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14-3-3σ Is a Cruciform DNA Binding Protein and Associates In Vivo With Origins of DNA Replication

David Alvarez,[1,2] Olivia Novac,[1,2] Mario Callejo,[1,2] Marcia T. Ruiz,[2] Gerald B. Price,[2] and Maria Zannis-Hadjopoulos[1,2]*

[1]Department of Biochemistry, McGill University, Montreal, Canada
[2]McGill Cancer Center, McGill University, Montreal, Canada

Abstract A human cruciform binding protein (CBP) was previously shown to bind to cruciform DNA in a structure-specific manner and be a member of the 14-3-3 protein family. CBP had been found to contain the 14-3-3 isoforms β, γ, ε, and ζ. Here, we show by Western blot analysis that the CBP-cruciform DNA complex eluted from band-shift polyacrylamide gels also contains the 14-3-3σ isoform, which is present in HeLa cell nuclear extracts. An antibody specific for the 14-3-3σ isoform was able to interfere with the formation of the CBP-cruciform DNA complex. The effect of the same anti-14-3-3σ antibody in the in vitro replication of p186, a plasmid containing the minimal replication origin of the monkey origin *ors*8, was also analyzed. Pre-incubation of total HeLa cell extracts with this antibody decreased p186 in vitro replication to approximately 30% of control levels, while non-specific antibodies had no effect. 14-3-3σ was found to associate in vivo with the monkey origins of DNA replication *ors*8 and *ors*12 in a cell cycle-dependent manner, as assayed by a chromatin immunoprecipitation (ChIP) assay that involved formaldehyde cross-linking, followed by immunoprecipitation with anti-14-3-3σ antibody and quantitative PCR. The association of 14-3-3σ with the replication origins was maximal at the G_1/S phase. The results indicate that 14-3-3σ is an origin binding protein involved in the regulation of DNA replication via cruciform DNA binding. J. Cell. Biochem. 87: 194–207, 2002.

Key words: 14-3-3σ; cruciform binding protein; DNA replication; cruciform DNA; G_1/S transition

Palindromes, or inverted repeat sequences, are a common feature of DNA regulatory regions [Panayotatos and Fontaine, 1987; Noirot et al., 1990; Zheng et al., 1991; Dayn et al., 1992]. Origins of replication in both prokaryotes and eukaryotes contain inverted repeats that have the potential to extrude into cruciform structures, and previous studies have demonstrated the involvement of cruciform structures in initiation of DNA replication [Hand, 1978; Zannis-Hadjopoulos et al., 1984, 1985, 1988; Ward et al., 1990, 1991; Landry and Zannis-Hadjopoulos, 1991; Boulikas, 1993; reviewed in Pearson et al., 1996].

A protein that is able to specifically recognize cruciform structures was previously purified from HeLa cells [Pearson et al., 1994]. The human cruciform binding protein, CBP, has been identified as a member of the 14-3-3 family of proteins [Todd et al., 1998]. CBP is a protein of approximately 66–70 kDa [Pearson et al., 1994], which is the size of the dimeric form of 14-3-3 molecules. Previous studies revealed the presence of 14-3-3β, γ, ε, and ζ isoforms in the CBP-cruciform DNA complex that was eluted from preparative band-shift gels, as well as the localization of these isoforms in HeLa cell nuclei [Todd et al., 1998].

14-3-3 proteins are conserved molecules encoded, in mammals, by seven different genes. The 14-3-3 family has been implicated in cell cycle control, and has been shown to interact with various signaling proteins [for reviews, see Aitken et al., 1992; Aitken, 1996; Fu et al., 2000]. 14-3-3 proteins have been reported to associate with receptors, such as the glucocorticoid receptor [Wakui et al., 1997], kinases, such as Raf-1 [Fu et al., 1994], death regulators, such as Bad [Zha et al., 1996], oncogene products, such

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*Correspondence to: Maria Zannis-Hadjopoulos, McGill Cancer Center, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6, Canada.
E-mail: maria.zannis@mcgill.ca

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as Bcr-Abl [Reuther et al., 1994], and cell division control proteins, such as Cdc25 [Yang et al., 1999]. It was recently shown that 14-3-3ε modulates topoisomerase II activity through protein–protein interaction during progression of the replication fork [Kurz et al., 2000]. Cruciform DNA binding is a new activity associated with the 14-3-3 family [Todd et al., 1998]. 14-3-3 proteins are present in a wide variety of different mammalian tissues, plant species, as well as in all eukaryotic organisms examined to date, including *Xenopus*, *Drosophila*, *C. elegans*, the budding yeast *Saccharomyces cerevisiae*, and the fission yeast *Schizosaccharomyces pombe* [Aitken, 1996]. A leading model of 14-3-3 function suggests that it facilitates critical protein–protein interactions and thus serves as a necessary component of a wide variety of cellular processes [Morrison, 1994].

Also known as stratafin, 14-3-3σ is known to sequester cyclin B-cdc2 complex in the cytoplasm during the G_2/M checkpoint [Chan et al., 1999] and its expression is markedly increased in a p53-dependent manner after DNA damage [Hermeking et al., 1997]. In this study, we show that 14-3-3σ is also present in the CBP complex with cruciform DNA. We also found that 14-3-3σ is present in HeLa cell nuclear extracts, participates in DNA replication, and associates in vivo with origins of replication in a cell cycle-dependent manner in CV-1 cells. The data suggest that 14-3-3σ is a nuclear factor involved in G_1/S transition via cruciform DNA binding at origins of DNA replication.

MATERIALS AND METHODS

Band-Shift Elution of CBP

Cruciform-containing DNA (pRGM21 × pRGM29) was constructed and end-labeled, as described previously [Pearson et al., 1994]. CBP was purified, as previously described [Todd et al., 1998], with some modifications. In brief, 70 ng of end-labeled cruciform DNA were used to purify CBP from 0.5 mg of an Affi-gel heparin column flow through (F_{TH}), which is enriched in CBP activity. The cruciform-CBP complex was resolved by electrophoresis for 1.5 h, in a 4% polyacrylamide gel at 180 V, and was then eluted from the gel by isotachophoresis, as previously described [Pearson et al., 1995], and concentrated with a YM-10 concentrator (QIAGEN) to a final concentration of approximately 0.5 μg/μl.

Western Blotting

Concentrated band-shift eluted CBP (1.5 μg), and 5 μg of HeLa purified nuclear extracts (NE) were mixed with 1 × SDS sample buffer (0.67 × Tris-Cl/SDS, pH 6.8, 5% glycerol, 1.67% SDS, 100 mM 1,4-dithiothreitol [DTT], and 0.002% bromphenol blue) loaded onto three 12% SDS–polyacrylamide gels, and subjected to electrophoresis at 200 V for 40 min. The gel contents were transferred to Immobilon™ P membrane (Millipore) at 100 V for 1 h at 4°C. Immunodetection was carried out as described in the ECL™ Amersham Pharmacia Biotech protocol, with the anti-rabbit or anti-goat secondary horseradish-peroxidase (HRP)-labeled conjugated antibodies (Santa Cruz Biotechnology, CA), using anti-14-3-3σ antibody (sc-7683, Santa Cruz Biotechnology), or anti-14-3-3ε antibody (sc-1020, Santa Cruz Biotechnology), used as a positive cccontrol, or anti-Ku70 antibody (sc-1486, Santa Cruz Biotechnology), used as a negative control. The anti-14-3-3σ antibody used in this and subsequent experiments was raised against a unique sequence at the C-terminus of the σ isoform (Santa Cruz Biotechnology), and does not cross-react with any other 14-3-3 isoforms. The same is true for the anti-14-3-3ε antibody, which was raised against a divergent domain that is unique to the ε isoform (Santa Cruz Biotechnology).

