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Intrinsically disordered human C/EBP homologous protein regulates biological activity of colon cancer cells during calcium stress

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Abstract

Intrinsically disordered proteins are emerging as substantial functional constituents of mammalian proteomes. Although the abundance of these proteins has been established by bioinformatics approaches, the vast majority have not been characterized structurally and functionally. C/EBP homologous protein (CHOP) is a proto-oncogene, traditionally shown as a dominant-negative inhibitor of C/EBPs and a transcriptional activator of Activating Protein-1. We report here the *in vitro* characterization of CHOP, where our computational analyses and experimental evidences show for the first time that CHOP is an intrinsically disordered protein. Intrinsic fluorescence, NMR spectroscopy, and analytical size exclusion chromatography studies indicate that CHOP contains extensive disordered regions and self-associate in solution. Interestingly, the disordered N-terminal region plays a key role in the oligomerization of CHOP and is vital for its biological activity. We report the novel mechanistic role of CHOP in the inhibition of Wnt/TCF signaling and stimulation of c-Jun and sucrase-isomaltase reporter activity in intestinal colon cancer cells. These findings are discussed in the context of oligomerization of intrinsically disordered proteins as one of the mechanisms through which they exert their biological function.

Keywords

Extra-cellular calcium-sensing receptor; colon cancer; natively unfolded protein; oligomerization equilibrium; transcription regulation

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Introduction

C/EBP Homologous Protein (CHOP) is a small nuclear protein that acts as a dominant-negative inhibitor of CCATT enhancer-binding proteins (C/EBPs) and a transcriptional activator of Activating Protein-1 (AP-1)¹. As a transcriptional regulator, CHOP plays a role in linking endoplasmic reticulum (ER)-stress to alterations in gene expression, which leads to apoptosis². CHOP expression is mainly regulated at the transcriptional level and is one of the highest inducible genes during ER-stress³. Aside from ER-stress mediated apoptosis, CHOP expression has been implicated in homo-cysteinemia, brain ischemia, diabetes and neurodegenerative disorders^{4,5}. Additional evidence for a role of CHOP in cellular growth and differentiation comes from a molecular analysis of human sarcomas³. Increased CHOP expression occurs in response to nutrient depletion, amino acid starvation, and during the unfolded protein response of hyper-stressed cells⁶. Although some stress-inducible genes associated with CHOP induction have been reported, molecular mechanism(s) linking CHOP induction to downstream signaling still remains elusive.

Many biologically active proteins lack a stable three-dimensional fold under apparently physiological conditions⁷⁻¹⁰. In contrast to ordered proteins, whose three-dimensional folds are relatively stable and whose Ramachandran angles vary slightly around their equilibrium positions with occasional cooperative conformational switches, intrinsically disordered (ID) proteins or regions exist as dynamic ensembles in which the atom positions and backbone Ramachandran angles vary significantly over time with no specific equilibrium values and typically undergo non-cooperative conformational changes^{9,11-15}. Importantly, these ID proteins and regions are known to carry out numerous biological functions including cell signaling¹⁶, molecular recognition¹⁷, and interactions with different proteins and nucleic acids^{10,15,18}. Computational studies have revealed that eukaryotes exhibit more disorder than either prokaryotes or archaea¹⁹⁻²². It is hypothesized that this higher abundance of intrinsic disorder is a consequence of the increased need for cell signaling and regulation in higher organisms^{16,19,23-25}.

Although biological functions of CHOP are relatively well characterized, little is currently known about the structural properties of CHOP and how they contribute to CHOP function. A recent study has implicated the involvement of N-terminal region in Wnt/Tcf signaling²⁶, yet the role of the N-terminal domain of CHOP largely remains elusive. To understand whether intrinsic disorder plays a role in CHOP structure and function, we have characterized CHOP using a variety of bioinformatics and biophysical techniques. We show that CHOP is an ID protein and that the N-terminal domain influences the oligomerization state of CHOP. It is important to note that like many transcription factors, DNA-binding ability of CHOP resides in its C-terminal which remains intact in our deletion studies; hence any change in observed activity is likely regulated by the N-terminal region of CHOP. Our data suggest that CHOP plays a critical role in the inhibition of Wnt/Tcf signaling and stimulation of c-Jun and sucrose-isomaltase reporter activity in intestinal colon cancer cells, the latter of which is dependent on the presence of the N-terminus of CHOP and its oligomerization state.

Results and Discussion

CHOP is predicted to be an intrinsically disordered protein

Statistical analysis shows that amino acid sequences encoding for ID proteins or regions are significantly different from those of ordered proteins on the basis of several factors, including local amino acid composition, flexibility, hydropathy, charge, and coordination number^{19, 27-29}. A signature of a probable ID region is the presence of low sequence complexity coupled with amino-acid compositional bias, characterized by a low content of “order-promoting” residues (Val, Leu, Ile, Met, Phe, Trp, Tyr, Cys, His and Asn), and a high proportion of

particular polar and charged amino acids (Arg, Gln, Ser, Pro, Glu, Lys and, on occasion, Gly and Ala), known as “disorder-promoting” residues^{23,30}. ID proteins and regions have been grouped into at least two broad structural classes – compact (molten globule-like) and extended (coil-like and pre-molten globule-like, so called natively unfolded (NU) proteins)^{9,12,18,31,32}.

To gain insight into CHOP structure, we compared its amino acid composition with averaged compositions of ordered proteins³⁰, ID proteins³⁰, and NU proteins⁹. The fractional difference in composition between an amino acid type in CHOP and an equivalent amino acid type in a set of ordered, intrinsically disordered and natively unfolded proteins is depicted by bars in Fig. 1A, which clearly shows that CHOP shares many characteristic amino acid features with ID and NU proteins¹⁷. Similar to ID and NU proteins, CHOP is depleted in general order-promoting residues (Cys, Phe, Ile, Tyr, Val, and His) and is also heavily enriched in some disorder-promoting residues (Arg, Gln, Ser, and Glu).

The sequence of CHOP was also used to predict whether this protein is likely to be mostly disordered using two binary predictors of intrinsic disorder: charge-hydrophathy plot (CH-plot)³³ and cumulative distribution function analysis (CDF)²⁰. Both these methods perform binary classification of whole proteins as either mostly disordered or mostly ordered, where mostly ordered indicates proteins that contain more ordered residues than disordered residues and mostly disordered indicates proteins that contain more disordered residues than ordered residues²¹. We have established that the CDF curve calculated for CHOP is located below a boundary, emphasizing that this protein would adapt a disordered conformation. Furthermore, the application of CH-plot analysis³⁴ to the CHOP amino acid sequence shows that this protein is characterized by the mean hydrophathy $\langle H \rangle = 0.3778$, the mean net charge $\langle R \rangle = 0.0955$, and the “boundary” mean hydrophathy value $\langle H \rangle_b = 0.4476$, therefore fulfilling the requirement for the natively unfolded proteins, namely that $\langle H \rangle < \langle H \rangle_b$ ($0.3778 < 0.4476$). Thus, CHOP is predicted to be mostly disordered by CDF and CH-plot analyses.

Fig. 1B illustrates the distribution of predicted disorder within the CHOP sequence using two classifiers of intrinsic disorder, PONDR VL-XT and VL3, which are non-linear neural network classifiers trained to distinguish order and disorder based on large feature space^{30,35}. Fig. 1B confirms that CHOP is expected to be disordered with 73.6% and 86.0% residues of this protein were predicted to be disordered by PONDR VL-XT and VL3, respectively. It is of interest to note that the PONDR VL3 plot is a smooth feature-less curve located mostly above the boundary, whereas PONDR VL-XT plot, being also mostly located above the boundary, possesses several distinctive features, like spikes and dips (Fig. 1B), corresponding to the disordered nature of CHOP.

CHOP C-terminal region

Coiled-coils are ubiquitous structural motifs composed of two or more α -helices, which entwine to form a rope-like structure that is often used to mediate protein oligomerization³⁶. It is found in many types of proteins, including transcription factors (e.g., GCN4 and C-fos), viral fusion peptides, SNARE complexes, and certain tRNA synthetases^{37,38}. CHOP sequence analysis by COILS (Fig. 1D) shows that the C-terminal domain of CHOP is strongly predicted to have a long coiled-coil region (residues 116–168), largely corresponding to a C-terminal helix predicted by secondary structural analysis (Fig. 1C).

