Human carbon catabolite repressor protein (CCR4)-associative factor 1: cloning, expression and characterization of its interaction with the B-cell translocation protein BTG1

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The human BTG1 protein is thought to be a potential tumour suppressor because its overexpression inhibits NIH 3T3 cell proliferation. However, little is known about how BTG1 exerts its anti-proliferative activity. In this study, we used the yeast 'two-hybrid' system to screen for interacting protein partners and identified human carbon catabolite repressor protein (CCR4)-associative factor 1 (hCAF-1), a homologue of mouse CAF-1 (mCAF-1) and *Saccharomyces cerevisiae* yCAF-1/POP2. *In vitro* the hCAF-1/BTG1 complex formation was dependent on the phosphorylation of a putative $p34^{cdc2}$ kinase site on BTG1 (Ser-159). In yeast, the Ala-159 mutant did not interact with hCAF-1. In addition, phosphorylation of Ser-159 *in vitro* showed specificity for the cell cycle kinases $p34^{CDK2}$/cyclin E and $p34^{CDK2}$/cyclin A, but not for $p34^{CDK4}$/cyclin D1 or $p34^{cdc2}$/cyclin B. Cell synchrony experiments with primary cultures of rat aortic smooth-muscle cells (RSMCs) demonstrated that message and protein levels of rat CAF-1 (rCAF-1) were up-regulated under conditions of cell contact, as previously reported for BTG1 [Wilcox, Scott, Subramanian, Ross, Adams-Burton, Stoltenborg and Corjay (1995) Circulation **92**, I34–I35]. Western blot and immunohistochemical analysis showed that rCAF-1 localizes to the nucleus of contact-inhibited RSMCs, where it was physically associated with BTG1, as determined by co-immunoprecipitation with anti-hCAF-1 antisera. Overexpression of hCAF-1 in NIH 3T3 and osteosarcoma (U-2-OS) cells was itself anti-proliferative with colony formation reduced by 67 % and 90 % respectively. Taken together, these results indicate that formation of the hCAF-1/BTG1 complex is driven by phosphorylation at BTG1 (Ser-159) and implicates this complex in the signalling events of cell division that lead to changes in cellular proliferation associated with cell–cell contact.

INTRODUCTION

The regulation of the cell cycle involves a subtle balance between the actions of proto-oncogene and tumour suppressor genes. The expression of these genes is affected by growth factors that bind cell-surface receptors and activate or deactivate specific target genes through secondary messenger systems. Changing the normal expression pattern of these genes can lead to uncontrolled cell proliferation, cell cycle arrest or apoptosis [1]. Proto-oncogenes have been widely studied as positive regulators of the cell cycle and might have therapeutic value in the treatment of cancer [2]. More recently, attention has shifted towards studying proteins involved in cell cycle checkpoint control such as cyclin-dependent kinases (CDKs), cyclins [3] and CDK inhibitors [4], and proteins involved in apoptosis, such as bcl2 and p53 [5].

The *BTG1* locus was discovered by mapping a c-*myc* chromosomal translocation [t(8:12) (q24, q22)] in a B-cell chronic lymphocytic leukaemia (CLL) [6]. The *BTG1* gene is a potential tumour suppressor gene [7], which is evolutionarily conserved and is expressed in most human tissues and cancer cell lines, with preferential expression in CLL cells and in tissues of lymphoid origin [8]. The cDNA encoding *BTG1* contains an open reading frame of 171 residues that has 60 % sequence similarity to *PC3*, an immediate-early gene induced by nerve growth factor in rat PC12 cells [9]. The *BTG1* gene contains a long 3′ AU-rich untranslated region, characteristic of unstable mRNA species such as those found in cell cycle control genes [10]. Furthermore the mature BTG1 protein contains PEST residues (Pro, Glu, Ser and Thr), characteristic of highly unstable proteins [11] including cyclins [12,13] and transcription factors [14]. BTG1 contains a potential $p34^{cdc2}$ kinase site (Ser^{159}), suggesting that BTG1 might be involved in phosphorylation events signalling cell division [15]. *BTG1* expression is high in cultured peripheral blood lymphocytes and mouse NIH 3T3 cells in early G_1 phase and decreases to a minimal level as cells enter S phase. *BTG1* is thought to represent a new class of anti-proliferative genes [8,16–18]. Transfection experiments demonstrated that the over-expression of *BTG1* inhibits NIH 3T3 [7] and rat aortic smooth-muscle cell (RSMC) [18] proliferation. The identification of BTG1 homologues, Tob [16,19], an anti-proliferative protein, and BTG2 [17], whose expression is induced through a p53-dependent mechanism, suggests that these related genes are also important regulators of cell growth. More recently, BTG1 has been shown to be involved in spermatogenesis, in particular terminal spermatid differentiation, which suggests a role consistent with that proposed for the BTG1 family of anti-proliferative genes [20]. These studies suggest that *BTG1* has an important function in the cell cycle.