For Western blotting of HeLa cell NE and cytoplasmic (CE) extracts, 5 μg of each sample were used as described above. Membranes were probed with anti-14-3-3σ, or anti-14-3-3ε antibodies, or anti-Cas/p130 and anti-Crk II antibodies (sc-860 and sc-289, respectively, Santa Cruz Biotechnology; kindly provided by Dr. Pankaj Tailor, McGill Cancer Center).

Band-Shift/Supershift Assays

Band-shifts assays were carried out as previously described [Todd et al., 1998]. In brief, 5 μg of F_{TH} (CBP) were pre-incubated with 5, 10, or 20 μg of anti-14-3-3σ antibody, 20 μg of normal goat serum (NGS; Santa Cruz Biotechnology), or phosphate buffered saline (PBS) for 4 h at 4°C, followed by incubation with 0.3 ng of labeled cruciform DNA in DNA binding buffer (20 mM Tris-Cl, pH 7.5, 1 mM DTT, 1 mM ethylenediaminetetraacetic acid [EDTA], and 3% glycerol) [Elborough and West, 1988] and 100 ng/μl of poly-dI-dC (Pharmacia) for 0.5 h at 4°C. The mixtures were subjected to polyacry-

lamide gel (4%) electrophoresis (PAGE) for 1.5 h at 180 V in 1 × TBE buffer, and the gel was dried and exposed to an imaging plate for 3 h. The intensity of the bands corresponding to the amount of free-labeled DNA was quantified by Image Gauche (Fuji Photo Film Co., Ltd.). After quantification, the dried gel was exposed for autoradiography overnight.

In Vitro DNA Replication Assays

HeLa purified NE and CE were prepared and used as previously described [Pearson et al., 1991] with some modifications. In brief, 120 μg of total protein were pre-incubated for 30 min with either hypotonic solution, or 5, 10, or 20 μg of anti-14-3-3σ antibody, or 20 μg of NGS. Pre-incubations were also performed in the presence of 20 μg of the antibody and saturating amounts (5-fold molar excess) of blocking peptide, or same amount of blocking peptide alone. Template DNA, 150 ng of p186, a pBR plasmid containing an insert of 186 bp from *ors*8 that functions as an origin of replication [Todd et al., 1995], and the rest of the in vitro replication reaction ingredients were then added, and the samples were incubated for 60 min at 30°C, as previously described [Pearson et al., 1991]. In addition, 100 ng of unmethylated pBluescript KS + (pBS) was added to each reaction to internally control for differences in DNA recovery [Novac et al., 2002]. The replication products were purified by passing them through QIAquick® PCR Purification Kit columns (QIAGEN) and one third of each sample was digested with 1.5 U of DpnI enzyme (New England BioLabs), as described previously [Matheos et al., 1998], for 1 h at 37°C. Both undigested and digested products were resolved in a 1% agarose gel for 15 h at 55 V, the dried gel was exposed to an imaging plate for 5 h, and the bands corresponding to the plasmid DNA forms II and III were quantified by Image Gauche (Fuji Photo Film Co., Ltd.), as previously described [Matheos et al., 1998], subtracting the background of each lane from the respective relevant band intensities. Quantification of the supercoiled (form I) band was not possible because it was: (1) inconsistently present, depending on the supercoiling quality (topoisomerase activity) of the NE, in agreement with previous data [Pearson et al., 1991]; and (2) overlapped (co-migrated) with the bands generated by DpnI digestion products.

The optimal amount (units) of DpnI required for the digestion reactions was determined by performing in duplicate similar in vitro DNA replication assays, using as template either 150 ng of p186 or equimolar amount of pBR322. The reaction products were purified, as described above, and one third of either sample was subjected to DpnI digestion with increasing amounts (0.5, 1.0, 1.5, and 2.0 U) of the enzyme. At 1.5 U of DpnI, the sample containing pBR322 was completely digested, whereas the sample containing p186 showed resistant bands, indicating the presence of replication products. At concentrations higher than 1.5 U of DpnI per reaction, both samples were completely digested, while at less than 1.5 U, pBR322 was partially digested.

CV-1 Cell Culture and Synchronization

CV-1 cells (monolayers) were cultured in MEMα (Gibco-BRL, NY) supplemented with 10% fetal bovine serum (Gibco-BRL; termed regular medium) at 37°C, and synchronized to G_1/S, S, and G_2/M, as previously described [Mah et al., 1993; Novac et al., 2002], with some modifications. In brief, for synchronization to the G_0/G_1 phase, 80% confluent CV-1 cells were placed in serum-free medium for 48 h; for synchronization to G_1/S, S, and G_2/M, 40% confluent CV-1 cells were exposed to 2 mM thymidine (Sigma, MT) for 12 h, then released for 9 h in regular medium lacking thymidine, followed with incubation for 12 h with 2 mM thymidine and 400 μM mimosine (Sigma); for S phase synchronization, the cells were released for 4 h in regular medium; and for synchronization to G_2/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 of CV-1 Cells

In vivo cross-linking was performed as previously described [Orlando et al., 1997; Novac et al., 2001, 2002]. In brief, CV-1 cells were washed twice with PBS and treated with formaldehyde (1%) in warm MEMα medium without serum for 10 min. Lysis buffer [50 mM HEPES/KOH, pH 7.5, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 capsule of protease inhibitors (Boehringer Mannheim)] was then added (at 4°C) and the cells were drawn into and out of a 21-gauge hypodermic needle three times. The cell lysates were then layered onto 4 ml of 12.5% glycerol in lysis

buffer, and centrifuged at 750*g* for 5 min in a benchtop centrifuge. The nuclear pellet was resuspended in 1 ml of lysis buffer.

Chromatin Fragmentation

Nuclei from cross-linked and non-cross-linked cells were sonicated ten times for 30 s each, and the chromatin size was monitored by electrophoresis [Hecht and Grunstein, 1999]. Fragments of approximately 20 kb were generated in this manner. The DNA was further digested with Sph1, HindIII, Pst1, and EcoR1 (100 U of each; New England Biolabs), in NEB2 buffer at 37°C for 6 h, to further reduce the chromatin size into fragments of 1.5–3.5 kb.

Immunoprecipitation and DNA Isolation

Lysed extracts with sheared chromatin were incubated with 50 μl of Protein A-agarose (Boehringer Mannheim; as per manufacturer instructions) and the cleared chromatin lysates were incubated, at 4°C for 6 h on a rocker platform, with either 50 μl of NGS, or 20 μg of anti-14-3-3σ or anti-NF-κB p65 (sc-372, Santa Cruz Biotechnology; against the transcription factor NF-κB p65) goat polyclonal antibodies, or anti-SC-35 (Sigma) rabbit monoclonal antibody against the splicing factor SC-35. The 50 μl of protein A-agarose (for rabbit antibodies) or protein G-agarose (for goat antibodies) was added, and the incubation was continued for a further 12 h. The precipitates were successively washed two times 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 (same as WB1, but with no NaCl), and 1 ml of TE (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and were finally resuspended in 200 μl of extraction buffer (1% SDS/TE). The samples were incubated at 65°C overnight to reverse the protein/DNA cross-links [Orlando et al., 1997; Novac et al., 2001, 2002], and then for 2 h at 37°C with 100 μg of proteinase K (Boehringer Mannheim). Finally, the DNA was purified by passage of each sample through QIAquick PCR purification columns (QIAGEN).