Interestingly, comparison of the results of different analyses of CHOP amino acid sequence revealed that the N-terminal dip in the disorder prediction (Fig. 1B) coincides with an increased probability of α -helix formation, as predicted by hierarchical neural network HNN³⁹ (Fig. 1C), and shows non-zero probability to form coiled-coil (Fig. 1D). Finally, a long C-terminal α -helix would be most likely stabilized via the formation of a dimeric coiled-coil structure.

Taken together, our bioinformatics analysis suggests that CHOP is an intrinsically disordered protein, and a coiled-coil structure may be formed through its C-terminal region.

Structural properties of CHOP

Recombinant full-length CHOP was obtained by overexpression of the protein in *E. coli*, followed by affinity purification and GST-tag removal via thrombin cleavage. One-dimensional (1D) ^1H and two-dimensional heteronuclear NMR spectra were collected to assess the folding properties of CHOP. The lack of upfield-shifted resonances associated with methyl protons and very little dispersion in the amide proton region of the 1D ^1H spectrum of CHOP, shown in Fig. 2A, suggest that CHOP comprises very little globular structure. Analysis of ^1H - ^{15}N HSQC spectrum of CHOP revealed well-resolved backbone amide resonances for 81 of the expected 168 backbone ^1H - ^{15}N correlations, which fell between 7.0 and 8.8 ppm (Fig. 2B). Although the limited ^1H chemical shift dispersion is suggestive of an unfolded protein, a transient secondary structure existing in fast exchange with a random coil conformation on the NMR time scale cannot be discounted. The observation of numerous $d_{\text{NN}}(i, i+1)$ NOEs in the ^{15}N -NOESY-HSQC spectrum of CHOP (data not shown) indicates that the associated residues reside in the α -region of ϕ/ψ conformational space, which can be satisfied by both an unstructured region or helical structure⁴⁰.

Structural features of CHOP were also assessed by near-UV CD and by the intrinsic fluorescence spectroscopy. The near-UV CD spectrum (Fig. 3A) of CHOP is dominated by a positive band in the vicinity of 275 nm (most likely associated with the asymmetric environment of the two tyrosine residues, Tyr24 and Tyr40) and has a shoulder in the vicinity of 280 nm (which can be attributed to the asymmetric environment of Trp18, Trp23, and Trp64). Typically, near-UV CD spectrum of tryptophan-containing proteins is dominated by this latter band. The fact that this is not the case for CHOP suggests that Trp18, Trp23, and Trp64 are located in the relatively symmetric environment and/or relatively accessible to solvent, indicating they are located in relatively flexible region. This hypothesis is supported by the red-shifted maximum of the intrinsic fluorescence, $\lambda_{\text{max}}=345$ nm (Fig. 3B). In fact, it is known that a tryptophan emission maximum is highly dependent on the polarity of its environment. A tryptophan residue in a non-polar environment (i.e., buried inside a protein), has an emission maximum close to 320 nm, while in a polar environment (i.e., solvent exposed) it has an emission maximum close to 350 nm, so-called Stokes shift⁴¹.

Oligomerization properties of CHOP and its N-terminal truncation construct

The hydrodynamic dimensions of full length CHOP and a N-terminal truncated CHOP construct (CHOP¹⁰¹⁻¹⁶⁸) were estimated by analytical gel filtration chromatography. Figure 4 shows that full-length CHOP elutes from the column as two peaks with the maximum at ~ 9.5 ml and ~ 13.7 ml, and CHOP¹⁰¹⁻¹⁶⁸ elutes from the column as predominantly one peak with the maxima at ~ 17 ml. Gel filtration analysis can provide useful information on the conformational state of the protein⁴², since proteins are separated based on their hydrodynamic dimensions, not on molecular mass. A calibrated gel filtration column also provides good estimates of the corresponding R_S values. For example, the formation of the molten globule is associated with a ~ 10 – 30% increase of the R_S ^{42,43}, whereas the pre-molten globule state is more expanded with a ~ 30 – 50% increase of the R_S ^{42,44,45}. This method has been successfully applied to determine the Stokes radius (R_S) values for proteins in different conformational states^{42,44,45}. The elution peaks observed for full-length CHOP correspond to R_S of 44.7 and 72.8 Å while CHOP¹⁰¹⁻¹⁶⁸ peak correspond to 32.3 Å. The experimentally determined Stokes radii of full-length CHOP and CHOP¹⁰¹⁻¹⁶⁸ were then used to evaluate potential conformations of CHOP by correlating Stokes radii with molecular mass of proteins in different conformational states using the equations described previously³¹. If the elution peaks of full-length CHOP and CHOP¹⁰¹⁻¹⁶⁸ are attributed to pre-molten globules, then the corresponding

molecular masses are 163.8 kDa and 48.7 kDa for CHOP and 21.8 kDa for the CHOP¹⁰¹⁻¹⁶⁸. Assuming the oligomers are comprised of monomers in the pre-molten globule conformation, the elution peaks of full-length CHOP and CHOP¹⁰¹⁻¹⁶⁸ correspond to an apparent octamer and dimer for CHOP and an apparent dimer for CHOP¹⁰¹⁻¹⁶⁸. For CHOP and CHOP¹⁰¹⁻¹⁶⁸, the R_S values exceeds more than 50% of the theoretical values calculated for a well-folded globular proteins with the molecular weight of the CHOP dimer or octamer and CHOP¹⁰¹⁻¹⁶⁸ dimer³¹. This significant increase in the R_S value is consistent with the assumption that when in the pre-molten globule-like conformation, CHOP associates to form oligomers.

To corroborate the findings of the analytical gel filtration chromatography and gain more comprehensive knowledge of the oligomeric state of CHOP and CHOP¹⁰¹⁻¹⁶⁸ in solution, we performed experiments using sedimentation velocity analytical ultracentrifugation (AUC). Fitting of the AUC data of CHOP resulted in no distinct peaks because of the large range of species of apparently high molecular mass, suggesting multiple oligomeric forms (data not shown). Conversely, when AUC data of CHOP¹⁰¹⁻¹⁶⁸ were evaluated by a continuous distribution $c(S)$ Lamm equation model, two distinct peaks (1.13S and 2.20 S) were observed (Fig. 4C). Integrating the $c(M)$ distribution, SEDFIT⁴⁶ estimated these two peaks with median values of 9.9 kDa, 23.4% of total population and 24.3 kDa, 68% of total population, respectively suggesting a large population of apparent dimer and a small population of monomer. Altogether, these data suggest that when N-terminal region of CHOP is deleted, it can no longer make large oligomers.

Effect of CaSR activation on expression of CHOP in adenocarcinoma cell lines

CHOP is an antiproliferative and proapoptotic protein^{47,48} and recently shown as a novel inhibitor of Tcf-4 binding to inhibit β -catenin activity²⁶. In this regard we wanted to evaluate if the extracellular calcium-sensing receptor (CaSR) activation may affect endogenous CHOP in colon cancer cells. First, we assessed whether activating the extracellular calcium-sensing receptor (CaSR) with a high Ca^{2+} challenge (3 mM) in the HT-29 adenocarcinoma cell lines, HT-29 and DLD-1, influenced expression of CHOP. Semi-quantitative RT-PCR revealed that extracellular Ca^{2+} [Ca^{2+}_o], increased intensity of CHOP amplicons (Fig. 5A). Western blot analysis of HT-29 and DLD-1 adenocarcinoma cells incubated with 3 mM Ca^{2+}_o revealed a substantial increase in CHOP protein levels (Fig. 5B). To understand the nature of the increased expression of CHOP, we assessed CaSR stimulated cell lysates on a native gel. As illustrated in Fig. 5C, we observed that the high Ca^{2+}_o challenge of these cell types increased the amount of CHOP present. Together these data suggested to us that CaSR activation in these cells increased the production of CHOP.

