The Human Cruciform-binding Protein, CBP, Is Involved in DNA Replication and Associates in Vivo with Mammalian Replication Origins*

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We previously identified and purified from human (HeLa) cells a 66-kDa cruciform-binding protein, CBP, with binding specificity for cruciform DNA regardless of its sequence. DNA cruciforms have been implicated in the regulation of initiation of DNA replication. CBP is a member of the 14-3-3 family of proteins, which are conserved regulatory molecules expressed in all eukaryotes. Here, the in vivo association of CBP/14-3-3 with mammalian origins of DNA replication was analyzed by studying its association with the monkey replication origins ors8 and ors12, as assayed by a chromatin immunoprecipitation assay and quantitative PCR analysis. The association of the 14-3-3β, -ε, -γ, and -ζ isoforms with these origins was found to be ~9-fold higher, compared with other portions of the genome, in logarithmically growing cells. In addition, the association of these isoforms with ors8 and ors12 was also analyzed as a function of the cell cycle. Higher binding of 14-3-3β, -ε, -γ, and -ζ isoforms with ors8 and ors12 was found at the G1/S border, by comparison with other stages of the cell cycle. The CBP/14-3-3 cruciform binding activity was found to be maximal at the G1/S boundary. The involvement of 14-3-3 in mammalian DNA replication was analyzed by studying the effect of anti-14-3-3β, -ε, -γ, and -ζ antibodies in the in vitro replication of p186, a plasmid containing the minimal replication origin of ors8. Anti-14-3-3ε, -γ, and -ζ antibodies alone or in combination inhibited p186 replication by ~50–80%, while anti-14-3-3β antibodies had a lesser effect (~25–50%). All of the antibodies tested were also able to interfere with CBP binding to cruciform DNA. The results indicate that CBP/14-3-3 is an origin-binding protein, acting at the initiation step of DNA replication by binding to cruciform-containing molecules, and dissociates after origin firing.

Inverted repeat sequences (IRs)1 are a common feature of prokaryotic and eukaryotic regulatory regions, and their distribution is nonrandom and clustered (1). Promoters (2, 3), terminators (4), amplified genes (5), and origins of DNA replication from prokaryotes (6–8), viruses (9), and eukaryotes (10) all contain IRs. Origins of DNA replication from higher eukaryotes, such as monkeys and humans (11–15), are also enriched in IRs. IRs have been implicated in the regulation of initiation of DNA replication in plasmids, bacteria, eukaryotic viruses, and mammals (reviewed in Ref. 16). IRs have the potential to form cruciform structures (stem loop or hairpin) through intrastrand base pairing and under conditions of torsional strain on the DNA (reviewed in Refs. 16–18). Such cruciform structures have been shown to form in vivo (reviewed in Ref. 16) in prokaryotes and lower eukaryotes (19–22), and in mammalian cells (23–25). Such structures are thought to serve as recognition signals for regulatory proteins involved in critical cellular processes such as transcription, recombination, and DNA replication (reviewed in Refs. 16 and 18).

We and others have previously demonstrated the involvement of cruciform structures in the initiation of mammalian DNA replication (20, 23–25) (reviewed in Ref. 16) We previously identified and isolated from human cells (HeLa) a cruciform-specific binding protein, CBP (26), of 66 kDa with binding specificity for cruciform-containing DNA (26). CBP binds at the base of four-way junctions (27), interacting with them in a different manner from other proteins known to bind such junctions (26, 27).

CBP is a member of the 14-3-3 protein family, a highly conserved family (28) of proteins through plants, invertebrates, and higher eukaryotes (reviewed in Ref. 29). 14-3-3 proteins are multifunctional, involved in diverse cellular processes, such as neurotransmitter biosynthesis, signal transduction pathways, and cell cycle control (reviewed in Ref. 30). 14-3-3-associated proteins include receptors (31), kinases (32), phosphatases (33), docking molecules (34), death regulators (35), and oncogene products (36). The interaction of CBP/14-3-3 with cruciform structures is functionally important for the regulation of DNA replication. Immunofluorescence studies using anti-cruciform DNA monoclonal antibodies showed that cruciforms are at a maximum number at the G1/S boundary (24, 25). The same antibodies had an enhancing effect on DNA replication in mammalian cells, presumably by stabilizing cruciform structures (23) and increasing DNA synthesis through continuing protein-protein interactions by signaling pathways essential to DNA replication.