Western Blotting of CV-1 Extracts Immunoprecipitates

Immunoprecipitates were resuspended in electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved on 10% SDS–polyacrylamide gels, transferred to Immobilon™ P membranes (Millipore) and probed with anti-14-3-3σ antibody. Protein-antibody complexes were visualized by enhanced chemiluminescence using the Amersham ECL system, with the anti-rabbit or anti-goat secondary horse-radish-peroxidase (HRP)-labeled conjugated anti-bodies (Santa Cruz Biotechnology).

Real-Time PCR Quantification Analysis of Immunoprecipitated DNA

PCR reactions were carried out in 20 μl with 1/200th of the immunoprecipitated material using LightCycler capillaries (Roche Molecular Biochemicals, Mannheim, Germany) and the LightCycler-FastStart DNA Master SYBR Green I from Roche Molecular Biochemicals, as previously described [Pfitzner et al., 2000; Novac et al., 2002], with slight modifications. In brief, the PCR reaction contained 3 mM Mg^{2+} and 1 μM of each primer of the appropriate primers set used (*ors*8 150, *ors*12 D2, EE′, or CD4 intron). Primer set *ors*8 150 and *ors*12 D2 (Fig. 5a, bars I and II) were used to amplify a 150-bp and a 251-bp corresponding genomic fragment of *ors*8 and *ors*12, respectively. Primer set EE′ was used to amplify a 250-bp genomic fragment which was located approximately 5 kb downstream of *ors*12 [Pelletier et al., 1999] (Fig. 5a, bar II). A control set of primers from the African Green Monkey *CV-1 CD4* gene (accession no.: AB052204; Matsunaga et al., 2000) were also used. Primer set CD4 intron amplifies a fragment of 258 bp from genomic CV-1 DNA. Primers were designed as 20–22mers with approximately 50% GC content. Sequence for the primers used:

Primer	Sequence	$T_{Annealing}$ (°C)
*ors*8 150F	5′-GACCCATAAAGGCAAAAGTACC-3′	45
*ors*8 150R	5′-GGAAGATATTAAGATAGATGG-3′	
*ors*12 D2	5′-CCTGAGAGCAAAACTCTGGC-3′	55
*ors*12 D2′	5′-CTTAAATCCTTGGCACCATC-3′	
E	5′-GGAATTCTGTCTTAGGCAAT-3′	50
E′	5′-TGATATTGCCAATCAGGATC-3′	
CD4 intron F	5′-AGCTCTGTTCTGTATCTTTG-3′	50
CD4 intron R	5′-CCACAGGCACTTTTATCTTC-3′	

Genomic CV-1 DNA (9.3, 18.6, 27.9, 37.2, and 55.8 ng), used for the standard curve reactions [Novac et al., 2001, 2002], (data not shown) was obtained from total cell lysates of non-cross-linked log phase cells (80% confluent). The PCR products were quantified by the LightCycler (Roche) instrument, using SYBR Green I dye for detection, as previously described [Novac et al., 2001, 2002]. Typically, an initial denaturation for 10 min at 95°C was followed by 35 cycles with denaturation for 15 s at 95°C, annealing for 15 s at 45°C, (primer set *ors*8 150), 50°C (primer sets, EE′ or CD4 intron) or 55°C (primer set *ors*12 D2), and polymerization for 15 s at 72°C. The specificity of the amplified PCR products was assessed by performing a melting curve analysis cycle with a first segment set at 95°C for 0 s and a temperature transition of 20°C/s, a second segment set at 45, 50, or 55°C (depending on the annealing temperature of the primer set used) with a temperature transition rate of 20°C/s, and, finally, a third segment set at 95°C with a temperature transition rate set at 0.2°C/s. A melting curve analysis verified that no primer-dimer interfered with the quantification of the products in the LightCycle instrument (data not shown). The PCR products were also resolved on 2% agarose gels, and visualized by staining with ethidium bromide and photographing with an Eagle Eye apparatus (Speed Light/BT Science-tech-LT1000) (data not shown).

RESULTS

14-3-3σ Is Present in HeLa Cell Nuclear Extracts

It was previously shown that HeLa cell nuclei contain 14-3-3 isoforms β, γ, ε, and ζ, as part of the CBP-cruciform DNA complexes eluted from a preparative band-shift gel [Todd et al., 1998]. Here, we tested HeLa cell nuclear extracts (NE) for the presence of the 14-3-3σ isoform. HeLa cell nuclear and cytoplasmic extracts were prepared as described previously [Pearson et al., 1991] and analyzed by Western blotting using an anti-14-3-3σ antibody specific for the σ isoform. Anti-14-3-3ε antibody was used as positive control, and anti-Crk II and anti-Cas/p130 antibodies as negative controls. Figure 1a shows that 14-3-3σ is present in both NE (lane 1) and CE (lane 2), as is 14-3-3ε (lanes 3 and 4), confirming previous data [Todd et al., 1998]. The extra band (35 kDa) detected with the anti-14-3-3ε antibody (Fig. 1a,b, lane 3) has also been observed previously and is characteristic of CBP [Todd et al., 1998]. The intensity of the bands corresponding to 14-3-3σ and 14-3-3ε in both NE and CE is approximately the same, suggesting that the concentration of these two isoforms is approximately equal in both the nuclear and cytoplasmic compartments, at least in log phase HeLa cells (same amount of total protein from both NE and CE was used in this assay). On the

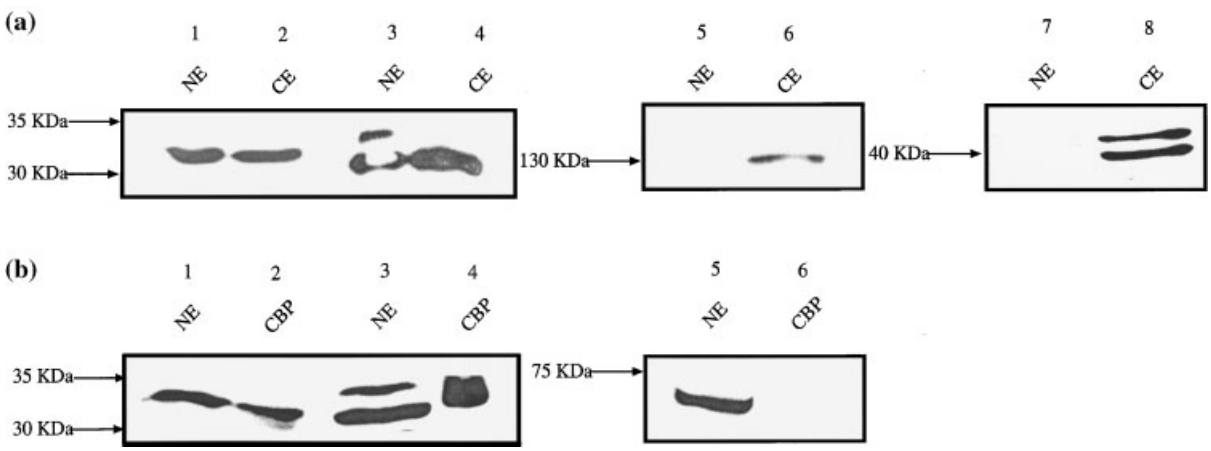

Fig. 1. 14-3-3σ is present in the HeLa cell nuclear extracts and in the CBP-cruciform DNA complex. **a**: Five micrograms of total protein of each nuclear (NE) and cytoplasmic (CE) extracts were subjected to electrophoresis in a 12% SDS polyacrylamide gel. The proteins were electro-transferred to Immobilon™ P membranes, followed by probing with the following antibodies: anti-14-3-3σ (**lanes 1** and **2**), anti-14-3-3ε (**lanes 3** and **4**), anti-Cas/p130 (**lanes 5** and **6**), and anti-Crk II (**lanes 7** and **8**). **b**: Five micrograms of HeLa nuclear extracts (NE) and 1 μg of band-shift eluted CBP were also subjected to electrophoresis in a 12% SDS polyacrylamide gel. After electro-transferring to an Immobilon™ P membrane, the proteins were probed with anti-14-3-3σ (**lanes 1** and **2**), anti-14-3-3ε (**lanes 3** and **4**), and anti-Ku70 (**lanes 5** and **6**) antibodies.

