding to the luciferase coding region located 1000-bp downstream to the luciferase translation start site top-primer 5'-CAGGATACGGCTGTGATATGG and bottom-primer 5'- GAGACTATACTGCC designed to generate a 320-bp PCR fragment. PCR reaction was performed with 30 cycles and 62°C annealing temperature.

**Cell viability assays**

Cell viability assays were performed using a CellTiter-GloTM luminescent cell viability assay kit (Promega Corp.). The kit is a homogeneous method of determining the number of viable cells in culture based on luminescent assay directed toward ATP content determination. The assay was performed according to the manufacturer’s instructions, with small modifications. Briefly, 24 h following the addition of thapsigargin the medium was removed. The lysis-luminescent reagent diluted 1:1 with PBS was applied directly onto the cells. Luminescence was measured using an IVIS-200 and analyzed using Igor pro version 2.5 (Xenogen Inc.).

**Electromobility shift assay (EMSA)**

*In vitro* translated rabbit reticulocyte lysate (3 μl) was used to bind Jun-TRE 32P-labeled double-stranded oligonucleotides in binding buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 70 mM NaCl, unless otherwise indicated) containing 1 μg poly(dI-dC). The sequence of the double-stranded Jun-TRE site (the core TRE site is underlined) was: TTCGGGTGGACA TCATGGGCTAT

A 6% non-denaturing gel was used to separate bound and free probe in 0.5× TBE. The gel was dried and exposed to autoradiography for 16 h.

**Co-immunoprecipitation**

Protein-A Sepharose beads (Sigma–Aldrich Inc.) were preincubated with antibodies against either HA or JDP2. Following two washes with PBS and one wash with the nuclear extraction buffer, 200 or 900 μg of the nuclear extract were added to the beads, which were then incubated overnight at 4°C. Following three washes with the nuclear extraction buffer, the precipitated proteins were eluted using SDS–PAGE sample buffer. Samples were boiled and then separated by 12.5% SDS–PAGE followed by western blot analysis.

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RESULTS AND DISCUSSION

Identification of CHOP10 as a JDP2-associated protein using the Ras recruitment system

To identify proteins that interact with JDP2, we used the Ras recruitment system (RRS) (7). In principle, a bait protein was designed that comprised the JDP2 leucine zipper domain fused to the C-terminus of cytoplasmic Ras (Ras-JDP2-LZ). The bait was used to screen a pMyr-based HeLa cDNA expression library (Stratagene Inc.). Fifty-three yeast colonies were selected and the prey DNA expression plasmids were isolated and reintroduced into Cdc25-2 yeast strain together with either nonrelevant bait or the original Ras-JDP2-LZ bait. 47 clones displayed bait-specific growth at the restrictive temperature. Sequence analysis of the plasmids revealed the isolation of novel JDP2-interacting partners among them; members of the basic leucine zipper family such as ATF4 and CHOP10, transcription regulators such as HDAC6, as well as known interacting proteins such as C/EBPγ (7). The current study focuses on CHOP10 protein interaction with JDP2. CHOP10, which is also known as GADD153 will be referred to hereafter as CHOP10. To verify that CHOP10 interacted specifically with JDP2, yeast cells were used to co-express different baits with the CHOP10 prey. The prey protein used was membrane-anchored CHOP10 (M-CHOP); whereas an empty expression plasmid (pMyr) was used as a control. Yeast transformants that co-expressed the bait Ras-JDP2-LZ together with membrane-anchored CHOP10 displayed efficient growth at the restrictive temperature, 36°C (Figure 1). JDP2 and ATF3 display a high degree of identity within the bZIP domain, and an association between ATF3 and CHOP10 has already been shown (17). Indeed, we were able to recapitulate the association of the bait encoding the full-length ATF3 fused to the cytoplasmic Ras (Ras-ATF3) with the membrane-associated CHOP10 prey using the RRS (Figure 1).