Our laboratory has also previously isolated origin-enriched sequences (ors) from early replicating monkey kidney (CV-1) cells (37–39), which are capable of conferring autonomous replication to plasmids in vivo (14, 40) and in vitro (41). In vivo mapping of ors12 by competitive PCR demonstrated that it acts

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1 The abbreviations used are: IR, Inverted repeat sequence; ors, origin-enriched sequence(s); NRS, normal rabbit serum; FACS, fluorescence-activated cell sorting.
as a chromosomal replication origin (42). Among the ors, ors8 and ors12 have been characterized in detail. They both contain inverted repeats that give rise to a cruciform structure (43) (reviewed in Ref. 39). Deletion analysis of ors8 and ors12 revealed that the minimal sequences required for origin function (186 bp for ors8 (44) and 215 bp for ors12 (45)) retain an intact IR. The IR present in ors8 is capable of extruding into a cruciform both in vivo (23) and in vitro (16, 46).

In the present study, we analyzed the cruciform binding activity of CBP/14-3-3 as a function of the cell cycle, its association in vivo with replication origin-containing sequences (exemplified by ors8 and ors12), and its involvement in mammalian DNA replication. The in vivo binding of CBP/14-3-3 to ors8 and ors12 was analyzed using the formaldehyde cross-linking technique (47), followed by immunoprecipitation of protein-DNA cross-links with antibodies against the 14-3-3β, -ε, -γ, and -ζ isoforms. PCRs were then performed using the immunoprecipitated material as template. CBP/14-3-3 was found to associate specifically with ors8 and ors12, since DNA fragments containing these ors were enriched in the immunoprecipitate compared with other portions of the genome. Furthermore, higher binding of 14-3-3β, -ε, -ζ, and -γ isoforms to ors8 and ors12 was found at the G1/S border by comparison with other stages of the cell cycle. The cruciform binding activity of CBP was also found to be maximal at the G1/S interphase. In addition, the anti-14-3-3β, -ε, -γ, and -ζ antibodies were able to interfere with CBP-cruiform complex formation and inhibit the in vitro DNA replication of p186. The anti-14-3-3ε, -γ, and -ζ antibodies had a greater inhibitory effect on the in vitro DNA replication of p186 DNA than did the anti-14-3-3β antibody. The data suggest an involvement of CBP in mammalian DNA replication as an origin-binding protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—HeLa (S3) cells growing in suspension were synchronized by serum starvation and treatment with aphidicolin 2 μg/ml (23) and mimosine 400 μM (48). CV-1 cells growing in monolayers were cultured in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) (termed “regular medium”) at 37 °C, as previously described (49). For synchronization to the G1/G0 phase, 80% confluent CV-1 cells were placed in serum-free medium for 48 h. For synchronization to G1/S, S (50), and M (51) phases, the procedure was modified as follows: 40% confluent CV-1 cells were treated with 2 mM thymidine (Sigma) for 12 h and then released in regular medium without thymidine and subsequently incubated for 12 h with 2 mM thymidine and 400 μM mimosine (Sigma). For S phase synchronization, the cells were released from the thymidine/mimosine block for 4 h in regular medium. For synchronization to M phase, the cells were released from the thymidine/mimosine block in regular medium supplemented with 1 μg/ml of nocodazole (Sigma), for 14 h. Cell synchronization was monitored by flow cytometry.

In Vivo Cross-linking—In vivo cross-linking was performed as described by Ritzi et al. (52) with some modifications. In brief, CV-1 cells, grown as previously described, were washed twice with phosphate-buffered saline to remove all traces of serum, and then formaldehyde (1%) in warm minimal essential medium α (Invitrogen) (termed “regular medium”) at 37 °C, as previously described (49). For synchronization to the G1/G0 phase, 80% confluent CV-1 cells were placed in serum-free medium for 48 h. For synchronization to G1/S, S (50), and M (51) phases, the procedure was modified as follows: 40% confluent CV-1 cells were treated with 2 mM thymidine (Sigma) for 12 h and then released in regular medium without thymidine and subsequently incubated for 12 h with 2 mM thymidine and 400 μM mimosine (Sigma). For S phase synchronization, the cells were released from the thymidine/mimosine block for 4 h in regular medium. For synchronization to M phase, the cells were released from the thymidine/mimosine block in regular medium supplemented with 1 μg/ml of nocodazole (Sigma), for 14 h. Cell synchronization was monitored by flow cytometry.