other hand, Cas, an exclusively cytoplasmic protein [Klemke et al., 2000] of 130 kDa, was only present in CE (Fig. 1a, lane 6) and not in NE (lane 5), indicating that NE were not significantly contaminated with cytoplasmic contents. Furthermore, anti-Crk II antibody was also used as a negative control, since the molecular weight of Crk II protein (40–42 kDa), another exclusively cytoplasmic molecule [Klemke et al., 2000], is between that of a 14-3-3 dimer (66–70 kDa) and monomer (33–35 kDa). Figure 1a shows the absence of Crk II from NE (lane 7), again indicating that cytoplasmic proteins did not leak into the nuclear compartment after cell disruption.

14-3-3σ Is Contained in the Band-Shift Eluted CBP Complex

To analyze whether 14-3-3σ is present in the CBP/14-3-3-cruciform DNA complex, CBP, in complex with labeled cruciform DNA, was eluted from a 4% polyacrylamide band-shift gel [Pearson et al., 1995; Todd et al., 1998], resolved by SDS polyacrylamide gel electrophoresis (SDS–PAGE) in a 12% gel, and electro-transferred to an Immobilon P membrane, which was subjected to Western blot analysis, using anti-14-3-3σ antibody. Again, anti-14-3-3ε antibody was used as positive control, while anti-Ku70 antibody was used as negative control. The results (Fig. 1b) show that 14-3-3σ is also present in the CBP complex (lane 2), as is 14-3-3ε (lane 4), and confirm the absence of Ku70, a nuclear protein, from it (lane 6). The presence of all three proteins, namely 14-3-3σ (lane 1), 14-3-3ε (lane 3), and Ku70 (lane 5) in the HeLa cell NE was confirmed by Western blotting.

Anti-14-3-3σ Antibody Inhibits the Cruciform-CBP Complex Formation

To further confirm that 14-3-3σ participates in the binding of CBP/14-3-3 to cruciform DNA, 3 μg of the CBP-enriched fraction, F_{TH}, prepared from HeLa cell total extracts [Pearson et al., 1994], was pre-incubated with 5, 10, or 20 μg of anti-14-3-3σ antibody, or with 20 μg of NGS prior to the addition of cruciform DNA in band-shift assays. The results are shown in Figure 2a,b. Two CBP complexes (CBP1 and CBP2) were detected, as previously documented [Todd et al., 1998]. The histogram plot (Fig. 2b) shows the quantification of free DNA under the various pre-incubation conditions shown in Figure 2a (lanes 2–6), relative to lane 1. When the band-shift reaction was carried out in the presence of F_{TH} only, without the addition of anti-14-3-3σ antibody, less than 5% of the total cruciform DNA remained unbound (Fig. 2a, lane 2; Fig. 2b, column 2), indicating that over 95% of the labeled cruciform molecules were bound to CBP. Addition of increasing amounts of the anti-14-3-3σ antibody, however, interfered with the CBP-cruciform DNA complex formation, resulting in a progressive increase in the amount of free cruciform DNA molecules (Fig. 2a, lanes 4–6; Fig. 2b, columns 4–6). Specifically, pre-incubation of F_{TH} with 5 μg of anti-14-3-3σ antibody increased the amount of free DNA to approximately 5–15% (Fig. 2a, lane 4; Fig. 2b, column 4), further increasing it to approximately 20–30%, when the pre-incubation was

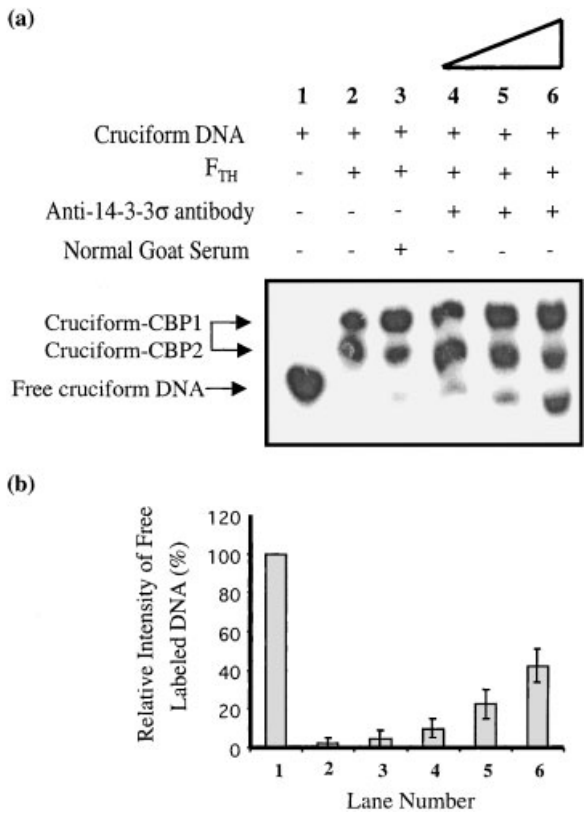

Fig. 2. Anti-14-3-3σ antibody interferes with the CBP-cruciform DNA complex formation. **a**: **Lane 1**, free cruciform DNA; 5 μg of CBP-enriched fraction, F_{TH}, were pre-incubated with buffer (**lane 2**), 20 μg of NGS (**lane 3**), or 5 μg (**lane 4**), 10 μg (**lane 5**) and 20 μg (**lane 6**) of anti-14-3-3σ antibody, and then used in band-shift assays, as described in Materials and Methods. The position of free cruciform DNA and the cruciform-CBP complexes (CBP1 and CBP2) is indicated (arrows). **b**: Histogram plot of the quantification of the free cruciform DNA bands shown in (a), in relation to lane 1, whose intensity is taken as 100% (**column 1**). Error bars represent three separate experiments.

done with 10 μg (Fig. 2a, lane 5; Fig. 2b, column 5), and to 35–50%, when the pre-incubation was done with 20 μg of antibody (Fig. 2a, lane 6; Fig. 2b, column 6), with respect to the total cruciform DNA (0.3 ng) used in each reaction (Fig. 2a, lane 1; Fig. 2b, column 1; 100%). In contrast, a modest interference with the formation of CBP-cruciform DNA complex was observed when 20 μg of NGS was used, the amount of free DNA being approximately 2–7% of the total DNA (Fig. 2a, lane 3; Fig. 2b, column 3).