JDP2–CHOP interaction in mammalian cells

To validate the interaction between CHOP10 and JDP2 in a different cell environment, we used a modified mammalian two-hybrid approach. NIH3T3 cells were transfected with plasmids encoding the full-length JDP2 protein fused to the yeast GAL4 DNA-binding domain (GAL4-DBD-JDP2) in the presence or absence of an expression plasmid that encoded CHOP10. A reporter plasmid was also included to drive the expression of a luciferase reporter gene under the control of five copies of the upstream-activated sequence (UAS) that corresponds to the GAL4 DNA-binding site. Because CHOP10 harbors a potent intrinsic activation domain at its N-terminus, it was not necessary to fuse CHOP10 to the GAL4 activation domain. We hypothesized that the interaction between CHOP10 and Gal4-DBD-JDP2 would be sufficient for the formation of a functional GAL4 transcription activator, and would lead to transcriptional activation of the 5 × UAS luciferase reporter gene.

Twenty-four hours following transfection, cells were harvested and luciferase activity was determined. The expression of GAL4-DBD-JDP2 resulted in very low basal transcription activity, which was even lower than that obtained with GAL4-DBD alone. This demonstrated the ability of JDP2 to repress transcription even when fused to a heterologous DNA-binding domain. Co-expression of Gal4-DBD-JDP2 with CHOP10 resulted in potentiation of the transcription activity of the reporter gene by 25-fold (Figure 2). This effect strongly confirms the specific association between full-length JDP2 and CHOP10 in mammalian cells (Figure 2). Because the original JDP2 bait used to isolate CHOP10 encoded the JDP2 leucine zipper domain, we were interested in examining which domain within CHOP10 is responsible for association with JDP2. We used the mammalian two-hybrid system to map the domains within CHOP10 that are responsible for its association with JDP2. Two expression plasmids encoding CHOP10 mutants, which lacked either the basic domain (CHOPΔBasic) or the leucine zipper domain (CHOPΔLZ), were used. Transfection of the CHOP10 mutant that lacked the basic domain resulted in luciferase activity similar to that of the wild-type CHOP10 protein, whereas the CHOP10 mutant that lacked the leucine zipper domain failed to stimulate transcription of the 5 × UAS luciferase reporter gene (Figure 2). Together, these data suggest that the association of JDP2 with CHOP10 is mediated by the leucine zipper domain of both proteins and that the basic domain of CHOP10 does not contribute to its association with JDP2.

JDP2 and CHOP10 co-immunoprecipitation

To demonstrate a direct association between JDP2 and CHOP10, we used a co-immunoprecipitation approach. NIH3T3 cells were transiently transfected with an
expression plasmid that encoded hemagglutinin-tagged (HA) JDP2 (HA-JDP2), or an empty HA-tagged expression vector plasmid. To induce endogenous expression of (HA) JDP2 (HA-JDP2), or an empty HA-tagged expression plasmid that encoded hemagglutinin-tagged (HA) JDP2 (HA-JDP2), or the empty vector (HA). For the induction of CHOP10 expression, transfected cells were exposed to 400 nM Tg for the indicated time. Nuclear extracts were used for immunoprecipitation with antibodies against the HA-tag. Western blots of 10% of the total input lysates (A) and precipitated proteins (B) were probed with the indicated antibodies. (C) NIH3T3 cells were either not treated or exposed to 400 nM Tg for 16 h. Nuclear extracts were precleared with a nonrelevant (aNR) polyclonal antibody (lanes 3 and 4), and subsequently an antibody against JDP2 was used for immunoprecipitation (lanes 5 and 6). Western blots of 5% of the total input lysates (lanes 1 and 2) and precipitated proteins using the indicated antibody are shown. Antibodies used were: anti-JDP2, mouse anti-CHOP10 and rabbit anti-c-Jun.

The level of endogenous expression of CHOP10 was increased considerably in the total cell lysates derived from cells exposed to Tg; the increase was related to the duration of exposure (Figure 3A). c-Jun expression level is slightly elevated during the first few hours following exposure to Tg while remained constant thereafter. In contrast, the level of the transfected HA-JDP2 was not significantly changed following Tg treatment. Following immunoprecipitation with anti-HA antibody, both CHOP10 and c-Jun were not precipitated by the antibody against the HA-tag in the vector control lysate, efficient precipitation was observed for both c-Jun and CHOP10 with cell lysates derived from HA-JDP2 transfected cells (Figure 3B). The amount of CHOP10 protein that co-precipitated with the antibodies against the HA-tag gradually increased with the time of exposure to ER stress, and attained its highest level at 16 h following Tg treatment (Figure 3B, middle panel). This is consistent with the elevation in CHOP10 expression levels that occurred with increased duration of exposure to Tg. In contrast, precipitation of c-Jun was reduced in an inverse relationship with the duration of exposure to Tg (Figure 3B, bottom panel). The increase in the level of precipitated CHOP10 protein, in parallel with the decrease in precipitated c-Jun (starting 3 h following Tg treatment), suggests that part of the association of JDP2 with CHOP10 that follows ER stress may be formed by replacement of the pre-existing JDP2-c-Jun heterodimers.

