Human Cruciform Binding Protein Belongs to the 14-3-3 Family†

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ABSTRACT: Cruciform DNA has been implicated in the initiation of DNA replication. Recently, we identified and purified from human (HeLa) cells a protein, CBP, with binding specificity for cruciform DNA. We have reported previously that the CBP activity sediments at approximately 66 kDa in a glycerol gradient. Here, photochemical cross-linking studies and Southwestern analyses confirm that a 70 kDa polypeptide interacts specifically with cruciform DNA. Microsequence analysis of tryptic peptides of the 70 kDa CBP reveals that it is 100% homologous to the 14-3-3 family of proteins and shows that CBP contains the ϵ, β, γ, and ζ isoforms of the 14-3-3 family. In addition to polypeptides with the characteristic molecular mass of 14-3-3 proteins (30 and 33 kDa), CBP also contains a polypeptide of 35 kDa which is recognized by an antibody specific for the ϵ isoform of 14-3-3. Cruciform-specific binding activity is also detected in 14-3-3 proteins purified from sheep brain. Immunofluorescene studies confirm the presence of the ϵ, β, and ζ isoforms of 14-3-3 proteins in the nuclei of HeLa cells. The 14-3-3 family of proteins has been implicated in cell cycle control, and members of this family have been shown to interact with various signaling proteins. Cruciform binding is a new activity associated with the 14-3-3 family.

Inverted repeat sequences (IRs)[1] are a common feature of prokaryotic and eukaryotic regulatory regions, including promoters (*1*−*5*), terminators (*6*), and origins of DNA replication in prokaryotes (*7*, *8*), viruses (*9*), eukaryotes (*10*, *11*), and mammalian organisms (*12*−*16*), as well as in amplified genes (*17*). They have been shown to be functionally important for the initiation of DNA replication in plasmids, bacteria, eukaryotic viruses, and mammalian cells (reviewed in ref *18*). IRs have the potential to form cruciform structures through intrastrand base pairing and under conditions of torsional strain on the DNA (reviewed in refs *18* and *19*). Cruciform formation in vivo (reviewed in ref *18*) has been demonstrated in prokaryotes (*20*−*23*) and in mammalian cells (*24*−*26*).

We and others have previously demonstrated the involvement of cruciforms in the initiation of DNA replication (*21*, *24*−*26*; reviewed in ref *18*). In support of the hypothesis that a cellular cruciform-specific binding protein may be involved in the regulation of DNA replication, we recently identified and isolated from human cell (HeLa) nuclei a cruciform-specific binding activity, CBP (*27*). This activity was enriched from HeLa cells and appears as a 66 kDa protein with binding specificity for cruciform-containing molecules (*27*). Hydroxyl radical footprinting studies demonstrated that the CBP binds at the base of four-way junctions (*28*), interacting with them in a manner different from that of other proteins known to bind such junctions (*27*, *28*).

In this study, we report the identity of CBP as 14-3-3 proteins. The 14-3-3 family of proteins is highly conserved through plants, invertebrates, and higher eukaryotes with several diverse functions, which include involvement in neurotransmitter biosynthesis, signal transduction pathways, and cell cycle control (*29*, *30*).

EXPERIMENTAL PROCEDURES

Band-Shift Assays. pRGM21 X pRGM29 is a stable cruciform formed by heteroduplexing the 200 bp *Hin*dIII−*Sph*I fragments of the plasmids pRGM21 and pRGM29 as described previously (*27*) (Figure 1a). C1 X C2 was made by annealing (*31*) the synthetic oligonucleotides C1 (69 bp) and C2 (68 bp). The 200 bp pRGM21 X pRGM29 cruciform has a stem−loop structure identical to that of the C1 X C2 cruciform, but longer flanking arms. These cruciforms were used to assay, purify, and compete with CBP. Band-shift analyses of 14-3-3 proteins purified from sheep brain were carried out as described previously for CBP (*27*) using 4 μg of 14-3-3 proteins purified from sheep brain to homogeneity by a combination of anion-exchange and hydrophobic-interaction chromatography steps as described by Toker et al. (*32*).

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[1] Abbreviations: CBP, cruciform binding protein; IR, inverted repeat; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CADMS, collisionally activated dissociation mass spectrometry; HPLC, high-pressure liquid chromatography; ESMS, electrospray mass spectroscopy; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease I; UV, ultraviolet; PVDF, polyvinylidene fluoride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; NP40, nonylphenoxypolyethoxyethanol; BLAST, basic alignment search tool; TBS, 10 mM Tris-HCl (pH 8.0) and 150 mM NaCl; TBS-T, TBS and 0.05% Tween-20; Blotto, TBS-T and 5% skim milk; ECL, enhanced chemiluminescence; PBS, 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4; βME, β-mercaptoethanol.

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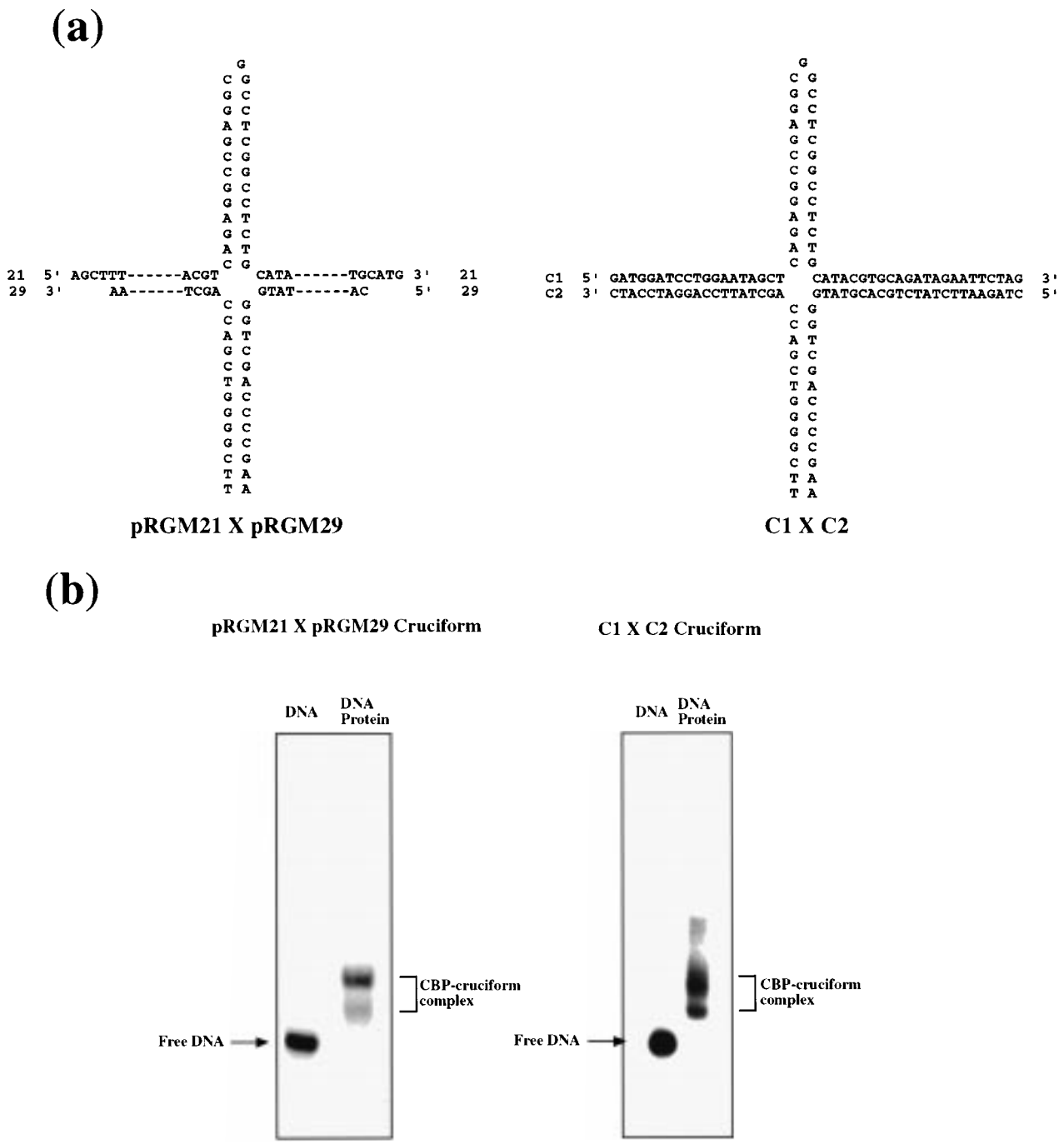