Anti-14-3-3σ Antibody Inhibits In Vitro DNA Replication of p186

In view of the interference of the anti-14-3-3σ antibody with the formation of the CBP-cruciform DNA complex and the previously found involvement of 14-3-3 isoforms β, γ, ε, and ζ in mammalian DNA replication [Novac et al., 2002], a mammalian in vitro replication system [Pearson et al., 1991] was used to analyze the effect of anti-14-3-3σ antibody on the replication of p186, a plasmid containing the minimal replication origin of *ors*8 [Todd et al., 1995]. HeLa cell extracts [Pearson et al., 1991] were pre-incubated with 5, 10, or 20 μg of anti-14-3-3σ antibody and then used in in vitro replication (see Materials and Methods). Figure 3a is a typical autoradiogram of the in vitro replication assays, before (−DpnI) and after (+DpnI) digestion with therestriction endonuclease DpnI. The p186 replication products (lanes 1–7 and 9–15) included relaxed circular (form II), and linear (form III) forms of the plasmid DNA, as previously observed [Pearson et al., 1991], while the supercoiled (form I) form was inconsistently present, as previously found, depending on the supercoiling activity of the NE [Pearson et al., 1991]. The migration position of form I p186 DNA also overlapped with both the unmethylated pBS (lanes 1–7 and 9–15) and the bands generated by the DpnI digestion products (lanes 9–15). Replicative intermediates (RI) and a series of topoisomeric forms were also present, as previously found [Pearson et al., 1991]. The unmethylated pBS plasmid, which lacks a mammalian replication origin, was included in the in vitro replication reactions (lanes 1–16) as an internal control for DNA recovery and completeness of the DpnI digestion [Novac et al., 2002]. pBS was unable to replicate in the mammalian in vitro replication system, and since it is unmethylated, it was not digested by DpnI (lanes 9–16). As additional control, the methylated pBR322 DNA, used as template in equimolar amounts (lane 8), also did not replicate in the HeLa cell extracts, since it too, like pBS, lacks a mammalian origin of DNA replication, and thus was fully digested by DpnI (lane 16). The quantification of the DpnI-resistant bands (forms II and III; lanes 9–15), resulting from replication of p186 DNA, is shown in Figure 3b. Pre-incubation of the extracts with increasing amounts of anti-14-3-3σ antibody progressively decreased the level of p186 in vitro DNA replication (Fig. 3a,b). Specifically, while pre-incubation with 5 μg of anti-14-3-3σ antibody had a modest effect on p186 in vitro DNA replication (Fig. 3a, lane 10; Fig. 3b, column 10), the replication level being comparable to the control reaction (100%), in which HeLa cell extracts were pre-incubated with hypotonic buffer (Fig. 3a, lane 9; Fig. 3b, column 9), pre-incubation with 10 μg (Fig. 3a, lane 11; Fig. 3b, column 11) and 20 μg (Fig. 3a, lane 12; Fig. 3b, column 12) of this antibody decreased the level of replication to approximately 55 and 30%, respectively, by comparison to the control reaction. In contrast, pre-incubation of the extracts with 20 μg of NGS only slightly decreased the in vitro replication of p186 (Fig. 3a, lane 13; Fig. 3b, column 13). Normal levels of replication were restored when the anti-14-3-3σ antibody was neutralized by the addition of 5-fold molar excess of a blocking peptide, specific for the anti-14-3-3σ antibody (Fig. 3a, lane 14; Fig. 3b, column 14). Using the same amount of blocking peptide alone gave no significant change in replication (Fig. 3a, lane 15; Fig. 3b, column 15).

Immunoprecipitation of 14-3-3σ From Lysed Cell-Extracts

To analyze whether formaldehyde crosslinking used in the chromatin immunoprecipitation (ChIP) assay (see below) would affect the immunoprecipitation of 14-3-3σ, the latter was immunoprecipitated with anti-14-3-3σ antibody from extracts of monkey (CV-1) cells that had been either untreated or treated with formaldehyde. As controls, antibodies against the spliceosome-specific protein, SC-35, a nuclear protein that does not bind DNA [Fu and Maniatis, 1990], or the transcription factor NF-κB p65 [Meyer et al., 1991], a nuclear DNA binding protein that does not associate with replication origins, or NGS were used. We have previously shown that the anti-NF-κB and the anti-SC-35 immunoprecipitates from CV-1

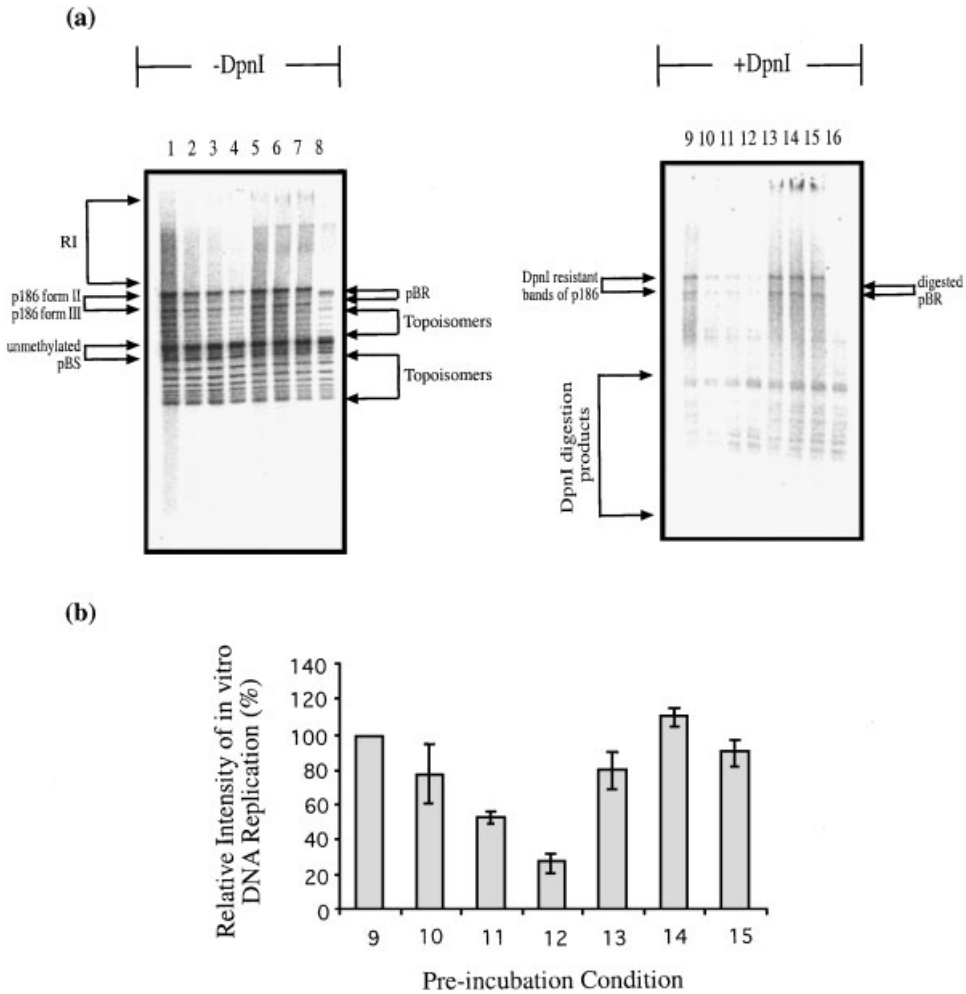

Fig. 3. Anti-14-3-3σ antibody downregulates in vitro replication of p186 plasmid. **a**: Representative autoradiogram of p186 in vitro replication assays (see Materials and Methods), before (**left panel**) and after (**right panel**) digestion with DpnI (1.5 U, 1 h, 37°C). HeLa cell extracts were pre-incubated with hypotonic buffer (**lanes 1** and **9**), 5 μg (**lanes 2** and **10**), 10 μg (**lanes 3** and **11**), and 20 μg (**lanes 4** and **12**) of anti-14-3-3σ antibody, 20 μg of NGS (**lanes 5** and **13**), 20 μg of antibody and 5-fold molar excess of its blocking peptide (**lanes 6** and **14**), or same amount of blocking peptide alone (**lanes 7** and **15**). Unmethylated pBS (+ve control; lanes 1–16); pBR322 (−ve control; **lanes 8** and **16**). The relaxed circular (form II), linear (form III), replicative intermediate (RI), and topoisomeric forms of DNA are indicated by arrows end brackets. **b**: Histogram plot of the quantification of the p186 in vitro replication assays after DpnI digestion (lanes 9–15, shown in (a)); the reaction in which the extracts were pre-incubated with hypotonic solution (lanes 1 and 9) was taken as 100% (lane 9, **column 9**). Error bars represent three separate experiments.