The fact that transfected HA-JDP2 was able to precipitate endogenous CHOP10 protein prompted us to examine the interaction between the endogenous JDP2 and CHOP10 proteins. NIH3T3 cells were treated for 16 h with 400 nM Tg to induce expression of JDP2. Pre-cleared nuclear extracts derived from treated and untreated cells were used for immunoprecipitation with antibodies against either THTR1 (NR) or JDP2.
Precipitated proteins, together with 5% of the total input lysate, were separated by 12.5% SDS–PAGE, and transferred to nitrocellulose filters. Membranes were probed with antibodies against either JDP2 or CHOP10. JDP2 expression levels remained unchanged in the total nuclear cell lysate derived from cells that were either untreated or treated with Tg. Consistently, CHOP10 expression level was significantly increased following Tg treatment (Figure 3C). Immunoprecipitation with the nonrelevant antibody did not result in precipitation of either CHOP10 or JDP2 proteins. In contrast, anti-JDP2 antibodies efficiently precipitated JDP2 in both cell lysates (Figure 3C). Significantly, CHOP10 was efficiently co-precipitated by the JDP2-antibody only in nuclear cell lysate that was derived from cells that had been induced by Tg for 16 h (Figure 3C).

### TRE-dependent transcription activation by JDP2–CHOP complex

To investigate the effect of CHOP10 on JDP2-induced transcription from different TRE and CRE sequences, NIH3T3 cells were co-transfected with plasmids encoding JDP2 and CHOP10 in different combinations, together with luciferase reporter plasmids that were under the control of different promoters known to harbor TRE/CRE DNA-binding elements. The promoters used for the reporter genes included a −1745 to +14 bp sequence derived from the c-Jun promoter (CycD1-luc), −79 to +170 bp derived from the human c-Jun promoter (−79-Jun-luc), −73 to +63 bp derived from the human collagenase promoter (−73-hCol-luc) and a synthetic CRE-pTAL luciferase reporter plasmid (CRE-luc, BD Bioscience Ltd.). Twenty-four hours following transfection, cells were harvested and luciferase activity was determined.

The transfection of either JDP2 or CHOP10 separately resulted in inhibition of transcription activity or a very minor effect on all the luciferase reporter plasmids examined. In contrast, the co-expression of CHOP10 with JDP2 resulted in strongly synergistically enhanced transcription of the CycD1, −79-Jun-luc and −73-hCol-luc sequences. The CRE-luciferase reporter plasmid showed only a mild additive effect of the proteins (Figure 4A). This result strongly suggests that the JDP2–CHOP complex potently enhances the transcriptional capability of both proteins; this effect is specific for promoters that contain a TRE and does not affect CRE-dependent promoters.

A previous report has demonstrated that the association of ATF3 and CHOP10 impairs the binding and repression activity of ATF3 towards CRE DNA-binding sequences (17). To examine the transcription capability of the ATF3-CHOP heterodimer, NIH3T3 cells were co-transfected with different combinations of plasmids encoding ATF3 and CHOP10 together with various luciferase reporter plasmids. Similar to the effect of JDP2, the co-expression of CHOP10 and ATF3 resulted in potentiation of the Jun, collagenase and cyclin D1 reporter plasmids, but consistent with the previous report (17), no significant increase in transcription was observed with the CRE luciferase reporter (Figure 4A).