FIGURE 1: (a) Stable cruciform molecules used to assay and purify CBP. Structure of the stable cruciform molecules pRGM21 X pRGM29 and C1 X C2. The 202 bp pRGM21 X pRGM29 cruciform has a stem−loop structure identical to that of the 68 bp C1 X C2 cruciform, but longer flanking arms. (b) Both pRGM21 X pRGM29 and C1 X C2 cruciforms share the same CBP band shift pattern. (Left) Migration of the band-shift assay using the CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. (Right) Migration of the band-shift assay using the same CBP as above with the C1 X C2 cruciform in a 6% polyacrylamide gel.

UV Cross-Linking. Protein−DNA complexes were formed for photochemical cross-linking (in volumes of 20−50 μL) essentially as they were for use in band-shift assays described elsewhere (*27*) with the following modifications. (1) Protein−DNA complexes were formed in 20 mM Tris-HCl (pH 7.5), 1 mM DTT, and 3% glycerol, in the absence of 1 mM EDTA. (2) The cruciform DNA was uniformly labeled with [α-^{32}P]dCTP. (3) Protein−DNA complexes were photochemically cross-linked for 5 min on ice, with reaction volumes placed on an aluminum foil-covered glass plate 2 cm from the UV light source. (4) The UV light source was a 15 W germicidal lamp (Fotodyne G15T8). (5) Following cross-linking, protein−DNA complexes were digested with 10 units of deoxyribonuclease I (DNase) and 15−20 units of micrococcal nuclease (both from Boehringer-Mannheim) in the presence of 10 mM $CaCl_2$ for 60 min at 37 °C. The resulting nuclease-digested protein−DNA complexes were then reduced using 5% β-mercaptoethanol and separated on 10% SDS−PAGE. After electrophoresis, the gels were dried and exposed for autoradiography. The pRGM21 X pRGM29 cruciform was used as a specific competitor, and the *Hin*dIII−*Sph*I fragment of the pRGM21 or pRGM29 plasmid

was used as a nonspecific competitor. Quantitation of ^{32}P-labeled bands was carried out using a Bio Image densitometer (MillGen/Biosearch).

CBP Purification. Briefly, an Affi-gel heparin column (Bio-Rad) flow through (F_{TH}) (*27*) from log phase HeLa S3 extracts was concentrated using Centriprep concentrators (30 000 molecular weight cutoff, Amicon) and lyophilization. This protein concentrate was used in several analytical and preparative scale band-shift reactions using the pRGM21 X pRGM29 cruciform, and run on 4% polyacrylamide gels. After electrophoresis, both protein−cruciform DNA complexes in which the cruciforms are specifically bound (*27*) were eluted from the gel as described previously (*28*). The combined eluates were precipitated using 4 volumes of ethanol, and the protein pellet was resuspended in distilled water to a concentration of 1−5 mg/mL.

Southwestern Analysis. Southwestern assays were carried out using a modification of the procedure described by Philippe (*33*). Approximately 5 μg of CBP purified by elution of protein−cruciform DNA complexes was separated on a 10% SDS−PAGE gel in the presence of 5% β-mercaptoethanol. The gel was electroblotted to Immobilon-P (Millipore) PVDF membrane in 192 mM glycine, 25 mM Tris base, and 0.01% SDS. Following transfer of the protein to the membrane, all manipulations were carried out at 4 °C while the mixture was gently rocked. The membrane was blocked overnight in binding buffer [25 mM Hepes/KOH (pH 7.7), 25 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT], 5% skim milk, and 0.05% NP40. Protein on the membrane was denatured using two 10 min incubations of 6 M guanidium hydrochloride followed by renaturation with five 10 min incubations of guanidine hydrochloride, each time halving the concentration (i.e., 3, 1.5, 0.75, 0.375, and 0.187 M). The final incubation was followed by two 10 min incubations in binding buffer, a 1 h incubation in binding buffer supplemented with 5% skim milk and 0.05% NP40, and a 30 min incubation in binding buffer supplemented with 1% skim milk and 0.05% NP40. ^{32}P-labeled pRGM21 X pRGM29 cruciform DNA (500−800 ng) was added at 2×10^5 cpm/mL in 5 mL of hybridization buffer [20 mM Hepes (pH 7.7), 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1% skim milk, and 0.05% NP40] containing 0.1 mg/mL double-stranded poly(dI-dC) (Pharmacia) and a 20-fold molar excess of the cold linear *Hin*dIII−*Sph*I fragment of pRGM21 DNA. Hybridization was carried out overnight followed by three 15 min washes with hybridization buffer and exposure of the membrane to a Fuji phosphoimager plate between two layers of plastic wrap.