whole cell extracts (CV-1 WCE), prepared from either cross-linked or untreated cells, contained NF-κB and SC-35, respectively, while the NGS immunoprecipitate did not contain either protein [Novac et al., 2001]. Here, Western blot analysis showed that the anti-14-3-3σ antibody immunoprecipitate from CV-1 WCE, prepared from either cross-linked or non-cross-linked cells, also contain the 14-3-3σ isoform (Fig. 4, lanes 1 and 2). In contrast, when NGS was used, 14-3-3σ protein was not detected (lane 9). Furthermore, Western blot analysis using anti-14-3-3σ antibody verified that the immunoprecipitated material from the cross-linked cells (logarithmically growing or synchronized at G_0, G_1/S, S, and G_2/M phases of the cell cycle) contained the 14-3-3σ protein (lanes 3–8), and that formaldehyde cross-linking did not affect the immunoprecipitation of 14-3-3σ, since the same amount of this protein was immunoprecipitated from both cross-linked and untreated cells (lanes 1–4).

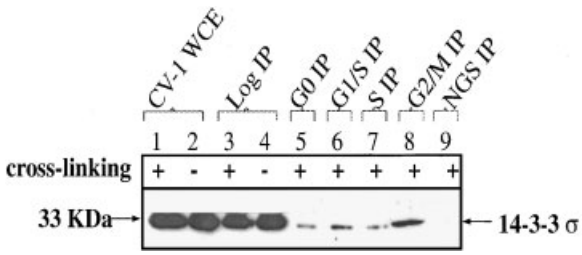

Fig. 4. 14-3-3σ isoform is present in both formaldehyde-treated (cross-linked) and untreated (non-cross-linked) cells. Western blot analysis of material immunoprecipitated with anti-14-3-3σ antibody from either cross-linked (+) or non-cross-linked (−) CV-1 cells. The membrane was probed with 1/400th dilution of anti-14-3-3σ antibody. **Lanes 1** and **2**: Fifty micrograms of cross-linked (+) or non-cross-linked (−) CV-1 whole cell extracts (WCE); (**lanes 3–9**) 1/20th of the immunoprecipitated material from log-phase cross-linked (lane 3) or non-cross-linked (lane 4) cells, from cross-linked (+) cells synchronized at G_0 (lane 5), G_1/S (lane 6), S (lane 7), and G_2/M (lane 8) phases; lane 9, 1/20th of material immunoprecipitated with NGS from cross-linked (+) cells.

14-3-3σ Association With Replication Origins *ors*8 and *ors*12

To analyze whether 14-3-3σ preferentially bound in vivo to DNA sequences that were associated with origins of replication, ChIP assays were performed, in which 14-3-3σ was immunoprecipitated with anti-14-3-3σ antibody from extracts of CV-1 cells that had been previously treated or not with formaldehyde for crosslinking proteins bound in vivo to DNA, as described previously [Novac et al., 2001]. Quantitative PCR of the immnoprecipitated DNA was then performed, using the LightCycler instrument (Roche Diagnostics), for real-time quantification of PCR products, obtained using specific primers for the monkey replication origins *ors*8 [Zannis-Hadjopoulos et al., 1985] (Fig. 5a, bar I) and *ors*12 [Pelletier et al., 1999] (Fig. 5a, bar II), and non-origin sequences EE′ [Pelletier et al., 1999] (Fig. 5a, bar II) and CV-1 CD4 intron [Matsunaga et al., 2000]. Genomic CV-1 DNA was used to generate the standard curves necessary for the quantification of the immunoprecipitated DNA in different genomic regions (data not shown), as previously described [Orlando et al., 1997]. The immunoprecipitated DNA obtained with anti-14-3-3σ antibody, from logarithmically growing CV-1 cross-linked cells, was enriched in *ors*8 and *ors*12 sequence (amplified by primer sets *ors*8 150 and *ors*12 D2, respectively) by approximately 14- and 8-fold, respectively, by comparison to a sequence located approximately 5 kb downstream of *ors*12 (amplified by primer set EE′) (Fig. 5b). In contrast, the abundance of the immunoprecipitated DNA with anti-NFkB and anti-SC-35 antibodies in the origin-containing regions *ors*8 and *ors*12 was similar to that in the non-origin-containing sequence (amplified by primer set EE′), as well as to that immunoprecipitated by NGS (approximately 4×10^{10} molecules/1.5×10^{13} cross-linked CV-1 cells; background level) (Fig. 5b). In addition, when the immunoprecipitation was performed with either anti-14-3-3σ, anti-NF-κB, anti-SC-35 antibodies, or with NGS the DNA abundance in the non-origin-containing sequences (amplified by primer sets EE′ and CD4 intron) corresponded to background levels (Fig. 5b).

Cell Cycle-Dependent Association of 14-3-3σ With *ors*8 and *ors*12

Finally, to quantitatively assess whether the 14-3-3σ isoform associated with replication origins as a function of the cell cycle, quantitative PCR was performed using the LightCycler on CV-1 cells that were synchronized to G_0, G_1/S, S, and G_2/M phases of the cell cycle (see Materials and Methods). The association of the 14-3-3σ isoform with *ors*8 and *ors*12 (primer sets *ors*8 150 and *ors*12 D2), was approximately 18- and 20-fold higher, respectively, at the G_1/S phase, by comparison to its association in serum-starved G_0 cells (Fig. 6), and decreased by 3- and 6-fold, respectively, after 4 h release into the S phase. A slightly higher association of 14-3-3σ with *ors*8 and *ors*12 was observed at G_2/M, by comparison to its association in the S phase (Fig. 6).

DISCUSSION

We previously purified a human cruciform binding protein, CBP [Pearson et al., 1994], and identified it as a member of the 14-3-3 family [Todd et al., 1998]. The direct binding of the CBP/14-3-3 to cruciform DNA has been shown by Southwestern studies and by the cruciform binding activity of purified sheep brain 14-3-3 isoforms [Todd et al., 1998]. By microsequence analysis of isolated peptides and Western blotting with different isoform-specific anti-14-3-3 antibodies, it was previously shown that the β, γ, ε, and ζ isoforms are present in the CBP-cruciform DNA complex [Todd et al., 1998].