Figure 4. The CHOP–JDP2 interaction strongly potentiates TRE- but not CRE-dependent transcription. (A) NIH3T3 cells were co-transfected with the indicated expression plasmids (1 μg each) together with the indicated luciferase reporter plasmid (1 μg). Twenty-four hours following transfection, cells were harvested and luciferase activity was determined. Expression plasmids used were: pcDNA empty vehicle (vector), pcDNA based Rat JDP2 (JDP2), human CHOP10 (CHOP), human ATF3 (ATF3) and JDP2 fused to the VP16 activation domain (VP16-JDP2). In transfections in which a single expression plasmid was used the total amount of expression plasmid was adjusted with the corresponding pcDNA empty expression vehicle. Reporters used were: CRE-luc, cyclic AMP response element; CycD-luc, −1850 cyclin D1 promoter; −79-Jun-luc, −79 c-Jun promoter; −73-hCol-luc, −73 collagenase promoter. Luciferase activity was determined and the results represent the average±SEM from three independent experiments. (B) NIH3T3 cells were co-transfected with the −79-Jun luciferase reporter plasmid as described above, except that the amount of JDP2 expression plasmid was kept constant (1 μg) whereas the amount of CHOP10 was varied as indicated. Luciferase activity was determined and the results obtained with the reporter plasmid in the presence of JDP2 only were designated 1.0. The results represent the average±SEM from three independent experiments. Total cell lysate was subjected to 12.5% SDS–PAGE followed by western blotting. The nitrocellulose membrane was probed with antibodies against CHOP10. Lane 1 represents JDP2-transfected cells in the absence of CHOP10 expression.

To examine whether or not the JDP2-CHOP complex displays a distinct DNA-binding specificity from that of the JDP2 homodimer, we investigated the transcription capability of JDP2 fused to a strong activation domain derived from the herpes simplex virus protein VP16 (VP16-JDP2). The fusion of a strong activation domain is dominant over JDP2 repression activity turning JDP2 into strong transcriptional activator while preserving JDP2 DNA binding specificity (21).
Cells were transfected with the VP16-JDP2 expression plasmid and the different luciferase reporter plasmids. Luciferase activity measured 24 h following transfection revealed that VP16-JDP2 was able to potentiate transcription efficiently from all the TRE and CRE reporter plasmids tested. Surprisingly, the CRE-luc reporter exhibited the most distinguishable activity. Whereas the JDP2–CHOP complex was only able to activate transcription from the CRE-luc reporter plasmid by a factor of two, it exhibited a significantly more potent transcription activity with the VP16-JDP2 protein (a 50-fold increase). Thus we conclude that JDP2–CHOP complex displays an altered DNA-binding specificity as compared with the JDP2 homodimer.

To test whether the association between JDP2 and CHOP10 is required for the potentiation of transcription from TRE-responsive promoters, NIH3T3 cells were co-transfected with increasing amounts of expression plasmids encoding either wild-type CHOP10 or a CHOP10 mutant that lacked the leucine zipper domain (CHOPΔLZ), together with a constant level of JDP2. Whereas the wild-type CHOP10 activated the −79-Jun-luc reporter in a dose-dependent manner, the addition of CHOPΔLZ failed to result in any significant alteration in the −79-Jun-luc reporter transcription activity (Figure 4B). Western blot analysis using cell lysates derived from the transfected cells revealed that both the wild-type CHOP10 and CHOPΔLZ proteins are expressed at similar levels (Figure 4B, bottom panels). Therefore, expression level differences cannot provide an explanation for the inability of the CHOPΔLZ to increase JDP2-TRE-dependent transcription. Collectively the above results strongly suggest that the leucine zipper domain of CHOP10 is necessary for the JDP2-CHOP association (Figure 2) and for the potentiation of transcription.

A tethered JDP2–CHOP chimera activates transcription from TRE-containing promoters

We hypothesized that the association between CHOP10 and JDP2 results in the generation of a complex that activates transcription. To study the specificity of binding to the promoter element at an optimal stoichiometric ratio, single chain dimers were generated in which CHOP10 was fused in frame with JDP2 via a flexible polypeptide linker (JDP2–CHOP). In addition, relevant monomer controls were designed (Figure 5A). JDP2–CHOP chimera force specific intramolecular pairing to occur that is expected to reduce the associations with other bZIP proteins in the cell, as well as to decrease the chances of homodimerization of the overexpressed proteins (33). Thus the use of leucine zipper chimera provides a reliable system to examine more directly the transcription capability of different pairs of proteins. To this end, NIH3T3 cells were co-transfected with increasing amounts of either the JDP2–CHOP chimera or the monomeric components as controls (His-JDP2 and His-CHOP10), together with plasmids encoding the luciferase reporter gene driven by various promoter elements: −79-Jun, CRE, or the CHOP10 site that corresponded to the CHOP10-C/EBP specific binding site (34). Twenty-four hours following transfection, cells were harvested and luciferase activity was determined (Figure 5B).