Microsequence Analysis. CBP purified by elution of protein−cruciform DNA complexes was run on a 7% SDS−PAGE gel under reducing conditions and electroblotted to Problott membrane (Perkin-Elmer). The membrane was stained with 0.2% Ponceau-S in 1% acetic acid. Two bands, with apparent molecular masses of 50 and 70 kDa (see Results), were excised from the membrane, and sequence analysis was performed at the Harvard Microchemistry Facility with collisionally activated dissociation (CAD) mass spectroscopy on a Finnigan TSQ 700 triple-quadrupole mass spectrometer. The peptide sequences obtained were subjected to homology searches using the BLAST program (*34*).

Western Blotting. Three micrograms each of CBP purified by elution of protein−cruciform DNA complexes and purified sheep brain 14-3-3 (*32*) was separated per lane on a 10% SDS−PAGE gel in the presence of 5% β-mercaptoethanol. The gels were electroblotted to Immobilon-P (Millipore) PVDF membrane in 99 mM glycine, 12 mM Tris base, and 10% methanol. Membranes were blocked for 1 h in Blotto [5% skim milk, TBS [10 mM Tris-HCl (pH 8.0) and 150 mM NaCl], and 0.05% Tween 20], incubated with primary antibody in Blotto for 45 min, washed twice for 7 min in TBS-T (TBS and 0.05% Tween 20), and incubated in a 1:2000 dilution in Blotto of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) for 30 min, followed by six 5 min washes in TBS-T. Bands were visualized using enhanced chemiluminescence as prescribed by the ECL kit (Amersham). Specific antisera against isoforms of 14-3-3 proteins were raised in rabbits as described previously (*35*). The following antibodies were used. β_T 2042, specific for the 14-3-3 β isoform, was raised against the acetylated N-terminal sequence of the alternative start β isoform, Ac-TMDKSELV (diluted 1:2000). ECT 1116, specific for the C terminus of the 14-3-3 ϵ isoform, was raised against the C-terminal 17 residues of ϵ, GEEQNKEALQDVEDENQ (diluted 1:2000). γ1006, specific for the 14-3-3 γ isoform, was raised against an acetylated N-terminal peptide of the γ isoform, Ac-VDREQLVQKAC (diluted 1:6000). ζ 1002, reactive with the 14-3-3 ζ isoform with slight cross reaction with the 14-3-3 β isoform, was raised against an acetylated N-terminal peptide of the ζ isoform, Ac-MDKNELVQKAC (diluted 1:2000).

Immunolabeling. HeLa cells were grown in log phase in α-MEM supplemented with 10% FCS, 2 mM glutamine, 2 mM asparagine, and 50 units/mL penicillin/streptomycin on 22 mm coverslips (whole cell preparations) or 90 mm cell culture dishes (nuclear preparations). Cells grown for whole cell and isolated nuclei preparations were washed and fixed with paraformaldehyde as described previously (*36*). Cells grown for preparations of nuclei were further processed as described previously (*25*). Coverslips with whole cells were washed in PBS, fixed, and transferred to separate wells in a six-well cell culture plate prior to immunostaining. Both nuclei and whole cell preparations were immunostained as described previously (*36*), and mounted with antifade (Oncor). The use of primary antibodies is described above for Western blotting, with the following exceptions: ϵ_2 2025 (*35*), specific for the N terminus of the 14-3-3 ϵ isoform, raised in rabbits against an acetylated N-terminal peptide of the ϵ isoform, Ac-MDDREDLVYQAK (diluted 1:2000); and anti-actin antibody, H-196, from Santa Cruz (diluted 1:100). The secondary antibody used was fluorescein-linked goat anti-rabbit antibody (Oncor), diluted 1:50.

RESULTS

CBP had previously been partially purified from HeLa cell extracts using chromatographic techniques (*27*). The CBP activity is assayed by performing band-shift reactions with stable cruciform DNA molecules and partially purified HeLa cell extracts (Figures 1b and 3a; *27*). Two stable cruciforms, pRGM21 X pRGM29 (*27*, *37*) and C1 X C2 (see Experimental Procedures), which differ in the lengths of their flanking arms (Figure 1a), were used in the assays and purification of CBP. The band-shift patterns are the same for both cruciforms (Figure 1b).

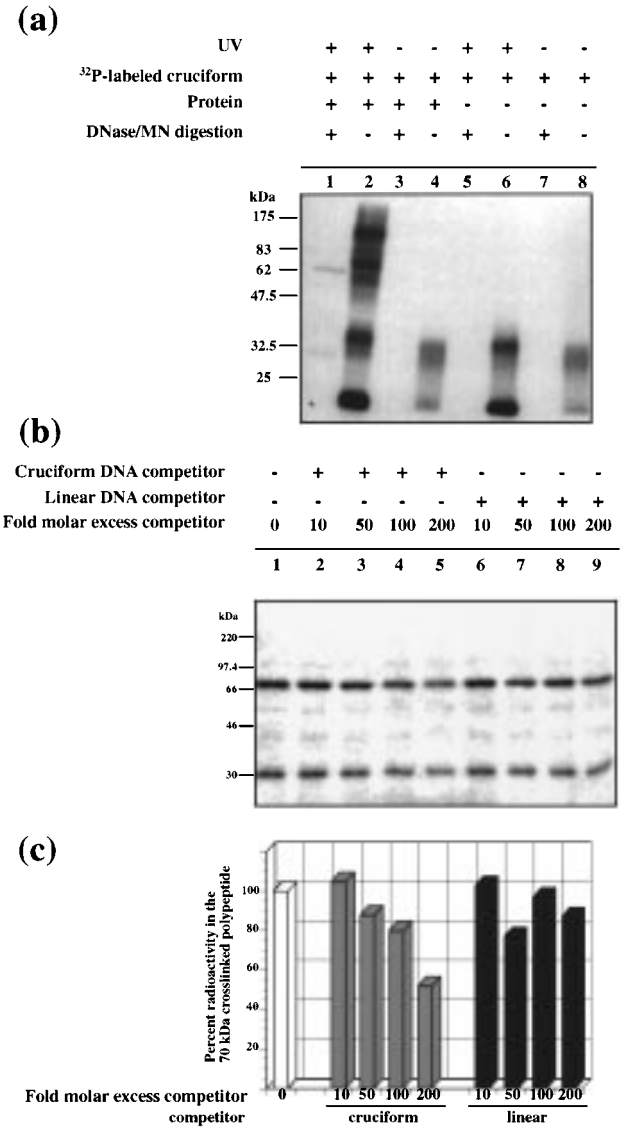

FIGURE 2: A 70 kDa polypeptide specifically cross-links to cruciform DNA. (a and b) Autoradiographs of UV-irradiated C1 X C2 cruciform−protein complexes (see Experimental Procedures) that have been separated on a 10% SDS−PAGE gel under reducing conditions. (b) The pRGM 21 X pRGM 29 cruciform was used as a specific competitor, and the *Hin*dIII−*Sph*I fragment of the pRGM 21 or pRGM 29 plasmid was used as a nonspecific competitor. The fold excess of each competitor is indicated. (c) The percent radioactivity of the 70 kDa polypeptide from the autoradiograph in panel b is expressed as a percentage relative to that of the cross-linking reaction without competitor (panel b, lane 1).