Chromatin Fragmentation—Cross-linked or non-cross-linked cell nuclei were sonicated 10 times for 30 s each time, and the chromatin size was monitored by electrophoresis (53). This treatment generated fragments of ~20 kb. To further reduce the chromatin size into smaller fragments of 1.5–3.5 kb, DNA was digested with SphiI, HindIII, PstI, and EcoRI restriction endonucleases in NE82 buffer (100 units of each; New England Biolabs) at 37 °C for 6 h. These enzymes were chosen because they did not cut in either the origin or non-origin-containing sequences chosen.

Immunoprecipitation and DNA Isolation—Sheared chromatin lysed extracts were incubated with 50 μl of protein A-agarose (Roche Molecular Biochemicals) to reduce background caused by nonspecific adsorption of irrelevant cellular proteins/DNA to protein A-agarose beads (as described below). These cleared chromatin lysates were incubated, at 4 °C for 6 h on a rocker platform, with 50 μl of preimmune rabbit serum (Santa Cruz Biotechnology, Inc.) and 20 μg of anti-14-3-3ε (sc-1020), anti-14-3-3β (sc-628), anti-14-3-3ε (sc-1019), anti-14-3-3γ (sc-731) rabbit polyclonal antibodies (Santa Cruz Biotechnology), anti-NF-κB p65 (C-20) goat polyclonal antibody (Santa Cruz Biotechnology), or anti-SC-35 (Sigma) rabbit monoclonal antibody. 50 μl of protein A-agarose, or protein G-agarose for anti-NF-κB p65 antibody, was added, and the incubation was continued for 12 h. The precipitates were successively washed twice for 5 min with 1 ml of each buffer: lysis buffer, WB1 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate), WB2 (as WB1 with no NaCl), and 1 ml of TE (20 mM Tris-HCl, pH 8.0, 1 mM EDTA). The precipitates were finally resuspended in 200 μl of extraction buffer (1% SDS/TE). The samples were then incubated at 65 °C overnight to reverse the protein/DNA cross-links, followed by 2 h of incubation at 37 °C with 100 μg of proteinase K (Roche Molecular Biochemicals). Finally, the samples were processed for DNA purification by passing them through QiAquick PCR purification columns (Qiagen).

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CBP Cruciform Binding Activity as a Function of the Cell Cycle—HeLa cells growing in suspension were synchronized by serum starvation and treatment with aphidicolin or dansamino (as described above). Extracts were prepared as before (41) from arrested and released cells as well as cells in G0 and phase (see FACS analysis, Fig. 5B). Bandshift reactions were performed with 32P-labeled isolated cruciform (pRGM21; Ref. 25), on ice for 15 min, with an equal number of control reaction. The specificity of these antibodies was assayed by blocking peptide analysis (data not shown). As negative control, antibodies against the transcription factor NF-κB p65 (56), a DNA-binding protein that does not associate with origins of replication, the spliceosome-specific protein, SC-35, a nuclear protein that does not bind DNA (57), or the NRS was used. Western blot analyses showed that CV-1 whole-cell extracts (CV-1 WCE), prepared from formaldehyde-treated or -untreated cells, contained the 14-3-3β, -ε, -γ, and -ζ isoforms (Fig. 1, A–D, lanes 1 and 2), the NF-κB p65, and SC-35 proteins (58), respectively. In contrast, when NRS was used, none of these proteins were immunoprecipitated in either the formaldehyde-treated or -untreated CV-1 cells (Fig. 1, A–D, lane 9). Western blot analyses using anti-14-3-3β, -ε, -γ, and -ζ antibodies verified that the immunoprecipitated material from the cross-linked cells (logarithmically growing or synchronized at G0, G1/S, S, and M phases of the cell cycle) did contain the respective 14-3-3 isoforms (Fig. 1, A–D, lanes 3–5, and 8), although at very low levels in G0 cells (Fig. 1, A–D, lane 5), and that formaldehyde cross-linking did not affect the immunoprecipitation of 14-3-3 isoforms, since equivalent amounts of 14-3-3β, -ε, -γ, and -ζ were immunoprecipitated in both cross-linked and untreated log phase cells (Fig. 1, A–D, lanes 3 and 4). Western blot analyses using anti-SC-35 antibody showed that the material immunoprecipitated from cross-linked cells with anti-SC-35 antibody contained ∼10 times less SC-35 protein than the untreated cells (Fig. 1, D and E, of Ref. 58), whereas...
Western blots performed with the anti-NF-κB p65 antibody showed that the immunoprecipitated material contained equivalent NF-κB p65 protein in both the formaldehyde-treated and untreated cells (Fig. 1, D and E, of Ref. 58).