As previously observed, single transfection with either JDP2 or CHOP10 encoding plasmids repressed or exhibited an insignificant effect on transcription, respectively, with the luciferase reporters containing the −79-Jun and CRE sequences, as well as with the CHOP10-C/EBP luciferase reporter plasmid. In addition, JDP2-CHOP chimera consistently displayed a negligible effect on transcription of the CRE and the CHOP10-C/EBP site. In contrast, the JDP2–CHOP chimera showed a significant dose-dependent transactivation of the −79-Jun-luc reporter plasmid; it induced transcription by ~20-fold when 1 μg of expression plasmid was used (Figure 5B).

These results are consistent with those obtained by transient co-transfection experiments with plasmids encoding CHOP10 and JDP2 (Figure 4), indicating the reliability of the tethered dimer method. The results strongly suggest that the forced dimer JDP2–CHOP has the same binding selectivity and activates the transcription activity of TRE-containing promoters. To examine the ability of JDP2–CHOP to associate physically with the TRE DNA site, we used in vitro translated protein products and an EMSA with P32-labeled Jun-TRE probe. Whereas unprogrammed reticulocyte lysate (Figure 5C) and CHOP10 (Figure 6B) displayed no binding to the Jun TRE probe, JDP2 exhibited efficient binding to the Jun TRE. Interestingly, the JDP2–CHOP chimera displayed efficient binding to the Jun TRE probe at 70 mM NaCl, and showed significantly slower migration compared with the JDP2–JDP2 homodimer (Figure 5C). Optimal DNA-binding activity of the in vitro translated JDP2 homodimer occurred at 150 mM NaCl. In addition, JDP2–CHOP displayed altered DNA-binding specificity compared with the JDP2 homodimer (data not shown). CHOP10 is unable to directly associate with DNA as a homodimer due to the presence of proline and glycine residues within the basic domain (32).

JDP2–CHOP associates with DNA in vivo

To examine whether JDP2–CHOP interaction with the Jun-TRE DNA also occurs in tissue culture cell lines, we used ChIP assay with NIH3T3 cells stably transfected with either CHOP10 or JDP2–CHOP chimera. The expression level of CHOP10 and JDP2–CHOP proteins in the NIH3T3 stable cell lines used for the assay relative to the endogenous JDP2 expression level is shown (Figure 5D). To increase the JDP2 level in NIH3T3-CHOP10 cell line, cells were co-transfected with an expression plasmid encoding for JDP2. Both cell lines were transfected with the human −79-Jun luciferase reporter plasmid. Twenty-four hours following transfection, cells were cross-linked with formaldehyde and DNA was fragmented by sonication. Immunoprecipitation was performed with either nonrelevant serum (anti-thiamine transporter 1, THTIR1) or with anti-CHOP10 polyclonal antibodies. Following extensive washes cross-link was reversed and DNA was extracted. The precipitated purified DNA was used as template for a PCR reaction with appropriate primers located 200 bp adjacent to the