In this study, we show the presence also of the 14-3-3σ isoform in the CBP-cruciform DNA

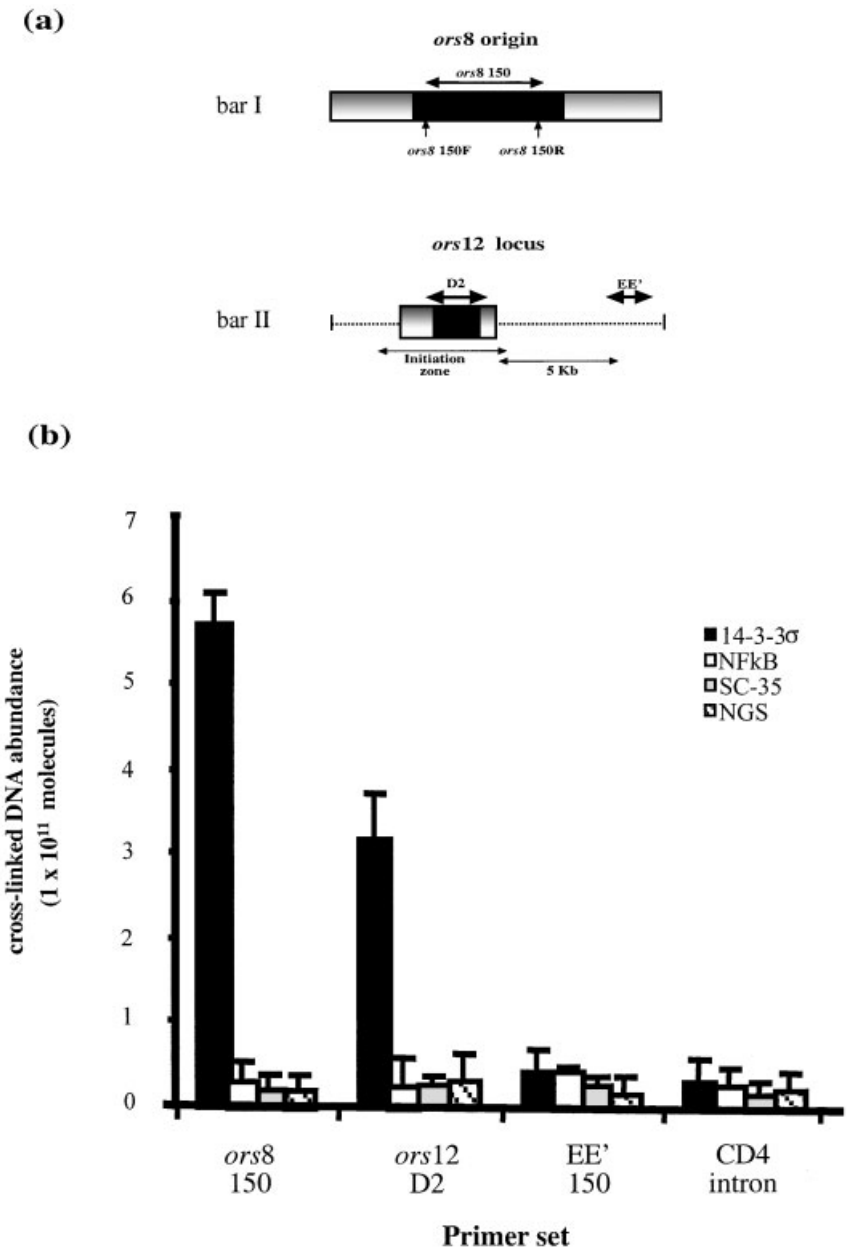

Fig. 5. Association of 14-3-3σ isoform with origins of DNA replication *ors*8 and *ors*12. **a**: Map of the *ors*8 origin (bar I) and the *ors*12 locus (bar II) in CV-1 cells. *Ors*8 origin bar shows location of expected target amplification products of *ors*8, generated by primer set *ors8* 150F and *ors*8 150R. *Ors*12 locus bar shows location of expected target amplification products of *ors12*, generated by primer set D2 or EE′. Primer set EE′ amplifies a fragment located 5 Kb from *ors*12. The black boxes represent the 186-bp or the 215-bp minimal origin of *ors*8 and *ors*12, respectively. **b**: Quantification of DNA abundance in origin-containing sequences and non-origin-containing sequences by real-time PCR using the LightCycler instrument. Total normalized DNA molecules detected by real-time PCR using the LightCycler instrument, from log-phase CV-1 cross-linked 14-3-3σ, SC-35, NF-κB p65, and NGS immunoprecipitates, with primer sets *ors*8 150, *ors*12 D2, EE′, and CD4 intron (see Materials and Methods). Each bar represents three experiments and one standard deviation is indicated.

complex, isolated by preparative band-shift elution. The band-shift eluted CBP had been previously shown to be recognized by anti-14-3-3β, anti-14-3-3γ, anti-14-3-3ε, and anti-14-3-3ζ antibodies [Todd et al., 1998] and, as shown here, the same complex was also recognized by a specific anti-14-3-3σ antibody (Fig. 1b). Thus, the CBP complex contains the 14-3-3 isoforms β, γ, ε, ζ, and σ. The remaining two isoforms, 14-3-3η and 14-3-3τ, have been reported to be particularly expressed in Purkinje cells in the cerebellum [Watanabe et al., 1991] and in T-cells

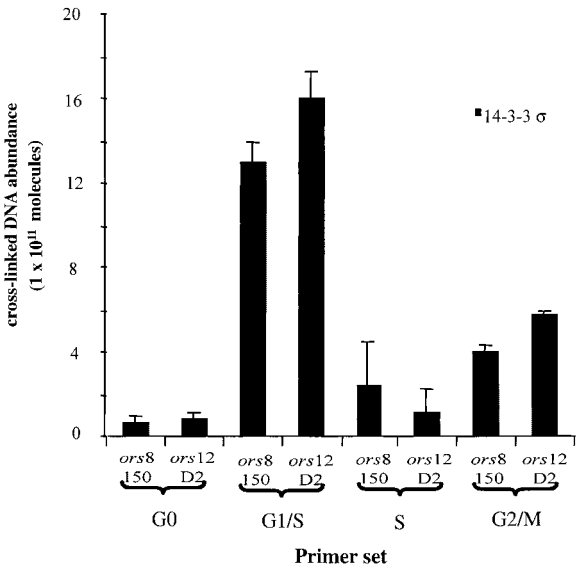

Fig. 6. Cell-cycle dependent association of 14-3-3σ with *ors*8 and *ors*12. Template DNA from log-phase CV-1 cells, and from G_0, G_1/S, or G_2/M synchronized CV-1 cells, with the origin-specific primer sets *ors*8 150 and *ors*12 D2. Total normalized cross-linked molecules detected by PCR, from cross-linked 14-3-3σ immunoprecipitates, at different points in the cell cycle, are shown in the diagram. Each bar represents three experiments and one standard deviation is indicated.

[Aitken, 1996], respectively, and were not included in this study, as it is unlikely that either of them would be found in the HeLa cell CBP complex. It is likely, however, that both 14-3-3η and 14-3-3τ would have CBP activity in these respective cells. In contrast, another nuclear protein, namely the Ku70 subunit of the heterodimeric Ku protein, whose molecular weight of 70 kDa is of approximately the same size as the dimeric molecular weight of functional CBP (60–70 kDa), was not detected in the CBP complex, although it reportedly binds to DNA ends and secondary DNA structures [Blier et al., 1993; Tuteja and Tuteja, 2000], as well as to origins of DNA replication [Novac et al., 2001].

The anti-14-3-3σ antibody specifically interfered with the formation of CBP-cruciform DNA complex in the band-shift reactions, resulting in an increase in the relative amount of free cruciform DNA of up to 50% (Fig. 2a,b). Similar results have also been previously obtained using anti-14-3-3β, anti-14-3-3γ, anti-14-3-3ε, and anti-14-3-3ζ antibodies [Novac et al., 2002], confirming that the CBP-cruciform DNA complex contains five 14-3-3 isoforms (β, γ, ε, ζ, and σ). The binding of 14-3-3 dimeric molecules to cruciform DNA structures was previously shown to be structure-specific and not sequence-specific, since the formation of both the human CBP-cruciform complex and the purified sheep brain 14-3-3-cruciform complex was competed with cruciform DNA, but not with the corresponding linear DNA template [Pearson et al., 1994; Todd et al., 1998]. We found an apparently greater effect of the anti-14-3-3σ antibody on binding to cruciform DNA. The observed high degree of interference of this antibody with the formation of the CBP-cruciform DNA complex might be explained by the fact that 14-3-3 molecules form both homo- and heterodimers [Aitken et al., 1992; Jones et al., 1995; Aitken, 1996; Woo et al., 1997], creating a highly conserved central groove [Xiao et al., 1995; Rosenquist et al., 2000], which is the proposed cruciform DNA binding region [Pearson et al., 1995]. Based on this model, all CBP molecules containing at least one 14-3-3σ polypeptide should be impaired in their binding to cruciform DNA in the presence of saturating amounts of anti-14-3-3σ antibody. Thus, the contribution to CBP, as measured by additive effects of a single specific antibody to a given specific isoform, may be greater than expected if only homodimers of 14-3-3 were involved.