UV Cross-Linking. Photochemical cross-linking studies were carried out with the CBP−cruciform DNA complex. Uniformly ^{32}P-labeled cruciform (C1 X C2) DNA was complexed with CBP and irradiated with UV light to form covalent protein−DNA adducts. The mixture was then digested with nuclease to remove flanking DNA that was not covalently attached to photochemically cross-linked proteins. The resulting ^{32}P-labeled proteins were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) (Figure 2a,b). Polypeptides of approximately 70 and 30 kDa were cross-linked to the cruciform DNA (Figure 2a, lane 1). The ^{32}P -labeled products obtained were UV- and protein-dependent, as there are no nuclease-resistant bands present in the absence of either UV irradiation, protein (Figure 2a, lanes 3 and 5, respectively), or both (Figure 2a, lane 7). When a 100- or 200-fold molar excess of cold pRGM21 X pRGM29 cruciform DNA was included in the cross-linking reaction as a specific competitor, the intensity of the 70 kDa band was reduced by 20 and 48%, respectively (Figure 2b, lanes 4 and 5, and Figure 2c). In contrast, a 100-fold molar excess of cold pRGM21 or pRGM29 linear DNA of the same length and sequence did not compete for the ^{32}P-labeled 70 kDa band (Figure 2b, lane 8, and Figure 2c), while a 200-fold molar excess of the same competitor DNA competed for only 13% of it (Figure 2b, lane 9, and Figure 2c). A cross-linked polypeptide of approximately 30 kDa is also seen in this experiment (Figure 2a, lane 1, and Figure 2b), which appears to competed for by a specific competitor. However, when the data of several experiments are averaged, less than 25% of the 30 kDa polypeptide can be competed away using a 200-fold molar excess cruciform, as compared to an average 50% competition for the 70 kDa polypeptide. The 30 kDa polypeptide is also not detected by Southwestern analysis (Figure 3b). Therefore, we do not believe the 30 kDa polypeptide is interacting specifically with the cruciform DNA. These results indicate that the 70 kDa polypeptide is specifically cross-linked to the cruciform DNA and are consistent with the CBP activity sedimenting at approximately 66 kDa in the glycerol gradient, as reported previously (*27*).

CBP Purification and Sequencing. As a first step toward the identification of CBP, we further purified the activity by performing preparative scale band-shift assays using the pRGM21 X pRGM29 cruciform (Figure 1a) on polyacrylamide gels and elution from the gel of the two protein−cruciform DNA complexes (Figure 3a, bracket) in which the cruciforms are specifically bound (*27*).

Aliquots of CBP purified by elution from protein−cruciform DNA complexes were subjected to electrophoresis on reducing SDS−PAGE, electroblotted onto membrane, and probed with the ^{32}P-labeled pRGM21 X pRGM29 cruciform in a Southwestern assay (Figure 3b) to identify the polypeptides involved in the binding of cruciform DNA. Among several protein bands present in CBP eluted from protein−cruciform DNA complexes (see Figure 4a), a polypeptide of approximately 70 kDa (CBP1) was identified in this manner as interacting directly with the labeled cruciform DNA (Figure 3b, arrow). A second band of approximately 50 kDa (CBP2) was also found to interact with cruciform DNA on some Southwestern assays (not shown). The CBP1 and CBP2 polypeptides were subjected to tryptic digestion and microsequence analysis by collisionally activated dissociation mass spectrometry (CADMS). The HPLC profiles of the tryptic digests of CBP1 and CBP2 suggested that the two are related, with the possibility of CBP2 being a degradation product of CBP1. The sequences obtained (Figure 3c) indicate that both the 50 (CBP2) and 70 kDa (CBP1) polypeptides share common sequence with the 14-3-3 family of proteins. The peptide sequences of neither CBP1 nor CBP2 are homologous with any other proteins outside the 14-3-3 family of proteins. One of the tryptic peptides common to both CBP1 and CBP2 (CBP1-PT41 and CBP2-PT48, respectively, Figure 3c) is identical to the β and zeta ζ isoforms of 14-3-3 proteins. The β and ζ isoforms are identical in sequence to the α and δ isoforms, respectively, which are post-translationally modified by phosphorylation at Ser^{185} (*38*). Electrospray mass spectrometry