Figure 5. An intramolecular dimer of JDP2–CHOP transactivates selectively the TRE-containing promoter element in a dose-dependent manner. (A) Schematic representation of the different monomer constructs and chimera used. The amino acid sequence of the linker used is indicated by a single letter code. The leucine zipper and the basic region of the corresponding encoded protein are indicated (ZIP and b, respectively) (B) NIH3T3 cells were co-transfected with expression plasmids, using the indicated amounts (µg), encoding the JDP2–CHOP chimera as well as their monomeric components, together with a luciferase reporter plasmid. Luciferase reporter plasmids used were: −79-Jun-luc, CRE-luc (as described in Figure 4) and CHOP-luc. The CHOP10 luciferase reporter corresponds to the CHOP-C/EBP-specific binding site (34). Twenty-four hours following transfection, cells were harvested and luciferase activity was determined. The luciferase activity obtained with the empty expression vector was designated 1.0 and all other luciferase activities were calculated relative to this. The results represent the average ± SEM of three independent experiments. (C) Electromobility shift assay with Jun-TRE DNA probe. Lysate used included; un-programmed reticulocyte lysate control (retic) or reticulocyte lysate programmed with either JDP2 or JDP2-CHOP chimera expression plasmids as indicated. Binding buffer contained 25 mM NaCl (lanes 1–3), 70 mM NaCl (lanes 4–6) or 150 mM NaCl (lanes 7–9). Protein–DNA complexes were separated on 6% nondenaturing gels, dried and exposed to autoradiography. (D) Western blot analysis of nuclear extract derived from NIH3T3 stably transfected with either CHOP10 (NIH-Chop, lanes 1 and 3) or JDP2–CHOP (JDP2–Chop lanes 2 and 4) probed with anti-JDP2 (left panel) and anti-CHOP10 (right panel) (E) NIH3T3–CHOP or NIH3T3–JDP2–CHOP stable cell lines were transfected with the human Jun-luciferase reporter in the presence or absence of JDP2 expression plasmid, respectively. 24 h following transfection, cells were formaldehyde fixed, lysed and DNA was fragmented by sonication. Cell lysate was either exposed to autoradiography. (D) Western blot analysis of nuclear extract derived from NIH3T3 stably transfected with either CHOP10 (NIH-Chop, lanes 1 and 3) or JDP2–CHOP (JDP2–Chop lanes 2 and 4) probed with anti-JDP2 (left panel) and anti-CHOP10 (right panel). (E) NIH3T3–CHOP or NIH3T3–JDP2–CHOP stable cell lines were transfected with the human Jun-luciferase reporter in the presence or absence of JDP2 expression plasmid, respectively. 24 h following transfection, cells were formaldehyde fixed, lysed and DNA was fragmented by sonication. Cell lysate was either incubated in high salt to reverse cross-link to assess total input (No-IP, lane 3) or used for immunoprecipitation. Immunoprecipitation was performed by either nonrelevant antibody (NR-anti-THTRI lanes 4 and 6) or anti-CHOP10 polyclonal antibody (lanes 5 and 7). Jun primers used for PCR corresponded to 200 bp adjacent the Jun-TRE sequence within the Jun-luciferase reporter plasmid (upper panel). Luciferase control primers located 1000-bp downstream to the luciferase translation start site corresponding to 320-bp fragment (bottom panel). DNA was extracted and used as template for PCR (23 cycles 63°C and 30 cycles 62°C, respectively). Control PCR reaction with either no DNA (−, lane 1) or with −79-Jun-Luciferase reporter plasmid (+, lane 2) were performed. The resulting PCR reaction was separated on 2% agarose gel, ethidium bromide stained and photographed.