Quantitative PCR with Template DNA from Immunoprecipitation with Anti-14-3-3β, -ε, -γ, or -ζ Isoforms, anti-NF-κB p65, anti-SC-35, or NRS—To analyze whether the DNA that was immunoprecipitated with the anti-14-3-3 antibodies, after cross-linking with formaldehyde, was enriched in origin-containing sequences and in order to quantify this association, real time PCR quantification was performed using the LightCycler instrument (Roche Molecular Biochemicals), utilizing specific primer sets for ors8 and ors12 (ors8 150 and ors12 D2) and primer sets specific for non-origin-containing sequences (EE' and CD4 intron) (Fig. 2A). Genomic CV-1 DNA was used to build the standard curves necessary for the quantification of the immunoprecipitated DNA in different genomic regions (Fig. 2B). In logarithmically growing CV-1 cross-linked cells, the DNA obtained by immunoprecipitating with anti-14-3-3β, -ε, -γ, and -ζ antibodies was enriched in ors8 and ors12 sequence by ~7- and 5-fold (for 14-3-3β), 8- and 11-fold (for 14-3-3ε), 11- and 9-fold (for 14-3-3γ), and 9- and 14-fold (for 14-3-3ζ), respectively, when primer sets ors8 150 and ors12 D2 were used, in comparison with primer set EE', which amplifies a sequence situated ~5 kb downstream of ors12 (Fig. 3A). In contrast, the DNA brought down by anti-NF-κB p65, anti-SC-35, or NRS antibodies, obtained from logarithmically growing cross-linked cells, in origin regions ors8 and ors12, corresponded to the DNA abundance found in the non-origin-containing sequence amplified by primer set EE' (~4 × 10^{10} molecules) (Fig. 3A). The DNA abundance in the non-origin-containing sequences, amplified by primer sets EE' and the CD4 intron, when the immunoprecipitation was performed with either anti-14-3-3β, -ε, -γ, or -ζ isoforms, anti-NF-κB, anti-SC-35 antibodies, or NRS, also corresponded to ~4 × 10^{10} molecules (background levels of DNA/1.5 × 10^{13} cross-linked CV-1 cells), which was considered to be the DNA that was brought down nonspecifically with anti-NF-κB, anti-SC-35 antibodies, or NRS, presumably as a result of the cross-linking (Fig. 3A). To verify that the PCR product generated by amplification, with the respective primer set (ors8 150, ors12 D2, EE', or CD4 intron) in the LightCycler instrument (Roche Molecular Biochemicals), using genomic CV-1 DNA as template, was amplified from sequences situated ~5 kb upstream of ors12, melting curve analysis of the PCR amplification products generated with their respective primer sets (see A and B) was performed (Fig. 3B).
Molecular Biochemicals) was of the expected size, reaction products were separated on a 2% agarose gel and then visualized by EtBr staining under UV light. All primer sets generated the expected corresponding 150-, 251-, 250-, or 258-bp DNA fragments (Fig. 3B), respectively. In addition, melting curve analysis of the amplification products verified that only the specific product was generated by the respective primer set and that no primer-dimers interfered with the quantification of the products in the LightCycler instrument (Fig. 2C). Primer set EE' generated a primer-dimer peak (−74 °C) when water was present instead of template DNA (Fig. 2C, EE'), which, however, did not interfere with the quantification of the specific product when template DNA is used instead of water. The melting temperatures of the PCR products generated with their respective primer sets were −78 °C (amplified by primer set ors8 150), −87 °C (amplified by primer set ors12 D2), −90 °C (amplified by primer set CD4 intron), or −81 °C (amplified by primer set EE'), respectively (Fig. 2C).