Effect of overexpression of full-length and N-terminal truncations of CHOP on β -catenin reporter activity in HT-29 cells

As a transcription factor, CHOP is similar to c-Myc in which the C-terminal domain binds to DNA and the N-terminal domain plays a regulatory role, hence termed transactivation domain⁴⁹. In light of the observation that the N-terminal region of CHOP influences its oligomerization state as shown by analytical gel filtration and AUC (Fig 4), we wished to examine the impact of the N-terminal truncation in a biological setting. In this quest, we prepared mammalian constructs of CHOP and its N-terminal truncations (Fig. 6A) in pcDNA 3.1 vector. As illustrated in Fig. 6B, deletions of the first 53 or first 100 amino acids of CHOP generated proteins that were proportionately reduced in molecular weight. Then we focused on comparing β -catenin signaling responses of overexpressed full-length CHOP the N-terminal CHOP¹⁰¹⁻¹⁶⁸ construct. The effects of overexpression of full-length CHOP or CHOP¹⁰¹⁻¹⁶⁸ on the β -catenin reporter TOPFLASH in the HT-29 cells are illustrated in Fig. 6C. In low-calcium medium (5 μM) proliferation rates and β -catenin luciferase activity are highest in

HT-29 cells and⁴². In low calcium medium, overexpression of full-length CHOP inhibited TOPFLASH activity by ~60% ($p < 0.05$, $n = 6$, Fig. 6C). Overexpression of CHOP¹⁰¹⁻¹⁶⁸ in low-calcium medium inhibited TOPFLASH ~29% ($p < 0.05$, $n = 6$). Because activating the CaSR on HT-29 cells will inhibit β -catenin signaling⁵⁰, we compared overexpression of these constructs in the presence of CaSR activation. Overexpression of full-length CHOP increased the amount of β -catenin reporter inhibition in the presence of CaSR activation (% inhibition of activity in 5 μ M Ca²⁺: 78 ± 5 vs 60 ± 4 , $p < 0.05$, $n = 6$, Fig. 6D). Overexpression of CHOP¹⁰¹⁻¹⁶⁸ in the presence of CaSR activation did not change the amount of inhibition of the β -catenin reporter (% inhibition: 56 ± 6 vs 60 ± 4 , NS, $n = 6$).

Effect of overexpression of full-length and N-terminal truncations of CHOP on c-Jun and sucraseisomaltase promoter activity in HT-29 cells

Having shown that full-length and truncated CHOP overexpression would decrease, by differing amounts, the activity of the β -catenin reporter, we measured the activity of reporters known to or predicted to be stimulated by CHOP. We first tested the activity of a c-Jun promoter in HT-29 cells in response to overexpressed full-length CHOP, CHOP¹⁰¹⁻¹⁶⁸ and MEKK4 as a positive control. As illustrated in Fig. 7A, full-length CHOP increased the activity of the c-Jun promoter (% of control RLA: 180 ± 33 , $p < 0.05$, $n = 6$). Overexpression of CHOP¹⁰¹⁻¹⁶⁸ had no effect on activity of the c-Jun promoter (% of control RLA: 99 ± 12 , NS, $n = 6$). Subsequently, we assessed the activity of a marker of intestinal epithelial differentiation, a sucrase-isomaltase (SI) promoter, and known to be responsive to p38 MAPK⁵¹. Overexpression of full-length CHOP increased activity of the SI promoter (% of control: 230 ± 16 , $p < 0.05$, $n = 6$) over control values (Fig. 7B). The overexpression of CHOP¹⁰¹⁻¹⁶⁸ had no effect on the activity of the SI promoter (% of control: 99 ± 23 , $p < 0.05$, $n = 6$) in the HT-29 cells (Fig. 7B).

Together these data suggest that the disordered N-terminal transactivation domain influences the oligomerization state of CHOP, which plays a key role in inhibiting β -catenin activity and stimulating c-Jun and SI reporter activity in a model intestinal epithelial cell line. While the primary function of CHOP as a transcription factor is mediated by its C-terminal domain akin to other transcription factors, we provide here first experimental evidence that the little known intrinsically disordered N-terminal region of CHOP regulates the multi-functional biological activity of CHOP, likely through its influence on oligomerization.

Further discussion and conclusions

The studies presented here demonstrate that CHOP is an intrinsically disordered protein. The ability of CHOP to interact with numerous binding partners and to modulate a variety of cellular processes suggests that in the exquisitely complex network of protein-protein interactions CHOP can be considered as a regulatory protein with a high connectivity level or a 'hub'. Interestingly, it has recently been suggested that intrinsic disorder may play a crucial role in these hub protein functions^{17,52}. ID regions can bind partners with both high specificity and low affinity⁵³, thus fulfilling the fundamental requirements of signaling interactions – specificity and reversibility³². Furthermore, ID proteins and regions possess tremendous binding diversity with the ability to interact specifically with numerous partners, including proteins, nucleic acids, polysaccharides and small molecules^{7-9,16,19,54}.

Our experiments suggest that the oligomerization of CHOP can be mediated by the disordered N-terminal domain of this protein. While intrinsically disordered proteins are widely recognized to possess versatile adaptability to bind numerous ligands, we show here that they can also exert their biological function through manifestation of their oligomerization state. Presumably, their structural fluidity or plasticity provides an advantage to allow easier manipulation of oligomerization state, thereby serving as another means to elicit their activity.

This is not surprising given the factor that oligomerization is known to play functional role in ordered or globular proteins^{36,55}. In this study, we found that the N-terminal domain of CHOP is a participant in CHOP oligomerization, which plays a key role in both inhibition of Wnt/Tcf signaling and stimulation of c-Jun and SI reporter activity in intestinal colon cancer cells.

Increases in dietary calcium supplementation have been shown to reduce recurrence of colonic adenomas⁵⁶ and calcium is known to be a chemoprotective agent against colon cancer⁵⁷. The extracellular calcium-sensing receptor (CaSR) has been shown to be present on the apical and basolateral membrane of human and rodent colonic epithelial cells⁵⁸⁻⁶⁰. It has been reported that activation of the CaSR on colonic cancer cell lines inhibits proliferative Wnt activity by increasing β -catenin degradation⁵⁰. Because CHOP is an antiproliferative and proapoptotic protein^{47,48} and can function as a novel inhibitor of Tcf-4 binding to reduce β -catenin activity²⁶, we speculated that CaSR activation might alter endogenous CHOP in colon cancer cells. Previously we have shown that Ca^{2+}_o (3–5 mM) stimuli may be blocked by transgenic expression of siRNA duplex against the CaSR in endogenously expressing cells, consistent with a reduction in CaSR transcript and protein^{50,61,62}. In the current experiments we have assumed the Ca^{2+}_o stimulation is mediated by the CaSR, consistent with previous reports. As such, CaSR activation of colon cancer cells resulted in a substantial increase in CHOP expression and dimeric/oligomerized protein. The further reduction of β -catenin signaling occurring in the presence of overexpressed full-length CHOP together with CaSR activation suggests to us that either the CaSR-mediated increases in CHOP protein become additive with the overexpressed CHOP, or additional mechanisms are activated by the CaSR to inhibit β -catenin signaling. The phytoalexin resveratrol will also induce upregulation of CHOP transcript and protein leading to apoptosis in colon cancer cells⁶³. These increases in CHOP were attenuated by a JNK inhibitor. Curcumin will also induce CHOP expression in full length APC expressing cells by activating PKC⁶⁴. We do not know the signal cascade activated by the CaSR which leads to increased CHOP or whether apoptosis occurs in these cells. Nevertheless, the current experiments suggest that the substantial upregulation of CHOP by CaSR activation may contribute to the chemoprotective effect of calcium against colon cancer.

Overexpression of CHOP in the current experiments stimulated expression of SI, an established marker of intestinal epithelial differentiation. The mechanism by which CHOP stimulates this marker may involve p38 MAP kinase as both CDX2 and p38 have been shown to regulate SI transcription^{51,65,66}. We do not know if the CaSR activation of CHOP is a manifestation of the Unfolded Protein Response (UPR) or is an ER stress-specific response since CHOP is the only UPR target known to be dually regulated by both responses⁶⁷. However, N-deletions of CHOP also influenced the activation of the SI promoter in a manner comparable to the inhibition of the β -catenin reporter, consistent with our interpretation that self-association of this unfolded protein is required for biological activity. Whether increases in CHOP result in increases in epithelial differentiation or lead to increases in apoptosis is not known at present time.