Jun-TRE within the human Jun-luciferase promoter. While no specific signal was obtained with DNA extracted from immunoprecipitation with the nonrelevant antibody following 20-25 PCR cycles (23 cycles are shown), the DNA extracted following immunoprecipitation with the anti-CHOP10 antibody resulted in a strong signal corresponding to a DNA fragment of the expected size (Figure 5E, top panel). To demonstrate that the precipitated DNA is specific to the DNA sequence adjacent the Jun-TRE element, we used oligonucleotides corresponding to the luciferase coding region located 1200-bp downstream of the Jun-TRE site and performed a PCR reaction using the precipitated DNA as template. A 320-bp fragment was efficiently generated using either 10 ng DNA plasmid corresponding to the Jun-luciferase reporter or total DNA extracted directly from the...
transfected cells. In contrast, no specific fragment was observed with the precipitated DNA derived from the immunoprecipitations performed with either the nonrelevant serum or the anti-CHOP10 (Figure 5E, bottom panel). These results, strongly suggest that the JDP2–CHOP complex was able to associate with the human Jun promoter directly in NIH3T3 cells.

The CHOP10 basic domain is crucial for transcription activation

The results shown in Figure 5 suggest that the JDP2–CHOP complex exhibits altered DNA-binding specificity as compared with the JDP2 homodimer. To examine whether the CHOP10 basic domain may support DNA binding of JDP2–CHOP and is crucial for transcription activation, a JDP2–CHOP chimera was designed that comprised JDP2 fused to a CHOP10 mutant protein lacking the entire basic region (JDP2–CHOPΔBasic). The transcription activity of the JDP2–CHOPΔBasic mutant was tested with the −79-Jun luciferase reporter (Figure 6). The JDP2–CHOPΔBasic chimera was unable to activate transcription of the −79-Jun luciferase reporter (Figure 6A). Western blot analysis revealed that JDP2–CHOPΔBasic is well expressed as compared with the other JDP2–CHOP chimeras (Figure 6A, bottom panel). Consistently, we used retic lysate programmed with different CHOP10 mutants lacking either the basic domain or the leucine zipper domain in EMSA with Jun-TRE. Although, the different JDP2–CHOP mutants were expressed to similar levels (Figure 6B, bottom), yet, JDP2–CHOP lacking the basic domain displayed reduced Jun-TRE binding (Figure 6B). In contrast, the JDP2–CHOP chimera lacking the CHOP10 leucine zipper domain showed potentiation of transcription similar to that of the wild-type JDP2–CHOP chimera (Figure 6A). In addition, the JDP2–CHOPΔLZ chimera exhibited efficient binding to the Jun TRE (Figure 6B, top panel), suggesting that the forced dimerization was sufficient to substitute for the leucine zipper domain and allowed the basic domain to support DNA binding of the complex. To ensure that the CHOP10 lacking the basic domain preserves its ability to associate with JDP2, we tested the interaction between JDP2 and CHOPΔBasic proteins. Co-immunoprecipitation of HA-JDP2 with CHOPΔBasic confirmed that the basic region is not required for JDP2 binding (data not shown and Figure 2). Collectively, the results suggest that the recruitment of CHOP10 to JDP2 is not sufficient for transcription activation, and that the basic domain of CHOP10 plays a role in the association of the JDP2–CHOP dimer with the TRE-DNA-binding site. This is in contrast to a situation previously described, in which tethering of CHOP10 to pre-existing Jun–Fos

Figure 6. The basic domain of CHOP10 is essential for transcription activation by JDP2–CHOP10. (A) NIH3T3 cells were co-transfected with expression plasmids encoding the indicated JDP2–CHOP chimeras, together with a −79-Jun-luciferase reporter plasmid. JDP2–CHOP chimeras used encoded for the wild type and mutants lacked either the basic domain (JDP2–CHOPΔBasic) or the leucine zipper motif (JDP2–CHOPΔLZ). JDP2 and CHOP10 expression vectors were used as controls. Twenty-four hours following transfection, cells were harvested and luciferase activity was determined. The activity of the reporter in the presence of the vehicle expression plasmid was designated 1.0, and all other activities were calculated relative to this. The results represent the average ± SEM of three independent experiments. The expression levels of CHOP10 and the JDP2–CHOP chimeras were analyzed by western blotting using antibodies against CHOP10. The level of α-tubulin serves as loading control. (B) Reticulocyte lysates programmed with the indicated expression plasmids were used in EMSA with 32P-labeled Jun-TRE as the DNA probe. The binding reaction was performed with 70 mM NaCl. The gel was dried and exposed to autoradiography. Retic lysate programmed with the corresponding plasmids used for EMSA in the presence of S35 methionine is shown to ensure that similar expression levels of the JDP2–CHOP wild type and mutants were used in the EMSA assay. Programmed and unprogrammed retic lysate were separated on 12.5% SDS-PAGE dried and exposed to autoradiography.
Ectopic expression of JDP2 and CHOP10 alters cell sensitivity to ER stress