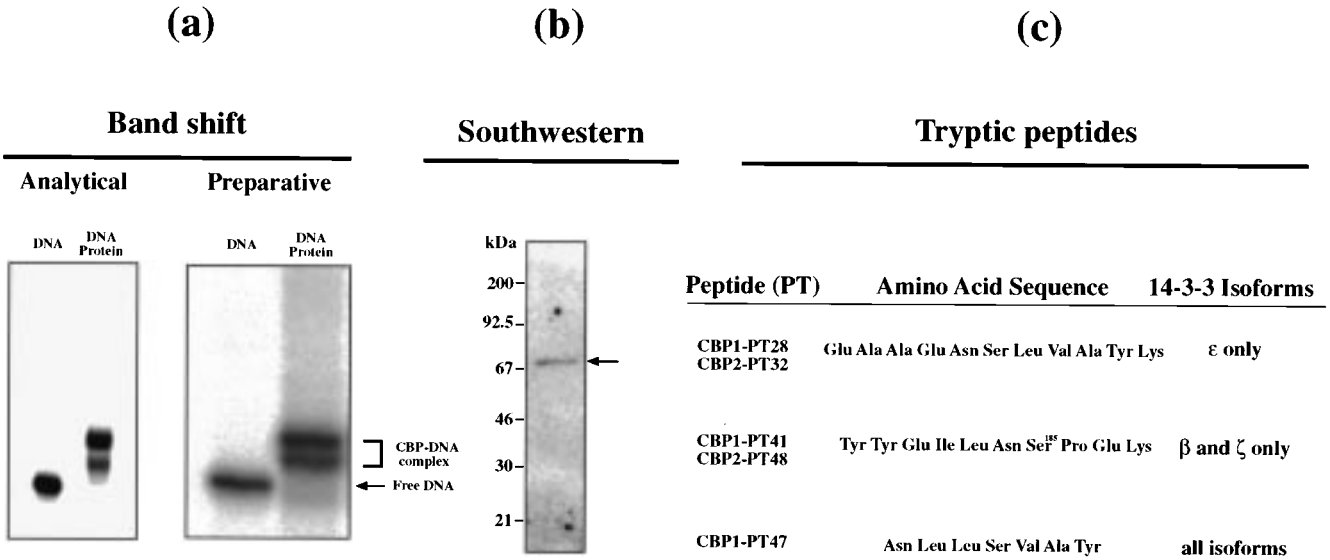

FIGURE 3: (a) (Left) Migration of analytical scale band-shift assays using the CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. (Right) Migration of preparative scale band-shift assays using the CBP activity recovered in the F_{TH} and the pRGM21 X pRGM29 cruciform in a 4% polyacrylamide gel. The position of the cruciform-bound complex is bracketed. Band-shift assays were performed as described previously (*27*) using the indicated amounts of ^{32}P-labeled DNA and protein. (b) Southwestern analysis of CBP eluted from cruciform DNA−protein complexes. The 70 kDa cruciform−DNA binding polypeptide is indicated by the arrow. (c) Sequence of tryptic peptides of the 70 (CBP1) and 50 kDa (CBP2) cruciform DNA-binding polypeptides. The position of Ser^{185} is indicated (see the text). All peptides show 100% identity with the indicated 14-3-3 protein isoforms.

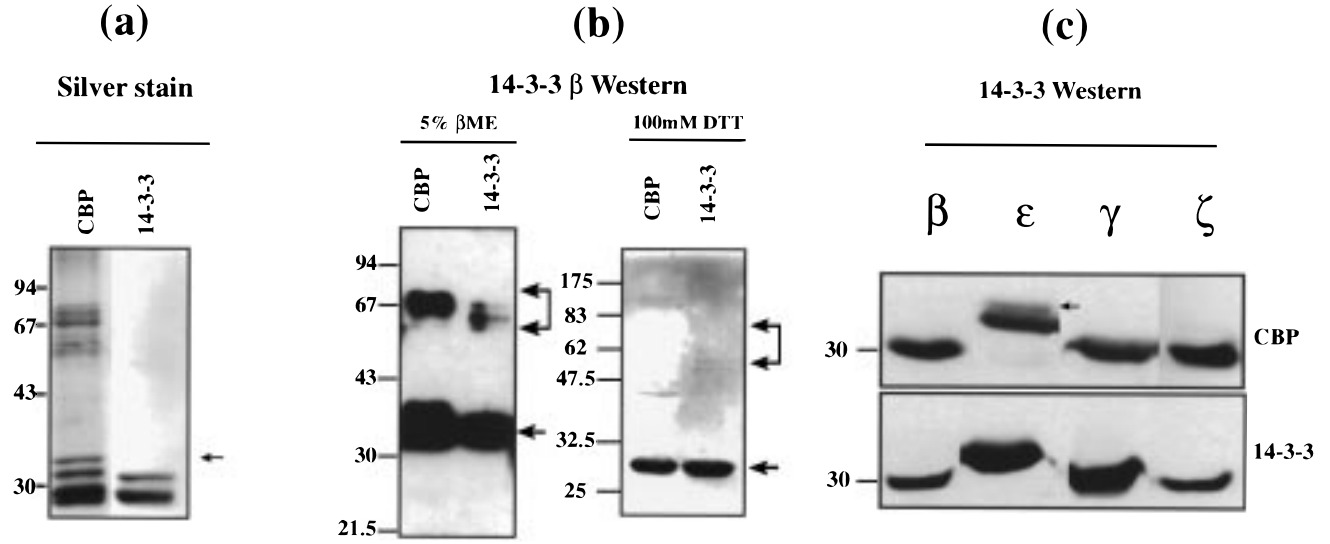

FIGURE 4: CBP contains a band with an apparent molecular mass of 35 kDa (arrow) not present in sheep brain 14-3-3 proteins. (a) Separation of 3 μg each of purified sheep brain 14-3-3 protein and CBP purified by elution from protein−cruciform DNA complexes (see the text) on a 10% SDS−PAGE gel using 5% β-mercaptoethanol as the reducing agent. The arrow indicates a 35 kDa band present in CBP but not clearly seen in sheep brain 14-3-3 proteins. (b) Anti-14-3-3 β Western blot of CBP purified by elution from protein−cruciform DNA complexes and 14-3-3 proteins purified from sheep brain. (Left) SDS−PAGE was carried out using 5% β-mercaptoethanol (5% βME) as the reducing agent. (Right) DTT (100 mM) was used as the reducing agent. The positions of the 14-3-3 monomer species are indicated by arrows, and the dimer species are indicated by brackets. (c) Western blots of CBP purified by elution from protein−cruciform DNA complexes and purified sheep brain 14-3-3 proteins. The Greek characters β, ϵ, γ, and ζ denote the antisera specific for each isoform of 14-3-3 proteins used on each lane of CBP and 14-3-3 proteins. The arrow indicates the presence of a 35 kDa band found in CBP but not in the sheep brain 14-3-3 proteins.

(ESMS) data show no indication of phosphorylation of CBP1-PT41 or CBP2-PT48 peptides, which include Ser^{185}, suggesting that these peptides originate from the unmodified β and/or ζ isoforms (data not shown). Phosphorylation at this site does not cause a shift in the apparent molecular mass on SDS−PAGE (*29*, *39*). The presence of the ϵ isoform in the CBP is confirmed by the sequence of the CBP1-PT28 and CBP2-PT32 peptides, which are 100% identical to a unique region of the ϵ sequence. Finally, a third peptide obtained from CBP1 (CBP1-PT47) is identical to a region common to all known 14-3-3 isoforms.

Comparison of Sheep Brain 14-3-3 Proteins to CBP. CBP was eluted from protein−cruciform DNA complexes, and compared by electrophoresis on 10% polyacrylamide gels (Figure 4a) to purified sheep brain 14-3-3 proteins, which is a mixture of all isoforms (*32*). In addition to bands with apparent mobilities of 30 and 33 kDa, which are characteristic of brain 14-3-3 monomers, CBP reveals a band with

an apparent mobility of 35 kDa (Figure 4a, arrow) not seen in purified sheep brain 14-3-3 proteins. There is also a series of higher-molecular mass bands visible in CBP corresponding to the dimer mass of 14-3-3 proteins (approximately 60−70 kDa, Figure 4a). These latter bands are recognized by 14-3-3 antibodies on an anti-14-3-3 β Western blot (Figure 4b, left panel). Bands of similar size are present in the 14-3-3 proteins purified from sheep brain (Figure 4b) but are not visible by silver staining (Figure 4a). These bands corresponding to the dimer molecular mass can be completely reduced when 100 mM DTT is used in place of 5% β-mercaptoethanol (Figure 4b, right panel). The CBP was identified as 14-3-3 on the basis of the sequence of proteins with approximate molecular masses of 50 and 70 kDa which interact with labeled cruciform DNA (see panels b and c of Figure 3). 14-3-3 proteins are known to form both homo- and heterodimers in vivo (*40*) and in vitro (*39*−*42*). Chemical cross-linking of 14-3-3 proteins also produces bands which migrate in this region on SDS−PAGE gels (*39*).