In summary, we have established that with N-terminal deletions the degree of self-association of natively unfolded CHOP protein is reduced as is the biological activity of either stimulating an epithelial marker of differentiation or inhibiting β -catenin signaling. This study highlights the oligomerization mediated activity of CHOP and reveals an important mechanism through which intrinsically disordered proteins elicit their biological function. This is particularly important since, as a whole, the large class of intrinsically disordered proteins is not well understood.

Materials and Methods

Cloning, expression and purification of recombinant CHOP

GST- fusion constructs containing CHOP (residues 1–168), CHOP (residues 54–168) and CHOP (residues 101–168) were generated from human cDNA (IMAGE:3530545; Open Biosystems, Huntsville, AL) using forward primers 5'- GCG GAT CCA TGG CAG CTG AGT CAT TGC CT -3' (1–168), 5'- GCG GAT CCG CAA TGG GAA ATG AAG AGG AAG AAT CA -3' (54–168), 5'-GCG GAT CCG CAA TGG ATT CCA GTC AGA GCT CCC TG -3' (101–168), in combination with primer with sequence 5'- GAG CGG CCG CAT GCT TGG TGC AGA TTC ACC AT -3' as reverse primer. The amplified PCR products were ligated into pCR 2.1-TOPO vector (Invitrogen) and subcloned as a BamH1-SalI fragment into the BamH1 and SalI sites of pGEX-4T-3 (Amersham Biosciences). Recombinant GST-CHOP protein was expressed in *Escherichia coli* strain BL21(DE3). Cells were grown in 1L Terrific Broth medium (Bioshop Inc.) containing 50 µg ml⁻¹ ampicillin at 37°C to an OD₆₀₀ of 0.8 and induced with 1 mM isopropyl-β-D-thiogalacto-pyranoside. Growth was continued for an additional 14 h at room temperature. The cells were harvested and stored at –20°C. Cell lysis was performed in 1 × PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) pH 7.3 containing 1 mM phenylmethane sulfonyl fluoride, 10 mM dithiothreitol and 0.1 mg mL⁻¹ DNase I using a cell disrupter (Cell Disruption System, UK) at 25000 psi. The lysate was cleared by centrifugation in a JA25.5 rotor at 18000 × g and loaded on a GSTrap column (Amersham Biosciences) for affinity purification using the glutathione-S-transferase (GST) tag attached to the protein. Multiple washes with 1 × PBS were performed and followed by on-column cleavage of the GST-tag using 80 units of thrombin (1 unit per µl) (Sigma) for 16 h at 4°C. CHOP was eluted with 1 × PBS and further purified on a HiLoad 16/60 G-200 gel filtration column attached to an AKTA FPLC (Amersham Biosciences). Peak fractions were pooled and concentrated to ~8 mg/mL in 1 × PBS buffer using a Centricon 10K concentrator (Millipore). Protein was extensively dialysed into 5 mM Na₂HPO₄-NaOH (pH 7.0) and stored at 4°C.

Amino acid composition profiling

Amino acid composition of CHOP was compared with averaged compositions of ordered³⁰, intrinsically disordered³⁰, and natively unfolded proteins⁹ based on the approach developed for intrinsically disordered proteins¹⁹. To visualize differences, the amino acid mole fractions, P_j , of each amino acid, j , for pairs of protein sets, a and b , are displayed as $(P_j^a - P_j^b) / P_j^b$, where set a varies and set b is a composition of ordered proteins³⁰. For these comparisons, the lower bound is –1 for cases for which $P_j^a = 0$ and the upper bound for amino acid j is equal to $(100 - P_j^b) / P_j^b$. The amino acids are arranged from the most rigid to the most flexible according to the scale of Vihinen et al.⁶⁸, which is based on the averaged B-factor values for the backbone atoms of each residue type as estimated from 92 proteins⁶⁸.

Cumulative distribution function (CDF) analysis

CDF analysis summarizes the per-residue disorder predictions by plotting Predictor Of Natural Disordered Regions (PONDR) scores against their cumulative frequency, which allows ordered and disordered proteins to be distinguished based on the distribution of prediction scores²¹. At any given point on the CDF curve, the ordinate gives the proportion of residues with a PONDR score less than or equal to the abscissa. For CDF analysis, order-disorder classification is based on whether a CDF curve is above or below a majority of boundary points.

Charge-hydrophathy plot

CH-plot analysis is based on the observation that naturally folded and intrinsically unstructured proteins occupy non-overlapping regions in the charge-hydrophathy plots, with natively

unfolded proteins being specifically localized within a particular region of charge-hydrophathy phase space, satisfying the following relationship^{21,33}:

$$\langle H \rangle \leq \langle H \rangle_b = \frac{\langle R \rangle + 1.151}{2.785}$$

where $\langle H \rangle$ and $\langle R \rangle$ are the mean hydrophathy and the mean net charge of the given protein, respectively, whereas $\langle H \rangle_b$ is the “boundary” mean hydrophathy value, below which a polypeptide chain with a given $\langle R \rangle$ will be most probably unfolded. The mean hydrophathy, $\langle H \rangle$, is defined as the sum of the normalized hydrophathy estimated by the Kyte and Doolittle approach³⁴ of all residues divided by the number of residues in the polypeptide. The mean net charge $\langle R \rangle$ is defined as the net charge at pH 7.0, divided by the total number of residues.

PONDR Analysis

PONDR is a set of neural network predictors of disordered regions on the basis of local amino acid composition, flexibility, hydrophathy, coordination number and other factors. These predictors classify each residue within a sequence as either ordered or disordered. CHOP is subjected to PONDR analysis using programs VL-XT^{30,35} VL3⁶⁹ and VSL1⁷⁰.

Secondary structure prediction

Two predictors, hierarchical neural network, HNN³⁹ and ALB⁷¹, were used to predict the propensity of CHOP to form secondary structure. HNN, being made up of two networks, a sequence-to-structure network and a structure-to-structure network, is based on X-ray protein structures, including local information, some physico-chemical data and multiple-alignment. On the other hand, the ALB secondary structure stability parameters and the residue to hydrophobic surface interaction energies are based on experimental data on poly- and oligopeptides, amino acids, and physical estimates of protein structures⁷¹. CHOP was also analyzed using COILS, a program that derives a similarity score of a given sequence to a database of known parallel two- stranded coiled-coils and calculates the probability that the sequence will adopt a coiled-coil conformation⁷².

Circular dichroism (CD)

CD experiments were performed on an OLIS RSM CD spectrophotometer at 25°C. The protein concentration and the path length used near UV CD analysis were 25 μ M and 1 cm, respectively. The spectrometer was purged with pure N₂ during the course of the experiment. Each CD plot was an average of 14 accumulated scans. Plots were baseline corrected for buffer.

NMR Spectroscopy

One-dimensional ¹H and two-dimensional ¹H-¹⁵N HSQC NMR spectra of 0.4 mM uniformly ¹⁵N-labeled protein samples was recorded on a Varian INOVA 600 MHz spectrometer equipped with a pulse field gradient triple resonance cryoprobe at 4°C. The experiments used the enhanced sensitivity pulsed-field gradient approach by Kay *et al.*⁷³. Carrier frequencies of 4.77 ppm (¹H), and 118 ppm (¹⁵N) were used and ¹H chemical shifts were calibrated relative to the trimethylsilyl resonance (0.0 ppm) of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The buffer conditions were 1 \times PBS pH 6.8 in 90% H₂O/10% D₂O. Spectra were processed and analyzed using NMRPipe⁷⁴ and NMRview⁷⁵, respectively.

Analytical gel filtration chromatography

Oligomerization states of CHOP and CHOP¹⁰¹⁻¹⁶⁸ were measured by analytical gel filtration. Chromatography was performed on the Superose-12 prepacked chromatography column

calibrated as per manufacturer's instruction using protein molecular weight standards (Amersham Biosciences). Protein solutions (~0.25 mg/ml) were loaded onto the column equilibrated with the same buffer. The elution was carried out isocratically at a flow rate of 0.5 ml/min and monitored by the absorbance at 280 nm.