CHOP10 is a transcription factor that is activated at multiple levels during ER stress and is responsible for ER stress-induced apoptosis (36). Cells in which CHOP10 has been disrupted are more resistant to ER stress-induced apoptosis. To investigate whether the JDP2–CHOP interaction may have some biological relevance, we generated NIH3T3 cell lines that stably expressed either JDP2 (NIH3T3-JDP2) or CHOP10 (NIH3T3-CHOP). NIH3T3-JDP2 expresses JDP2 to about 3-fold higher levels as compared with vector transfected cells whereas, NIH3T3-CHOP expressing cells expresses CHOP10 to about 2-fold higher as compared with NIH3T3 cells exposed to 200 nM Tg for 6 h (Figure 7A). Stably transfected NIH3T3 cells were exposed to increasing concentrations of Tg for 24 h, and the viability of the cells was examined by measuring the cellular ATP content. NIH3T3 cells with ectopic expression of JDP2 displayed increased viability compared with wild-type NIH3T3 cells (Figure 7B). In contrast, NIH3T3 cells with CHOP10 overexpression displayed increased sensitivity to Tg treatment. This result may suggest that JDP2 functions to counteract the toxic effect of CHOP10 following ER stress. Alternatively, JDP2 over-expression may increase cell survival in a CHOP10-independent manner. Indeed, previous work has suggested that JDP2 is a cellular survival protein necessary for normal cellular function (37). However, some recent study demonstrating that JDP2 knockout mice are viable and JDP2−/− MEF exhibit no growth defect, strongly argues against a role of JDP2 as a survival factor (14). To distinguish between these two possibilities, we used SV40 large T antigen immortalized CHOP10−/− MEF cells exposed to different concentrations of Tg. The expression level of the different stable cell lines used is shown (Figure 7C). CHOP10−/− MEF cells are more resistant to Tg treatment as compared with the parental MEF cells [(29) and Figure 7D]. In addition, CHOP10 ectopic expression displayed sensitivity to Tg treatment similar to wild-type MEF cells (Figure 7D). Whereas JDP2 expressing cells exhibited increased resistance to Tg treatment in NIH3T3 (Figure 7B) and in the parental MEF cells (Figure 7D), JDP2 overexpression in CHOP10−/− MEF cells failed to alter the Tg sensitivity (Figure 7D). Suggesting that the increase in Tg sensitivity observed following JDP2 overexpression is CHOP10 dependent. To examine whether or not JDP2–CHOP transcription complex is responsible for the transcription of target genes (survival genes) that are responsible for the increased resistance towards Tg treatment, we over-expressed JDP2–CHOP chimera in the parental MEF cells and examined their sensitivity to Tg. JDP2–CHOP chimera expressing cells displayed similar sensitivity to Tg treatment as compared with the parental MEF cells (Figure 7D). Collectively, these results suggest that JDP2 protects cells from ER stress in a CHOP10-dependent manner by counteracting CHOP10 toxic effect. Yet, we cannot exclude the possibility that JDP2 may have some other beneficial effects acting as a homodimer or heterodimer with CHOP10. Since JDP2 protein is constitutively expressed at variable levels in all cells tested, the level of JDP2 expression may play a role in determining the cellular threshold response to ER stress-induced cell death. In contrast, ATF3 is highly induced following ER stress. ATF3 was shown to control CHOP transcription and functions in a feedback inhibition of translation control following ER stress by mediating GADD34 transcription (38). Our data may suggest that ATF3 is able to cooperate with CHOP10 to result in increase in GADD34 transcription. Both JDP2 (11) and CHOP10 (39) serve as substrates for the p38 kinase. The precise role of JDP2 and CHOP10 phosphorylation following ER stress is currently under investigation.

CONCLUSIONS

Here we described for the first time the role of JDP2 in TRE-dependent transcription activation. JDP2 association with CHOP10 results in activation of transcription from TRE but not CRE containing promoters. The basic leucine zipper domain of both proteins is necessary for the DNA binding and transcription activation. JDP2 expression counteracts CHOP10 pro-apoptotic activity and may serve to set a threshold for ER response.

The role of JDP2 in carcinogenesis is controversial. On the one hand, JDP2 inhibits TRE-dependent transcription and inhibits NIH3T3 cell transformation by activated Ras, H-RasV12 (22). On the other hand, JDP2 has been found, during screening by insertional mutagenesis, to be an oncogene cooperating with cMyc and the cell cycle inhibitor p27 (23–25). The ability of JDP2 to associate with CHOP10, resulting in transcription activation of TRE-responsive genes, may explain this discrepancy. For example, the cyclin D1 promoter has been shown to be a direct target for JDP2 regulation (12). Indeed, both VP16-JDP2 and JDP2-CHOP complexes strongly potentiated the transcription of the cyclin D1 promoter (Figure 4). However, in NIH3T3 cells, H-RasV12 results in down-regulation of CHOP10 expression via post-transcriptional mechanism involving decrease in mRNA stability (40).
Therefore, JDP2 inhibition Ras-mediated transformation is consistent with JDP2 repression of the Ras–MAPK–Jun axis independent of its ability to interact with CHOP10 (22). On the other hand, solid tumors suffer from nutrient and glucose deprivation, which results in an ER stress response that involves the elevation of the CHOP10 expression. ER stress response in human breast carcinoma cell lines was shown to result in the increase in pro-angiogenic factors such VEGF and IL-8 production (41). CHOP10 association with bZIP proteins such as JDP2 and ATF3 that results in potentiation of transcription, may provide the molecular basis for the promotion of angiogenesis. Further research is required to elucidate the dual role of JDP2 in angiogenesis and oncogenic processes.

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