In Western analyses of SDS−PAGE gels run under reducing conditions, the CBP purified by elution from protein−cruciform DNA complexes is recognized by antibodies specific for the β, γ, and ϵ isoforms, as well as by an antibody which recognizes the ζ isoform and cross reacts with the β isoform (Figure 4c, CBP, lanes β, ϵ, γ, and ζ). Furthermore, the 35 kDa band (Figure 4a, arrow) is recognized by an antibody that is specific for the C terminus of the ϵ isoform (Figure 4c, CBP, lane ϵ, arrow). A band with an apparent mobility of 35 kDa is not seen in the sheep brain 14-3-3 proteins (Figure 4c, 14-3-3, lane ϵ) or in 14-3-3 proteins from a large number of other tissues (*43*). However, a faint band at 35 kDa can often be seen on SDS−PAGE of heavily loaded purified brain protein after HPLC purification (unpublished results). There is a specific variant of the ϵ isoform found in hematopoietic cells of a wide range of mammalian species, which contains a single amino acid change from the ϵ isoform found in sheep, rat, and human placenta and brain (*44*).

Band-shift analysis of purified sheep brain 14-3-3 proteins with the pRGM21 X pRGM29 stable cruciform reveals cruciform-specific binding activity (Figure 5). When a 50- or 100-fold molar excess of cold specific (cruciform) competitor is added to the sheep brain 14-3-3 band-shift reaction mixture, the cruciform-specific binding activity is competed for by 66 and 87%, respectively (Figure 5, lanes 8 and 9). In contrast, a 50- or 100-fold molar excess cold linear competitor has no effect on the sheep brain 14-3-3 cruciform binding activity (Figure 5, lanes 5 and 6). The migration of the sheep CBP−cruciform complexes is retarded relative to that of the HeLa CBP−cruciform complexes, possibly reflecting the species and tissue differences in the protein sources.

Detection of 14-3-3 Isoforms in HeLa Cell Nuclei. The subcellular distribution of 14-3-3 proteins was analyzed by immunofluorescence studies performed on whole HeLa cells (Figure 6a) and on isolated HeLa cell nuclei (Figure 6b). Rabbit polyclonal antibodies specific for the β, ϵ, and γ isoforms, as well as an antibody that recognizes the ζ isoform and cross reacts with β, show uniform extents of labeling of whole HeLa cells fixed in paraformaldehyde (Figure 6a, β, ϵ, γ, and ζ, respectively). Due to the possibility that the intensity of the signal generated by the cytosolic 14-3-3 proteins may be obscuring the nuclear signal, islolated HeLa nuclei were stained in a similar manner. Antibodies specific for the β and ϵ isoforms, as well as an antibody that recognizes the ζ isoform and cross reacts with β, show nuclear labeling in isolated HeLa nuclei (Figure 6b, β, ϵ, and ζ, respectively). Antibodies directed toward the γ isoform may also show faint nuclear labeling (Figure 6b, γ); the detection of the γ isoform in the cruciform−protein complex (Figure 4c) lends support to the likelihood of a nuclear presence. The control antibody directed toward the cytoplasmic protein actin shows no labeling in the isolated HeLa nuclei (Figure 6b, panel A).

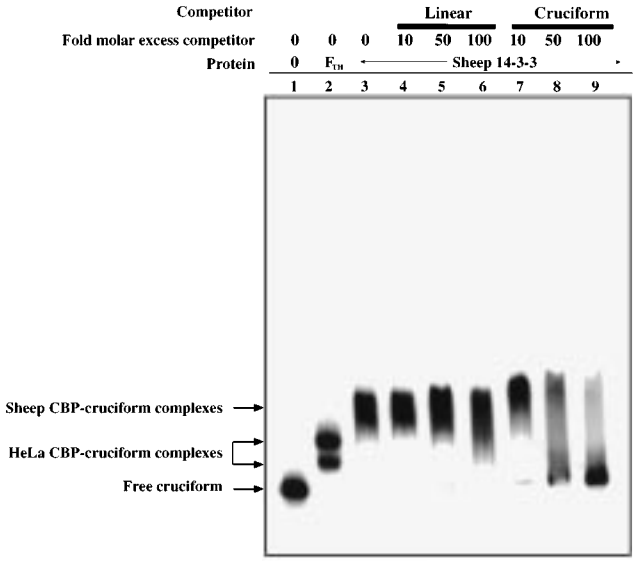

FIGURE 5: Sheep brain 14-3-3 proteins have CBP activity. Migration on a 4% polyacrylamide gel of 14-3-3 proteins subjected to competition band-shift analysis. Lane 1 shows the migration of the labeled pRGM21 X pRGM29 cruciform in the absence of protein. CBP activity recovered from a heparin column flow through (F_{TH}, see Experimental Procedures) is shown in lane 2. Lanes 4−7 show the CBP activity present in 14-3-3 proteins purified from sheep brain. The *Hin*dIII−*Sph*I fragment of the pRGM21 or pRGM29 plasmid was used as a linear nonspecific competitor (lanes 4−6), and the pRGM21 X pRGM29 cruciform was used as a specific competitor (lanes 7−9). The migration positions of free cruciform, HeLa CBP−cruciform complexes, and sheep CBP−cruciform complexes are indicated.

DISCUSSION

Cruciform structures, which can form transiently in vivo from IR sequences (*20*−*26*), have been shown to be involved in the initiation of DNA replication (*18*, *21*, *24*−*26*).

We have previously isolated and partially purified from HeLa cell extracts a novel cruciform-specific binding protein (CBP) which sedimented on a glycerol gradient with an apparent molecular mass of 66 kDa (*27*). Hydroxyl radical analysis of the protein−DNA interaction revealed binding of the protein at the base of the four-way junction, making contacts with the sugar−phosphate backbone, and inducing structural alterations in the DNA (*28*).