Analytical ultracentrifugation

Full length CHOP and CHOP¹⁰¹⁻¹⁶⁸ samples used in the analytical ultracentrifugation (AUC) were dialyzed extensively against 1X PBS buffer pH 7.0. The dialysate of each sample was used for making protein dilutions (OD₂₈₀ of 0.2, 0.4 and 0.8) and also for the reference solution. All sedimentation velocity AUC experiments were performed at 20 °C using a Beckman XL-I analytical ultracentrifuge with a four-hole An-60Ti rotor fitted with double-sector Epon charcoal center pieces. Experimental runs on samples were conducted at 55 000 rpm by use of Rayleigh interference optics. Interference sedimentation coefficient distributions (c(S) and c(M)) were calculated from the sedimentation velocity data using SEDFIT⁴⁶.

Mammalian constructs and cell lines

Mammalian constructs for CHOP¹⁻¹⁶⁸ (full-length CHOP), CHOP⁵⁴⁻¹⁶⁸ (N-terminal deleted), and CHOP¹⁰¹⁻¹⁶⁸ (only C-terminal containing basic helix-loop-helix region of CHOP) was generated by performing a restriction enzyme digestion on respective GST-fusion constructs (described above) using *Bam*H1 and *Not*I enzymes and sub-cloning into pcDNA 3.1 vector (Invitrogen Inc., Carlsbad, CA). Sequence of the vectors were validated by restriction digestion and sequencing of positive clones.

The human colon adenocarcinoma HT-29 and DLD-1 cell line were purchased from the American Type Culture Collection (ATCC; Rockville, MD). HT-29 and DLD-1 cells were cultured in Dubelco's Modified Eagle Media supplemented with 10% FBS or RPMI media supplemented with 10% FBS respectively, and penicillin 100µU/mL, and grown at 37°C in a humidified 5% CO₂ atmosphere. The Cos-1 line was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin 100µU/mL. Cells were passaged weekly with 0.25% trypsin and used for experimentation within the first 6 passages.

Transient transfection and luciferase reporter assays

Equal amounts of HT-29 and DLD-1 cells were seeded into 24-well tissue culture plates, and grown in DMEM or RPMI for 24 h. At 50–60% confluent stage the cells were transfected with plasmid DNAs using superfect transfection reagent (Qiagen, Inc., Mississauga, ON) according to manufacturer's recommendations. At least, three independent experiments were performed in triplicates. The following amounts of plasmids were used per well of a 24 well plate: 0.5 µg sucrose-isomaltase-Luc (Upstate USA, Inc., Chicago, IL), and 2 ng pRL-SV40 (Promega, Co., Madison, MI), and 0.3 µg CHOP¹⁻¹⁶⁸, CHOP⁵⁴⁻¹⁶⁸, CHOP¹⁰¹⁻¹⁶⁸, respectively. The control plasmid phRL-SV40 was used as internal control for transfection efficiency of SI promoter reporter. Eighteen hours post-transfection, cells were incubated in serum-free, Ca²⁺ free DMEM containing 4 mM L-glutamine, 0.2% BSA, penicillin 100µU/mL and 0.5 mM CaCl₂ for 18 h. This medium was removed and substituted with the same medium supplemented with 0.5 mM Ca²⁺_o for another 18 h. Cells were harvested and lysed in 100 µl of reporter lysis buffer (Promega, Co., Madison, MI). For separate experiments, to determine the effect of overexpressing N-terminus truncation of CHOP on Tcf/Lef reporter, HT29 colon cancer cells were transfected with 1 µg TopFlash and 2 ng phRL-null, and 0.5 µg CHOP¹⁻¹⁶⁸, 0.5 µg CHOP⁵⁴⁻¹⁶⁸, or 0.5 µg CHOP¹⁰¹⁻¹⁶⁸. Twenty four hours after transfection cells were incubated for 18 h in serum free, Ca²⁺_o free DMEM containing 4 mM L-glutamine, 0.2% BSA, 100 µU/ML penicillin, and 0.005 mM CaCl₂. This medium was removed and was substituted with the same medium supplemented with 0.005 mM Ca²⁺_o or 5 mM Ca²⁺_o for

18h. Luciferase activity was measured using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wilbad, Germany).

Reverse Transcription PCR (RT-PCR)

Differential gene expression analysis was performed by semi quantitative PCR using a Mastercycler (Eppendorf, Hamburg, Germany). Total RNA from HT-29 cells was isolated by the TRIzol reagent (Invitrogen Inc., Carlsbad, CA) as per manufacture's instructions. The recovered RNA was quantitated by spectrophotometry, and aliquots of 1 µg total RNA from low Ca²⁺_o (0.5 mM) or high Ca²⁺_o (3 mM), was used for cDNA synthesis at 37°C for 1 h in a volume of 20 µL containing the following reagents: 5 mM dNTP mix; 10 µM Oligo-dT primer; 10 U RNase inhibitor; 4 U Omniscript Reverse Transcriptase; 1× Buffer RT (all from Qiagen Valencia, CA). The PCR reaction was prepared in a volume of 50 µL containing the following reagents: 0.2 mM dNTP Mixture; 1.5 mM MgCl₂; 1 µL template cDNA; 1.0 U platinum taq DNA polymerase (Invitrogen Inc., Carlsbad, CA). The PCR cycle conditions were: initial denaturation at 94°C for 60 s followed by 35 amplification cycles denaturing at 94°C for 30 sec, annealing at an amplicon dependent temperature for 30 s and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 min. The primers for CHOP were: sense 5' – GCG GAT CCA TGG CAG CTG AGT CAT TGC CT - 3'; antisense 5'- GAG CGG CCG CAT GCT TGG TGC AGA TTC ACC AT -3'. The resulting bands were visualized on a 1.0% agarose gel stained with ethidium bromide and compared to a 100 bp DNA ladder to confirm the predicted size. For positive amplification control we amplified the GAPDH gene using sense: 5' - TTA GCA CCC CTG GCC AAG G - 3' and antisense 5' - CTT ACT CCT TGG AGG CCA TG - 3' primers.

Western blotting and Native PAGE

Western blotting was performed in whole cell lysates which were prepared as described above and flash frozen as single-use aliquots till further use. A volume of 2X SDS sample buffer containing 5 % β-mercaptoethanol was added to an equal volume of cell lysate and boiled for 5 min. Samples were subjected to 16 % SDS-PAGE and were subsequently transferred to Immobilon P membrane. Western blotting was performed with the indicated anti-human HGF antibodies, followed by chemiluminescence detection (Western Lightning, Perkin-Elmer Life Sciences, Boston, MA).

For the identification of self-association states of overexpressed CHOP (20 µg) of whole cell lysate was resolved on 6% NATIVE-PAGE gels under non-reduced conditions. Under non-reduced conditions, samples were not boiled and sample loading buffer did not contained SDS and β-mercaptoethanol. A lane containing 2 µg of bovine serum albumin served as molecular weight protein marker. For the visualization of protein bands, the gel was stained with SYPRO orange as per manufacturer's instructions (Molecular Probes, Invitrogen Inc., Carlsbad, CA) and visualized on a UV transilluminator.

Statistics

Data are presented as means ± SE of at least 3 separate experiments. Data were analyzed by Student's *t*-test or ANOVA when appropriate. *P* < 0.05 was set as the statistically significant difference level.