Cell Cycle-dependent Association of 14-3-3β, -ε, -γ, and -ζ with ors8 and ors12—Real time PCR was also used to quantitatively assess whether the 14-3-3β, -ε, -γ, and -ζ isoforms associated with origins of DNA replication, ors8 and ors12, as a function of the cell cycle. CV-1 cells were synchronized to G0, G1/S, S, and M phases (see “Experimental Procedures”), and synchronization was monitored by FACS analysis (Fig. 4A). The association of 14-3-3β, -ε, -γ, and -ζ isoforms with ors8 and ors12, amplified by primer sets ors8 150 and ors12 D2, respectively, was 9- and 11-fold higher (for 14-3-3β), 20- and 14-fold higher (for 14-3-3ε), 21- and 18-fold higher (for 14-3-3γ), and 24- and 20-fold higher (for 14-3-3ζ), respectively, at the G1/S boundary, by comparison with their association in G0 (serum-starved) cells (Fig. 4B). The association of all four 14-3-3 isoforms with ors8 and ors12 was the highest at the G1/S boundary, and, after 4-h release into S phase, decreased by 6- and 7-fold, respectively, for isoforms ε, γ, and ζ, and by 4- and 3-fold, respectively, for the 14-3-3ζ isofrom (Fig. 4B). Furthermore, there was a significantly lower association of 14-3-3β, -ε, -γ, and -ζ with both ors8 and ors12 in the M phase of the cell cycle, by comparison with its association in G1/S phase (Fig. 4B). Western blot analyses were performed to assess the total amount of 14-3-3β, -ε, -γ, and -ζ isoforms immunoprecipitated from nuclear extracts with their respective antibodies at each cell cycle point, regardless of their association with DNA or protein-protein complexes (Fig. 1, A–D, lanes 5–8). Small amounts of 14-3-3 isoforms were immunoprecipitated in G0 cross-linked CV-1 cells (Fig. 1, A–D, lane 5), by comparison with immunoprecipitations in G1/S phase (Fig. 1, A–D, lane 6).

Fig. 3. Quantification of DNA abundance in origin-containing and non-origin-containing sequences by real time PCR. A, total normalized cross-linked molecules detected by real time PCR using the LightCycler instrument, with primer sets ors8 150, ors12 D2, EE', and CD4 intron, from log, G0, G1/S, or M phase synchronized cells, using primer sets ors8 150, ors12 D2, EE', or CD4 intron, respectively. Lanes 1–7 (left panel) and lanes 2–5 (right panel), template DNA from cross-linked 14-3-3β, -ε, -γ, and -ζ isoforms, SC-35, NF-κB p65, or NRS immunoprecipitates. Lane 8 (left panel) and lane 1 (right panel), template DNA from CV-1 total genomic DNA from untreated cells. Lanes 9 (left panel) and lane 6 (right panel), negative control to verify primer contamination; no template DNA added to PCR.

Fig. 4. Cell cycle-dependent association of 14-3-3ε, β, -ζ, and -γ isoforms with ors8 and ors12. A, FACS analysis of DNA contained in logarithmically growing or synchronized CV-1 cells at G0, G1/S, or M phase of the cell cycle. B, PCR with template DNA from log, G0, G1/S, or M phase synchronized cells, using primer sets ors8 150 and ors12 D2. Total normalized cross-linked molecules detected by PCR are shown, from cross-linked 14-3-3β, ε, γ, and ζ immunoprecipitates, at different points in the cell cycle, as indicated. Each bar represents three experiments, and one S.D. is indicated.
The amount of 14-3-3ε isoform immunoprecipitated from G1/S phase was ~10-fold higher than in S and M phases of the cell cycle in cross-linked cells (Fig. 1B, lanes 6–8), while that of the 14-3-3ζ isoform immunoprecipitated in G1/S phase was equivalent to the amount immunoprecipitated in S and M phases (Fig. 1C, lanes 6–8). The 14-3-3β and -γ immunoprecipitations from G1/S phase cells were equivalent to the ones in M phase, whereas their S phase immunoprecipitations were ~5-fold lower than those in G1/S (Fig. 1, A and D, lanes 6–8). The observed differences in the amount of the four 14-3-3 isoforms immunoprecipitated at different cell cycle stages could either be due to differences in cross-linking efficiencies or in subcellular localization, since whole-cell extracts from different cell cycle points showed the presence of equivalent amounts of each 14-3-3β, -ε, -γ, and -ζ isoform in CV-1 cells (data not shown).