In this study, we have estimated the molecular mass of the CBP to be approximately 70 kDa by photochemically cross-linking partially purified HeLa cell extracts to uniformly labeled cruciform DNA (Figure 2). The 70 kDa band is specifically cross-linked to cruciform DNA, as a 200-fold

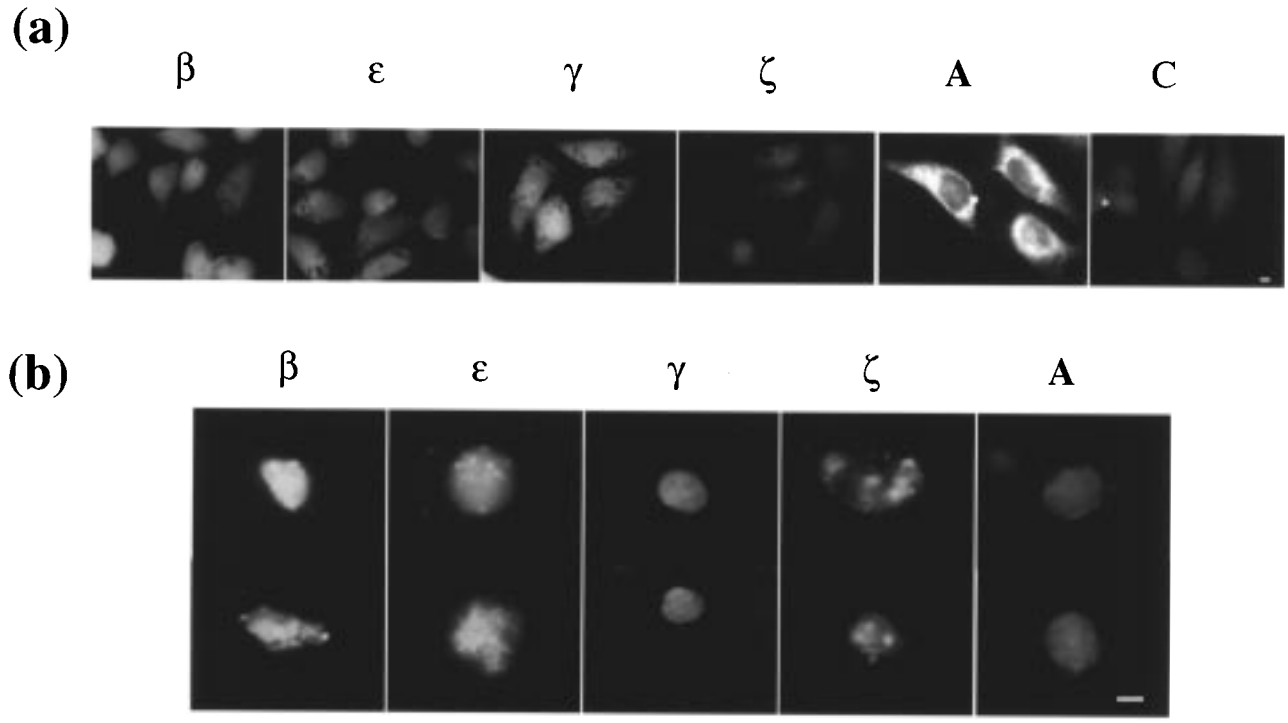

FIGURE 6: Nuclear localization of 14-3-3 proteins. HeLa whole cell preparations (a) and isolated nuclei (b) were immunostained using polyclonal rabbit antisera specific for four 14-3-3 isoforms (indicated along the tops of each panel) and for the cytoplasmic protein actin (labeled with A). C in panel a denotes a negative control prepared without primary antibody. White bars in boxes C (panel a) and A (panel b) represent 1 μm.

molar excess of cold crucifrom DNA can compete with it, but not with an equivalent amount of cold linear DNA with the same sequence (Figure 2b,c). We have purified CBP on the basis of two functional assays. The first assay is a band-shift assay where the cruciform−CBP complexes (Figure 3a, bracket) are eluted from the native band-shift gel. In the second assay, the protein eluted from the band-shift gel is again subjected to a cruciform binding assay in the form of a Southwestern assay. The Southwestern assay identifies a polypeptide of 70 kDa that is interacting with ^{32}P-labeled cruciform DNA in the presence of excess nonspecific competitor DNA (Figure 3b). A second polypeptide of approximately 50 kDa is also occasionally found to interact with cruciform DNA in the Southwestern assay, and is thought to be a degradation product of the 70 kDa polypeptide by virtue of its identical amino acid sequence in two different peptides (Figure 3c). Sequence analyses of the tryptic peptides of the two CBP polypeptides of approximately 70 and 50 kDa reveal 100% identity to isoforms of the 14-3-3 family of proteins (Figure 3c).

Electrophoretic analysis of the CBP isolated from the CBP−cruciform complex on reducing SDS−PAGE gels reveals three bands corresponding to the monomer size of 14-3-3 proteins (30, 33, and 35 kDa, respectively; see below) as well as a series of bands of 60−70 kDa, corresponding to the dimer molecular mass of the 14-3-3 proteins (Figure 4a). These dimer bands, although not visible by silver staining, are also present in a lesser amount in 14-3-3 proteins purified from sheep brain (Figure 4b). In addition to the monomer and dimer 14-3-3 bands, CBP contains a series of bands of approximately 50−60 kDa which are not recognized by 14-3-3 antibodies (Figure 4a,b). These bands are thought to be degradation products of the dimeric 14-3-3 proteins (such as the 50 kDa CBP2 polypeptide) and therefore may not be recognized by the 14-3-3 β antibody used in Figure 4b, since it is directed against the acetylated N terminus of the 14-3-3 protein.

Western analyses of the CBP isolated from two different CBP−cruciform complexes (using either the pRGM21 X pRGM29 or C1 X C2 cruciform, Figure 1a) reveal the presence of the β, ϵ, γ, and ζ isoforms of 14-3-3 proteins (Figure 4c).

Our observation of at least four different isoforms of 14-3-3 proteins interacting with cruciform DNA is not suprising given the high degree of conservation between isoforms of 14-3-3 proteins, both in primary structure (*29*, *30*) and at the level of three-dimensional structure (*41*, *42*). In addition, the phosphoserine binding affinity for phosphorylated Raf-1 peptides is the same for several isoforms (*45*). Other 14-3-3-interacting proteins, such as the apoptotic promoter BAD, lack isoform selectivity and interact with multiple isoforms of 14-3-3 proteins (*46*). The apoptotic inhibitor A20 also interacts with several isoforms, although there is a preference for the η isoform (*47*). Furthermore, the ϵ and ζ isoforms show redundancy in their effects on Ras signaling in *Drosophila* (*48*). This overlap in function between isoforms appears to transcend species, since four isoforms of 14-3-3 proteins from the plant *Arabidopsis thaliana* are able to complement the lethal double disruption of the *Saccharomyces cerevisiae* 14-3-3 homologues *bmh1* and *bmh2* (*49*).