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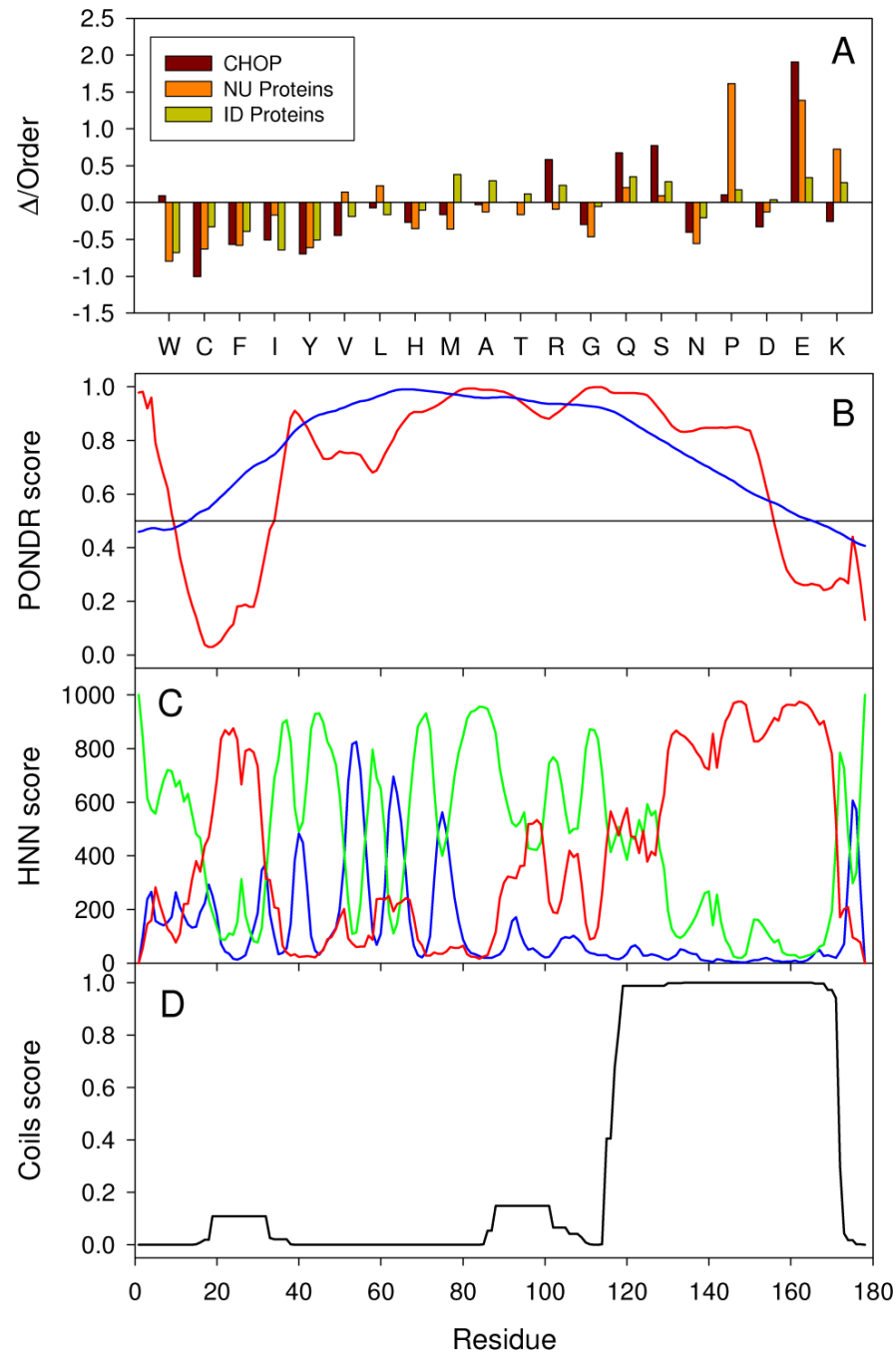


Fig. 1. The amino acid composition of CHOP predicts a disordered structure. **(A)** Bar graph showing amino acid composition of CHOP in comparison to averaged composition of natively unfolded and intrinsically disordered proteins. **(B)** Assessment of CHOP disorderedness using PONDNR VL-XT and VL3. **(C)** Secondary structure gaining propensity of CHOP is measured here by HNN³⁹ and ALB⁷¹. **(D)** Represents sequence analysis of CHOP using COILS⁷².

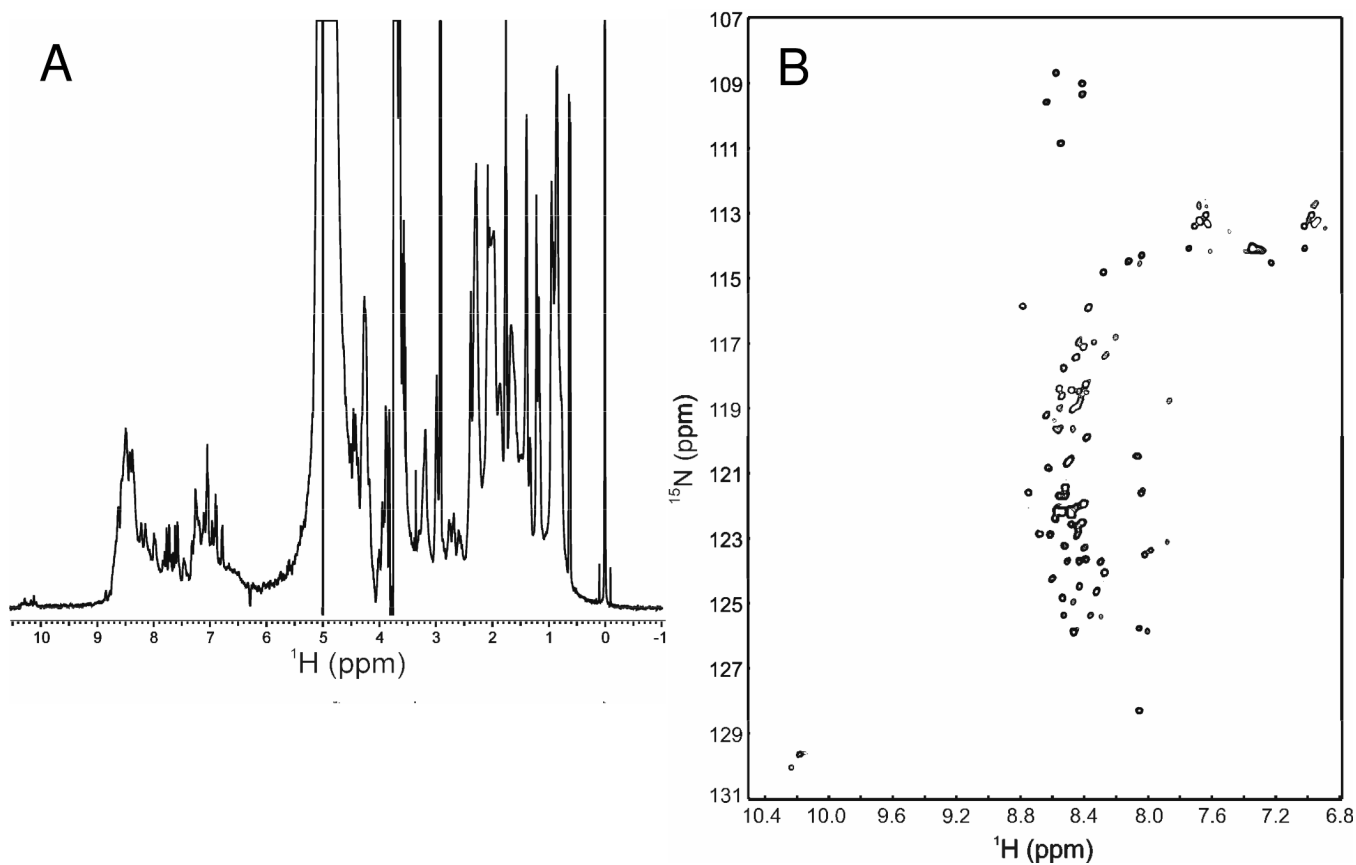


Fig. 2. NMR analyses of the folding properties CHOP. (A) 1D ^1H and (B) 2D ^1H - ^{15}N HSQC spectra of 0.4 mM CHOP (PBS, pH 6.8) at 600 MHz and 4°C. The lack of chemical shift dispersion suggests that CHOP lacks stable secondary structure.