**CBP Cruciform Binding Activity Is Maximal at the G1/S Boundary**—HeLa cells growing in suspension were synchronized by serum starvation and treatment with aphidicolin (23) or mimosine (48). Extracts were prepared as before (41) from arrested and released cells as well as cells in G0 and log phases (see FACS analysis in Fig. 5B). Bandshift unique to the cruciform (D complexes) molecules were obtained in all reactions from the indicated treatment, except for the serum-starved (G0) cells (Fig. 5A, see D complexes). The cruciform binding activity of CBP was maximal at G1/S cells (arrested with either mimosine or aphidicolin) and in early S phase cells that had been released from the mimosine or aphidicolin block for 45 min (Fig. 5A).

**Effect of Anti-14-3-3 Antibodies on the CBP/14-3-3-Cruciform Complex Formation**—Preincubation of a CBP-enriched protein fraction with increasing amounts of anti-14-3-3β, -ε, -γ, and -ζ antibodies, prior to the addition of cruciform DNA in a bandshift assay, resulted in a reduction of detectable CBP-cruciform complex formation. The anti-14-3-3ε, -γ, and -ζ antibodies were more effective in inhibiting CBP-cruciform complex formation, since even at the lowest amount (5 µg) of antibody used, free cruciform DNA (Fig. 6A, lane 1) could be detected (Fig. 6A, lanes 6, 9, and 12), the corresponding band becoming more prominent as higher amounts of the antibody were used (10 µg (Fig. 6A, lanes 7, 10, and 13) and 20 µg (Fig. 6A, lanes 8, 11, and 14, and Fig. 6B, lanes 4, 10, and 13)). In contrast, the anti-14-3-3β antibody started to inhibit CBP-cruciform complex formation only at the highest amount (20 µg) used (Fig. 6A, lane 17) and B (lane 7), while the same amounts of NRS had no effect (Fig. 6, A (lanes 3–5) and B (lane 3)). The effect of combining anti-14-3-3 antibodies was also tested in order to assess whether it would result in a greater interference with DNA binding. The inclusion of anti-14-3-3β antibody in different combinations of anti-14-3-3ε, -γ, and -ζ antibodies (Fig. 6B, lanes 5, 6, 8, and 15) gave approximately the same effect on inhibiting CBP-cruciform complex formation as did the various combinations of two of the antibodies (anti-14-3-3γ and -ζ or anti-14-3-3ε and -γ) (Fig. 6B, lanes 11 and 14) or the antibodies alone (anti-14-3-3ζ or anti-14-3-3ε) (Fig. 6B, lanes 4 and 13). In contrast, combinations of two of the antibodies (anti-14-3-3γ and -ζ and anti-14-3-3ε and -γ) were more effective than either one of these antibodies alone (Fig. 6B, lanes 11 and 14). The combinations that had the greatest effect (~80% of free cruciform DNA) denote bandshifts common to both homoduplexes and heteroduplex cruciform molecules; D denotes bandshifts unique to the cruciform as described in Ref. 26. B, FACS analysis of log phase and serum-starved (G0), mimosine-arrested (late G1), mimosine-released (G1/S), aphidicolin-arrested (G1/S), and aphidicolin-released (early S) cells, as indicated.
form DNA detected) were those of anti-14-3-3e, -γ, and -ζ and anti-14-3-3β, -ε, -γ, and -ζ (Fig. 6B, lanes 12 and 16).