The results presented here show that the CBP, although containing identical sequence, differs in some aspects from the purified sheep brain 14-3-3 proteins (Figure 4a,c). One notable difference is the presence in CBP of a polypeptide with an apparent molecular mass of 35 kDa (Figure 4a, arrow), which is recognized by antibodies specific for the ϵ isoform of 14-3-3 proteins (Figure 4c, arrow) and is not usually seen in sheep brain 14-3-3 proteins, although a faint

band at 35 kDa is often observed on HPLC of heavily loaded purified brain proteins (unpublished results).

Band-shift competition analysis of 14-3-3 proteins purified from sheep brain (*32*) reveals cruciform-specific binding activity (Figure 5). The migration of the sheep brain 14-3-3 protein−cruciform complex is retarded relative to that of the CBP−cruciform complex from HeLa cells, reflecting potential differences between CBP from HeLa cells and 14-3-3 proteins from sheep brain (Figure 4). The purification protocols for the two proteins also differ (*27*, *32*). The nonproliferative status of brain tissue might also contribute to the differences we have noted between the purified sheep brain 14-3-3 proteins and the CBP (Figures 4 and 5).

Preliminary studies indicate that the CBP activity is regulated with the cell cycle and is maximal at the G1/S boundary but absent at G_o (unpublished data). This pattern correlates with the profile of cruciform distribution in the cell cycle (*25*).

The crystal structures of 14-3-3 proteins (*41*, *42*) reveal highly helical proteins that dimerize. The dimer forms a large channel which is lined by amino acids that are very highly conserved throughout the family. Interestingly, the described structure of members of the 14-3-3 family (*41*, *42*) is similar to the model previously proposed for the CBP−cruciform complex (*28*).

This is the first report of a mammalian 14-3-3 protein with DNA binding activity. 14-3-3 protein homologues in the plants *A. thaliana* and maize participate in DNA−protein complexes (*50*, *51*). These plant 14-3-3 homologues are part of a complex that binds a G-box promoter element of inducible genes and are thought to regulate transcription. However, they do not bind directly to DNA. The sequence of the plant G-box promoter, 5′-CCACGTGG-3′, is not present in either of the cruciform molecules used in the isolation of CBP, nor does CBP bind DNA in a sequence-dependent manner (*27*). The nuclear localization of the plant 14-3-3 homologues has been confirmed in *A. thaliana* and *Zea mays* by scanning laser microscopy and immunocytochemistry (*52*).

Mammalian 14-3-3 proteins have previously been described as cytosolic with a small proportion of brain 14-3-3 proteins tightly associated with some membranes (*53*). The ϵ and γ isoforms have also been shown to bind the cytoskeleton (*54*), as well as centrosomes and spindle apparatus (*44*). The association of various isoforms of 14-3-3 proteins with several different signaling molecules known to interact at or near the cell membrane (see refs *55* and *56* for reviews) also suggests a cytoplasmic location. Cell fractionation studies of rat brain have provided indirect evidence for the nuclear localization of 14-3-3 proteins (*53*). Furthermore, the presence of 14-3-3 proteins in mammalian nuclei is supported by a recent report which documents the p53 protein interacting with 14-3-3 proteins following exposure to ionizing radiation in nuclear extracts from several cell lines (*57*). The authors also show that the p53−14-3-3 interaction increases the affinity of p53 for sequence-specific DNA binding. The CBP activity is enriched at least 4-fold in HeLa cell nuclei as determined by quantitative band-shift assays (not shown). Immunostaining analyses performed here demonstrate the presence of the β, ϵ, ζ, and possibly γ isoforms in HeLa cell nuclei, in both whole cells and isolated HeLa nuclei (Figure 6). These immunolocalization studies now provide direct evidence for the presence of at least three isoforms of 14-3-3 proteins in HeLa cell nuclei. The nuclear localization of several 14-3-3 isoforms supports the hypothesis that the HeLa cruciform-specific binding protein, CBP, recognizes cruciform structures in nuclei.

A wide array of unrelated functions have been ascribed to 14-3-3 proteins (*29*, *55*, *56*, *58*). These proteins associate with a number of oncogene and proto-oncogene products, including middle T antigen of polyoma, Raf-1, and Bcr-Abl (*29*, *55*, *56*). Although 14-3-3 proteins have been convincingly shown to associate with signaling molecules, the functional role of these proteins in these signaling pathways remains unsolved. However, the recent description of two 14-3-3 consensus binding motifs, RSXpSXP and RXY/FXpSXP, where pS is phosphoserine, may explain why so many diverse proteins associate with 14-3-3 proteins (*45*, *59*). Many of the 14-3-3-interacting proteins contain one or more of these consensus binding motifs, and 14-3-3 proteins have been documented to interact with many proteins through one or more of these phosphoserine-containing consensus sites. Some of these 14-3-3-interacting proteins are involved in processes such as apoptosis, through interactions with the BAD protein (*46*, *60*); cell cycle regulation, by interacting with the Cdc25 (*61*−*63*) and A20 proteins (*47*); and signal transduction, by interacting with the Raf-1 protein, among others (*64*, *65*). The nature of interaction with 14-3-3 proteins is determined by the extent to which the binding motif(s) matches the consensus, and the nature of this interaction determines whether 14-3-3 proteins will act as an adapter, chaperone, or sequestering molecule (*59*). In addition, a second site for binding to 14-3-3 proteins exists and confers isoform specificity of interaction. We have shown, for example, that phosphorylation of 14-3-3 ζ Thr^{233} affects the interaction with Raf (*66*). These findings, along with the crystallographic data (*41*, *42*), point strongly toward a role of a scaffold or stabilizing protein for 14-3-3 proteins. A 14-3-3 protein that functions in such a manner would be ideally suited for recognizing cruciforms at origins of replication, to serve as a mediator in signaling pathways essential to DNA replication and cell cycle progression. 14-3-3 proteins participate in cell cycle control at the level of the DNA damage checkpoint in the budding yeast *Schizosaccharomyces pombe* (*67*). The gene products of the *rad24* and *rad25* genes are 14-3-3 homologues and participate in the radiation damage control checkpoint which prevents cells from entering mitosis after radiation-induced DNA damage. Cells carrying mutations in the *rad24* gene enter mitosis prematurely, suggesting a link between the DNA damage control checkpoint and the cell cycle machinery (*67*). 14-3-3 proteins are also involved in cell cycle regulation at the G2 checkpoint through the interaction with human cdc25 phosphatases in vivo and in vitro (*61*). Recent evidence shows that the 14-3-3 σ isoform can inhibit G2/M progression in a p53-dependent manner (*68*).

In summary, we have demonstrated here that the human cruciform binding protein (CBP) is a nuclear 14-3-3 protein, composed of permissible combinations of four of the known 14-3-3 isoforms, but exhibits characteristics different from those of purified sheep brain 14-3-3 proteins.

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