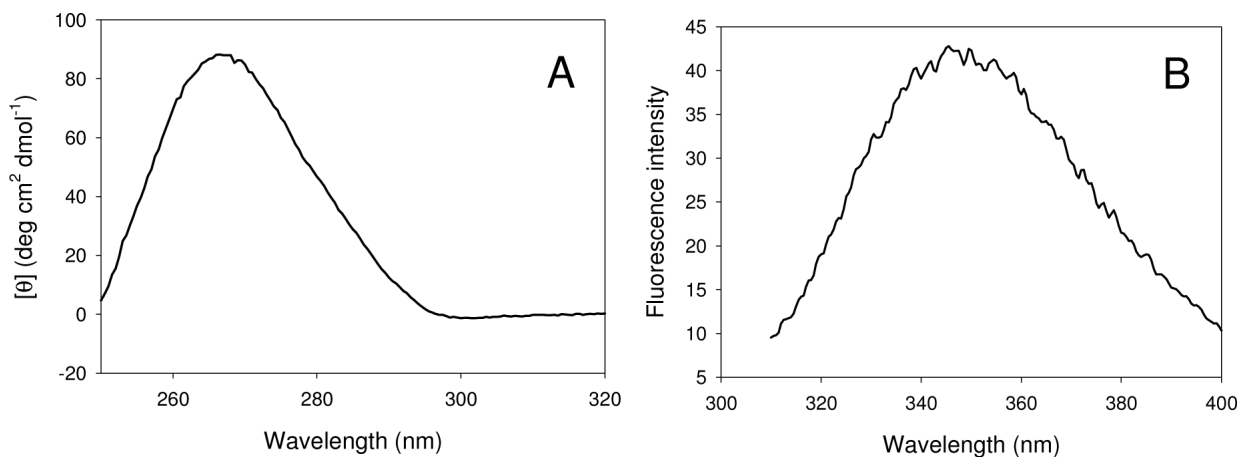
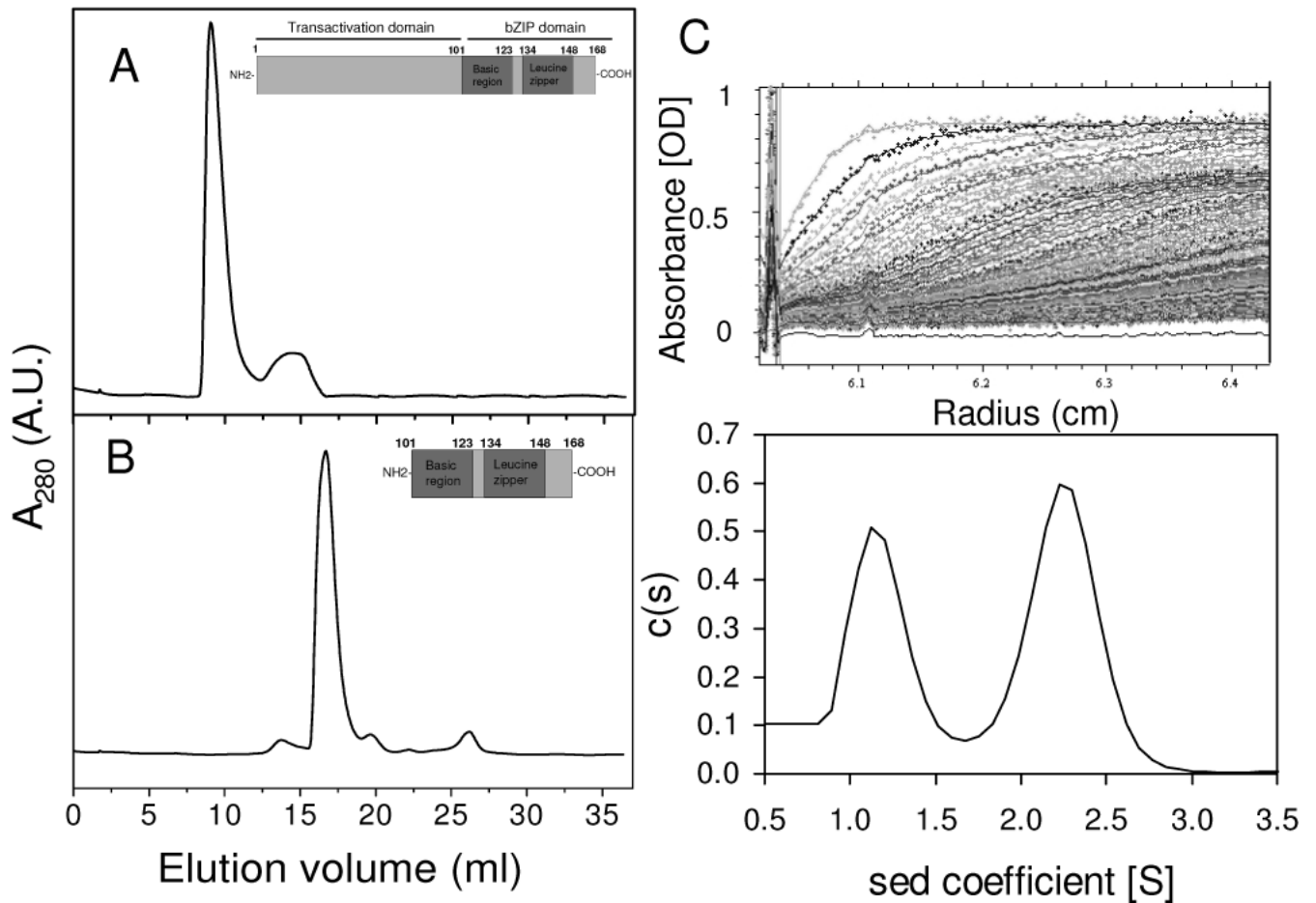


Fig. 3.

Structural properties of CHOP measured as measured by CD and fluorescence. **(A)** Near-UV CD spectrum of CHOP. **(B)** Intrinsic tryptophan fluorescence of CHOP measured on an LS50B fluorescence spectrometer (Perkin-Elmer). **(C)** Far UV CD Spectrum of CHOP. **(D)** Far UV CD Spectrum of CHOP¹⁰¹⁻¹⁶⁸. CD spectra are recorded on an OLIS RMS CD Spectrophotometer at 25°C. The samples contained 0.16 mg/ml CHOP in 10mM phosphate buffer, pH 7.0. An average of 8 scans was conducted, each corrected against buffer blanks.

**Fig. 4.**

Gel filtration profile of (A) CHOP and (B) CHOP¹⁰¹⁻¹⁶⁸ on an AKTA FPLC Superose-12 (10/300 GL) column (Amersham Biosciences) in 5 mM sodium phosphate buffer, pH 7.0 eluted at a flow rate of 0.5 ml/min. Samples (0.25 mg/ml) was passed through a 0.2-mm filter immediately before injection onto the column. Inset in the upper and lower panels show the domain organization of CHOP constructs used in the experiment. (C) Sedimentation velocity analysis of CHOP¹⁰¹⁻¹⁶⁸. Upper panel shows the raw AUC data concentration profiles from a sedimentation velocity run on the CHOP¹⁰¹⁻¹⁶⁸ sample (OD 0.8). Lower panel show the plot of the distribution of sedimentation coefficients ($c(s)$ vs s , where s is plotted in Svedberg units, S) calculated from the concentration profiles shown in upper panel using SEDFIT⁴⁶.