Effect of Anti-14-3-3 Antibodies on the in Vitro DNA Replication of p186—In view of the association of 14-3-3β, -ε, -γ, and -ζ isoforms with ors8 and ors12 and the observed interference of their respective antibodies with CBP-cruciform complex formation, the effect of the same antibodies on the in vitro DNA replication of p186 was analyzed. A representative autoradiogram of the in vitro replication experiment, before and after digestion with DpnI, is shown in Fig. 7A (left panel (+DpnI) and right panel (+DpnI)). The in vitro replication reaction products included relaxed circular (form I) and linear (form III) forms of p186 as well as replicative intermediates and topoisomeric forms, as previously observed (41), whereas the supercoiled (form I) forms overlapped with the unmethylated pBluescript plasmid DNA (Fig. 7A). The unmethylated pBluescript plasmid was included in each in vitro replication reactions to control for DNA recovery and completeness of the DpnI digestion. As expected, this plasmid was not digested by DpnI (Fig. 7A, right panel, lanes 1–9), whereas the methylated pBR322 control was fully digested by DpnI (Fig. 7A, right panel, lane 9), indicating that the presence of a mammalian replication origin is required for replication of the plasmid DNA. Quantitation of the DpnI-resistant DNA showed that preincubation of the HeLa cell extracts with 20 μg of anti-14-3-3e, anti-14-3-3γ, or anti-14-3-3ζ antibodies decreased the level of p186 in vitro DNA replication to ∼30–40% of control reactions, in which the HeLa cell extracts were preincubated with the same amount of either hypotonic buffers (since the nuclear extracts are resuspended in hypotonic buffer) or NRS (Fig. 7B), while preincubation with the anti-14-3-3β antibody had a lesser effect (50–75% of control) on p186 replication (Fig. 7B). The combination of either anti-14-3-3e, -γ, and -ζ or anti-14-3-3β, -ε, -γ, and -ζ isoforms decreased the level of replication to ∼25 and 20%, respectively, of control reactions (Fig. 7B). These combinations where chosen because of their profound effect on the CBP-14-3-3-cruciform complex formation (Fig. 6, A and B).

DISCUSSION

The multifunctional 14-3-3 family of proteins consists of seven known mammalian isoforms (β, ε, γ, ζ, η, σ, and ς) that are highly conserved molecules and are expressed in a broad range of tissues and cell types (59). In eukaryotes, 14-3-3 proteins are largely found in the cytoplasm, but they can also be detected at the plasma membrane and in intracellular organelles, including the nucleus (34, 60). 14-3-3 proteins participate in the regulation of essential cellular processes, including cell cycle control, survival signaling, cell adhesion, neuronal plasticity, and DNA replication (reviewed in Refs. 29 and 30).

In the present study, we have investigated the association of 14-3-3β, -ε, -γ, and -ζ isoforms with specific genomic regions, containing origins of replication (ors8 and ors12) or non-origin-containing sequences (EE’ and CD4 intron), by the formaldehyde cross-linking approach. The efficiency of this approach has been demonstrated in a number of studies (47, 52, 58, 61–70). Formaldehyde treatment of cells readily produces protein-protein and protein-DNA cross-linked complexes. Here, after treatment of cells with formaldehyde, antibodies were then employed to immunoprecipitate the 14-3-3β, -ε, -γ, and -ζ isoforms and analyze the DNA recovered from the immunoprecipitates by PCR amplifications. Real time PCR was than used as a method to quantitatively assess whether the recovered template DNA was enriched in origin-containing sequences.

The in vivo association of 14-3-3β, -ε, -γ, and -ζ isoforms with origins of replication was investigated with specific primer sets from the monkey origins ors8 and ors12 (ors8 150 and ors12 D2, respectively). All four 14-3-3β, -ε, -γ, and -ζ isoforms were found to associate specifically with these origins, since DNA fragments recovered from the 14-3-3β, -ε, -γ, and -ζ immunoprecipitates were enriched ∼9-fold in origin-containing sequences compared with other portions of the genome (Fig. 3A). A number of controls were included to ensure that the amplification signals obtained were due to specific protein/DNA interactions.
and quantitative PCR\(^2\) and was not found to contain any DNA fragments from the origin regions under investigation, showing that cross-linking is required prior to immunoprecipitation and that immunoprecipitations with the anti-14-3-3, -\(\gamma\), -\(\zeta\), and -\(\xi\) antibodies did not bring down considerable amounts of contaminating DNA. The background signal arising from DNA that was immunoprecipitated nonspecifically by the anti-SC-35, anti-NF-\(\kappa\)B \(p65\), and NRS antibodies was quantified in origin-containing sequences (\(\text{ors}8\) and \(\text{ors}12\)) as well as non-origin-containing sequences, amplified by primer sets EE' and CD4 intron (Fig. 3A). In addition, the DNA immunoprecipitated with anti-14-3-3 antibodies in these non-origin-containing genomic regions was also quantified (Fig. 3A). All of these

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negative controls permitted us to estimate the background nonspecific DNA to be \(4 \times 10^{10}\) molecules/1.5 \(\times 10^{13}\) CV-1 cells (Fig. 3A).