We also report in this study that 14-3-3σ was present in the HeLa nuclear extracts (Fig. 1a). Although 14-3-3 proteins are largely cytoplasmic, we recently showed that the β, ε, ζ, and to a lesser extent γ isoforms are also present in the nucleus [Todd et al., 1998]. Cell fractionation studies with rat brains had previously also provided indirect evidence for 14-3-3 nuclear localization [Martin et al., 1994]. Furthermore, 14-3-3ε was recently found to specifically interact with and modulate the activity of topoisomerase II during DNA replication [Kurz et al., 2000], while upon cell irradiation, 14-3-3 molecules were shown to interact with p53 in nuclear extracts from several cell lines, presumably to increase p53 sequence-specific DNA binding [Waterman et al., 1998]. In contrast, Cas protein (Crk-associated substrate), which functions as an adapter factor for Crk protein to elicit cell migration responses [Klemke et al., 2000] and interacts with 14-3-3 proteins upon cell adhesion [Garcia-Guzman et al., 1999], as well as Crk protein, both exclusively cytoplasmic [Klemke et al., 2000], are absent from the HeLa cell NE (Fig. 1a, lanes 5 and 7).

Previous studies have suggested that a cruciform DNA structure is involved in the regulation of DNA replication [reviewed in Pearson et al., 1996]. Furthermore, previous evidence,

in vivo [Bell et al., 1991] and in vitro [Price et al., 1992; Zannis-Hadjopoulos et al., 1992] suggested that the inverted repeat (IR) present in the 186-bp minimal replication origin of *ors*8 [Todd et al., 1995] extrudes into a cruciform structure. In addition, single-stranded (ss) phages ΦX174 and G4, double-stranded (ds) plasmids R1162 and pT181, mitochondrial origins of replication, as well as Epstein-Barr virus are examples where cruciforms, or inverted repeats, are essential in DNA replication [reviewed in Pearson et al., 1996]. Finally, when anti-cruciform antibodies were introduced into permeabilized cells, a 2–11-fold increase in DNA synthesis was observed, and attributed to the stabilization of cruciforms present at replication origins [Zannis-Hadjopoulos et al., 1988]. Here, results of in vitro replication assays (Fig. 3) suggest that 14-3-3σ is involved in DNA replication, since pre-incubation of HeLa cell extracts with up to 20 μg of anti-14-3-3σ antibody decreased the efficiency of p186 plasmid replication by approximately 70%. A similar inhibitory effect on in vitro replication assays was also previously obtained using anti-14-3-3β, anti-14-3-3γ, anti-14-3-3ε, and anti-14-3-3ζ antibodies [Novac et al., 2002]. The decrease in the levels of replication was specifically caused by the presence of anti-14-3-3σ antibody, since restoration of normal replication levels was observed when saturating amounts of a specific blocking peptide neutralized the antibody. The inhibitory effect of anti-14-3-3σ antibody was also higher than expected if only homodimers of 14-3-3 molecules would be binding to cruciform DNA. The data suggest that the mechanism by which 14-3-3σ modulates the in vitro DNA replication of p186 is by binding to the cruciform forming at the IR sequence present in the 186-bp sequence [Todd et al., 1995]. A possible mechanism might be that in the presence of anti-14-3-3σ antibody, CBP molecules containing at least one 14-3-3σ polypeptide are unable to bind to the cruciform at the origin, resulting in decreased initiation of replication from that origin. As mentioned before, monoclonal antibodies raised against cruciform DNA increased the levels of in vitro DNA replication, presumably by stabilizing cruciforms at replication origins, thereby increasing the frequency of initiation from those origins [Zannis-Hadjopoulos et al., 1988]. The present results further support the hypothesis [Zannis-Hadjopoulos et al., 1988] that cruciform stabilization at replication origins may facilitate origin recognition by proteins of the replication machinery, promoting DNA replication.

Finally, using a ChIP assay [Novac et al., 2001, 2002], 14-3-3σ was found associated with the monkey origins of replication *ors*8 and *ors*12 in vivo, as was also shown recently for isoforms β, γ, ε, and ζ [Novac et al., 2002]. The 14-3-3σ isoform was found to be associated with *ors*8 and *ors*12 origins approximately 14- and 8-fold higher, respectively, than it was associated with other non-origin-containing sequences in log-phase cells (Fig. 5b). The association of 14-3-3σ with these origins was similar to that of the γ, ε, and ζ, as determined in previous studies [Novac et al., 2002]. Furthermore, the association of 14-3-3σ with *ors*8 and *ors*12 was cell cycle regulated. In G_1/S phase, it was approximately 19-fold higher than in G_0 cells, decreasing by approximately 5-fold upon entry in S phase, and increasing slightly at G_2/M compared with its S phase association (Fig. 6). 14-3-3σ is an intrinsic cytoplasmic regulator for entry into M phase [Hermeking et al., 1997; Chan et al., 1999], while 14-3-3 proteins, in general, normally interact with p53 upon irradiation during the G_2/M checkpoint [Waterman et al., 1998]. Furthermore, topoisomerase II, an essential protein for chromatin condensation [reviewed in Withoff et al., 1996] was found to be modulated by 14-3-3ε during DNA replication [Kurz et al., 2000]. Thus, a possible explanation for the slightly higher association of 14-3-3σ with origins of replication in G_2/M, by comparison to that in S phase, might be that its interaction with other factors plays a role in repressing the replication origins and/or contributing to chromatin condensation via its binding to cruciform structures.

The role of 14-3-3σ seems similar to that of the γ, ε, and ζ isoforms in cruciform DNA recognition and stabilization at origins of DNA replication. 14-3-3β, although also detected in the CBP complex, seems to be less represented in both in vivo binding of origins and in in vitro DNA replication [Novac et al., 2002].

Here, we propose that 14-3-3σ, in its role as a cruciform binding protein, increases the efficiency of initiation of DNA replication via cruciform DNA binding. This role is not in contradiction with the others that have been assigned specifically to this isoform. Its specific downregulation in breast cancer cells [Vercoutter-Edouart et al., 2001], for example, would

not necessarily preclude initiation of replication from happening in these cells, since other isoforms present in the nucleus that are not downregulated might perform this same task. Thus, apart from being an essential G_2 arrest regulator [Hermeking et al., 1997; Chan et al., 1999; Vercoutter-Edouart et al., 2001], 14-3-3σ may be an enhancing factor for G_1/S transition at the level of initiation of DNA replication.

Overall, the data suggest that homo- or heterodimeric combinations [Jones et al., 1995; Woo et al., 1997] of the 14-3-3 isoforms that are present in the cell nucleus, collectively called CBP, can recognize and bind to cruciform structures that form at origins of replication. The lack of sequence-specificity in CBP binding [Pearson et al., 1994] suggests that the size and shape of the conserved groove formed by dimerization of 14-3-3 proteins are the only essential factors for cruciform DNA recognition and binding.

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