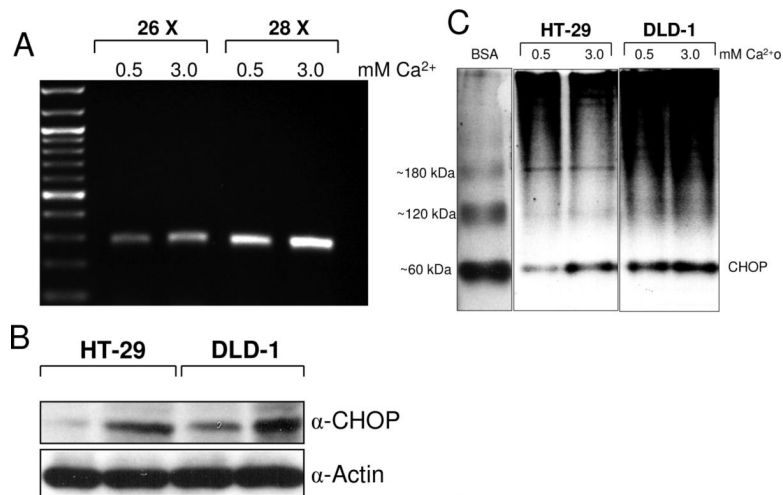
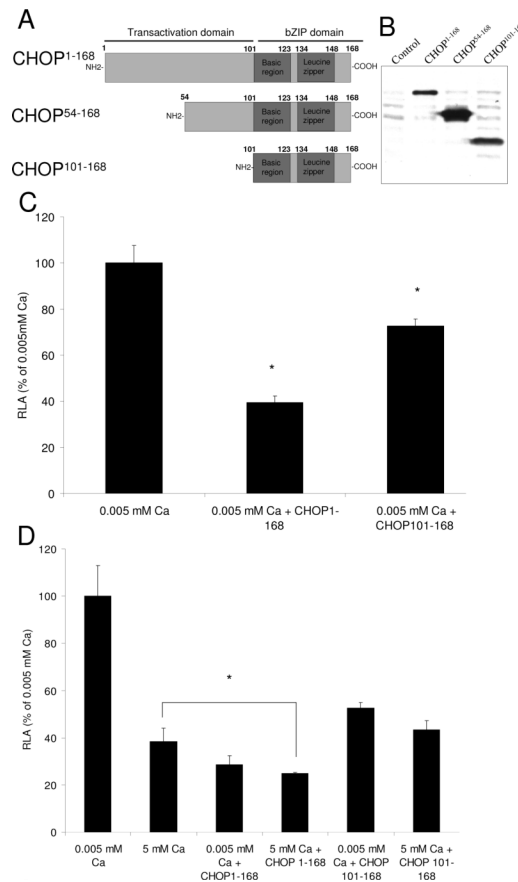
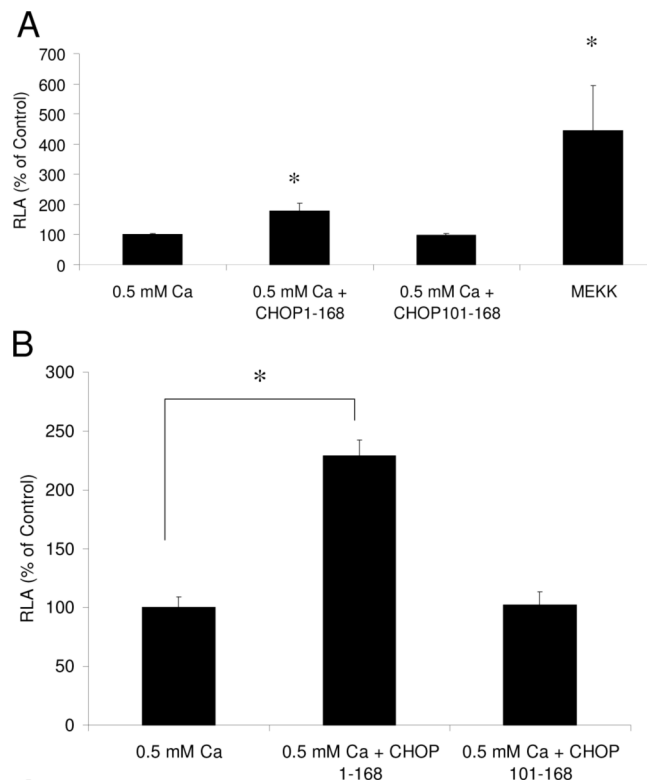


Fig. 5. CHOP expression in colon carcinoma cell lines is upregulated by CaSR activation. **(A)** HT-29 cells were cultured for 48 h in growth media, supplemented with 10% FBS. Cells were then serum starved overnight in DMEM containing 0.5 mM Ca²⁺_o, and later treated with high Ca²⁺_o (3 mM) for 18 h. Semi-quantitative RT-PCR demonstrated that high Ca²⁺_o stimulated the expression of the transcript for CHOP from HT-29. Similar results were obtained in cells transfected with the non-canonical Wnt5a. Figure shown here is representative of 3 independent experiments demonstrating similar results. **(B)** CHOP protein expression is upregulated by high Ca²⁺_o (3 mM). Western blot analysis for CHOP demonstrated a stronger immunoreactive band in whole cell lysates of cells treated with 3 mM Ca²⁺_o for 18 h. Immunoblotting using α-actin antibody was performed as internal loading control. Figure shown here is representative of 3 independent experiments demonstrating similar results. **(C)** High calcium addition increases the expression of CHOP in colon cancer cells. Cells were treated as above and a non-reducing 6% Native-PAGE was used to determine changes in the aggregation state of CHOP protein in HT-29 and DLD-1 cells. A marked upregulation of CHOP by 3 mM Ca²⁺_o treatment was observed in both cell lines.

**Fig. 6.**

(A) Shows the domain organization of CHOP and its truncation constructs. (B) Cos-1 cells were transiently transfected with different CHOP constructs, whole cell lysate was resolved on 16% SDS-PAGE and immunoblotted using α -CHOP antibody. Transfection with full length CHOP (CHOP¹⁻¹⁶⁸), N-terminus deletion of the first 53 amino acids (CHOP⁵⁴⁻¹⁶⁸) and N-terminus deletion of first 100 amino acids (CHOP¹⁰¹⁻¹⁶⁸) clearly demonstrated the presence of immunoreactive bands with different molecular weights consistent with the size truncations. Western blot shown is representative of three different experiments with similar results. (C) N-terminus truncation of CHOP diminishes the CHOP repression of a Tcf/Lef reporter in HT-29 cells. Cells were cotransfected with TopFlash (Tcf/Lef reporter), full length CHOP (CHOP¹⁻¹⁶⁸) or N-truncation (CHOP¹⁰¹⁻¹⁶⁸); 24 h later grown in 0.005 mM Ca²⁺ containing DMEM for additional 18 h. A greater reduction of TopFlash was observed with full length CHOP than with the N-truncation construct. TopFlash firefly luciferase RLU values were first normalized to Renilla RLU values and then results were expressed as a fraction of activity in 0.005 mM Ca²⁺. Data shown are representative of three independent experiments performed in triplicate. (D) CaSR activation in HT-29 transfected with full length CHOP further reduced the inhibition of Tcf/Lef reporter activity by CHOP alone. In contrast, high Ca²⁺ treatment (3 mM) to cells transfected with CHOP¹⁻¹⁶⁸ failed to inhibit Tcf/Lef reporter activity to the levels achieved by 3 mM + full length CHOP. TopFlash firefly luciferase RLU values were first normalized to Renilla RLU values and then results were expressed as a fraction of activity in 0.005 mM Ca²⁺. Data shown are representative of three different experiments performed in triplicate.

**Fig. 7.**

(A) N-terminus of CHOP modulates the up-regulation of c-Jun promoter in HT-29 cells. Cells were transiently co-transfected with c-Jun transcriptional activator in the presence of pFR-Luc reporter and CHOP¹⁻¹⁶⁸ or CHOP¹⁰¹⁻¹⁶⁸. MEKK4 was transfected as positive control for c-Jun promoter activation. Full length CHOP protein increased c-Jun promoter activity nearly 2 fold. In contrast, CHOP¹⁰¹⁻¹⁶⁸ had no effect on the c-Jun reporter activity. PhRL-null (renilla) luciferase was used as an internal transfection control, and the firefly luciferase RLU values were normalized against Renilla RLU values. Results were expressed as fold of increase from control cells. Data shown are representative of three different experiments performed in triplicate. **(B)** The increase of SI promoter activity expression is regulated by CHOP's N-terminus. HT-29 cells were transiently co-transfected with Sucrase-isomaltase (SI-Luc) promoter in the presence of CHOP¹⁻¹⁶⁸ or CHOP¹⁰¹⁻¹⁶⁸ and 48 h later luciferase activity was determined. Expression of the full length CHOP protein induced a >2 fold increased in SI-Luc reporter activity. Overexpression of the N-terminus truncation of the CHOP protein (CHOP¹⁰¹⁻¹⁶⁸) had no effect on SI-Luc reporter. PhRL-SV40 renilla was used as an internal transfection control, and the firefly luciferase RLU values were normalized against Renilla RLU values. Results were expressed as fold of increase from control cells. Data shown are representative of three different experiments performed in triplicate.