Finally, the cell cycle studies indicated that the association of 14-3-3\(\beta\), \(-\epsilon\), \(-\gamma\), and \(-\zeta\) with ors8 and ors12 was the highest at the onset of S phase, being \(\sim 10\)-fold (for the 14-3-3\(\beta\) isofrom) or \(\sim 20\)-fold higher (for 14-3-3\(\epsilon\), \(-\zeta\), and \(-\gamma\)), in cells synchronized at the G1/S boundary, compared with that in cells that were blocked at G0 phase by serum starvation (Fig. 4B). The higher association of 14-3-3 isoforms with ors8 and ors12 at the onset of S phase was specific and occurred at a time when the origin becomes activated (37). In addition, the cruciform binding activity of CBP/14-3-3 was also maximal at the G1/S phase of the cell cycle (Fig. 5A).

The same anti-14-3-3 antibodies that were used in the chromatin immunoprecipitation assays were also used in an in vitro replication assay to investigate their effect on the in vitro replication of the minimal origin of ors8, p186. The anti-14-3-3\(\epsilon\), \(-\zeta\), and \(-\gamma\) were each capable of reducing replication activity of p186 to 30–45% of control levels, while the anti-14-3-3\(\beta\) antibody gave a lesser decrease in replication activity (50–80% of control levels) (Fig. 7B). Thus, 14-3-3\(\beta\) appears not to be involved in DNA replication in the same way as 14-3-3\(\epsilon\), \(-\gamma\), and \(-\zeta\). A possible explanation might be that the \(\beta\) isoform is phosphorylated and hence unable to bind DNA. Phosphorylation analyses are ongoing. Alternatively, the heterodimers and homodimers of 14-3-3 involved in DNA replication are composed of different combinations of 14-3-3\(\beta\), \(-\epsilon\), \(-\gamma\), and \(-\zeta\) isoforms, where the 14-3-3\(\beta\) is represented in lesser amounts.

Furthermore, in bandshift/supershift experiments, the same anti-14-3-3 antibodies interfered with the formation of the CBP-cruciform complex. Antibodies to all of the four 14-3-3\(\beta\), \(-\epsilon\), \(-\gamma\), and \(-\zeta\) isoforms tested were able to interfere with the ability of CBP/14-3-3 to bind to cruciforms, suggesting that the epitopes of these antibodies may overlap with sites within 14-3-3 that are important for cruciform binding or that there is steric interference of 14-3-3 binding due to the large immunoepitopes of these antibodies may overlap with sites within the N-terminal part of each monomer that are involved in DNA replication. 14-3-3 proteins function as homo- or heterodimers (71, 72). The combination of all four anti-14-3-3\(\beta\), \(-\epsilon\), \(-\gamma\), and \(-\zeta\) antibodies reduced DNA replication to \(\sim 20\%\) of control levels, perhaps suggesting the presence of additional 14-3-3 isoforms involved in DNA replication.

A large number of 14-3-3 isoforms and a large number of their target proteins have been identified (29) (reviewed in Ref. 30) in many organisms. A common hypothesis is that all 14-3-3 isoforms bind with more or less specificity to a defined target and that the isoform-specific ligand interactions observed by others are due to either particular subcellular localization or transcriptional regulation of isoforms rather than to fundamental differences in their ability to bind to specific ligands. Furthermore, structural studies have not supported isoform-specific interactions with different targets (see Ref. 75 and references therein) (29), and there is little indication that such isoform specificity exists.

The data presented in this study suggest that all four 14-3-3 isoforms studied (\(\beta\), \(\epsilon\), \(\gamma\), and \(\zeta\)) are associated with mammalian origins of replication and are involved in the initiation of DNA replication, 14-3-3\(\beta\) being less important for both origin binding and in vitro replication. Taken together, the data suggest that CBP/14-3-3 is, as an origin-binding protein, acting at the initiation step of DNA replication by binding to cruciform-containing (activated) origins and that it dissociates after origin firing. The higher association of 14-3-3\(\beta\), \(\epsilon\), \(\zeta\), and \(\gamma\) with origins at the G1/S phase of the cell cycle suggests that these isoforms act at the level of initiation of replication, presumably by binding and stabilizing the cruciform structure.