Abbreviations used: AD, activation domain; BD, binding domain; CCR4, carbon catabolite repressor protein; CAF-1, CCR4-associative factor 1; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; h, human; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; m, mouse; r, rat; RSMC, rat aortic smooth-muscle cell; wt, wild-type; y, yeast.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number L46722.

To understand the function of BTG1 better, we decided to identify proteins that interact with BTG1 by using a yeast 'two-hybrid' screen. Here we report the isolation and characterization of human carbon catabolite repressor protein (CCR4)-associative factor 1 (hCAF-1) through its association with BTG1. We focused on characterizing hCAF-1, its expression and its interaction with BTG1 because of its similarity to the *Saccharomyces cerevisiae* protein, yCAF1/POP2, a transcription factor that regulates gene expression in yeast in stationary phase [21].

MATERIALS AND METHODS

Yeast 'two-hybrid' screen

S. cerevisiae strains YPB2 [Mat**a**, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, can^{R}, gal4-542, gal80-538, LYS::GAL1-HIS3, URA3::(GAL17mers)-lacZ] and Y153 (Mat**a**, ura3-52, his3, ade2-101, lys2-801, trp1-901, leu2-3,112 gal4, gal80, LYS2::GAL1 HIS3, URA3::GAL lacZ) were used in library screening and retesting. Transformation was performed by using a modified method of Schiestle and Gietz [22], followed by plating of transformants on synthetic complete (SC) medium [23]. The plasmid pGal4 binding domain (BD)–BTG1 was used as the 'bait' in screening a gal4 activation domain (AD) HeLa cDNA library (Clontech Laboratories, Palo Alto, CA, U.S.A.).

Library screening and retesting

Positive library clones showed β-galactosidase activity in plate and liquid assays. For the plate assay, yeast transformants were transferred to Whatman no. 3 paper, permeabilized by placing in liquid nitrogen, and placed in Z-buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM MgCl/50 mM 2-mercaptoethanol) containing 1.0 mg/ml 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside. For the liquid-assay cultures, yeast extracts were prepared by resuspending yeast cell pellets obtained from a culture growing exponentially in lysis buffer [100 mM potassium phosphate (pH 7.8)/0.2% (v/v) Triton X-100/1 mM dithiothreitol/1 mM PMSF] containing 300 mg of 425–600 μM acid-washed glass beads (Sigma Chemical Co., St. Louis, MO, U.S.A.) and centrifuged for 1 min at high speed in a microcentrifuge. β-Galactosidase activity was detected in the resulting supernatants by using the Galacto-light™ chemiluminescence reporter assay (Trophix, Bedford, MA, U.S.A.). For retesting, total yeast DNA was extracted by the method of Hoffman and Winston [24] from yeast transformants and used in the transformation of competent *Escherichia coli* HB101 cells (Gibco–BRL, Gaithersburg, MD, U.S.A.). Plasmids encoding interacting gal4(AD)–cDNA fusions were isolated by plating the resulting transformants on M9 minimal essential medium [25].

DNA sequencing and sequence analysis

Plasmids containing *hCAF-1* and *BTG1* were analysed by double-stranded sequencing with Sequenase version 2.0 (United States Biochemical, Cleveland, OH, U.S.A.). Protein sequences were analysed with the GCG Wisconsin Package, version 8.0, September 1994.

Identification of additional hCAF-1 clones

An *hCAF-1*-specific 1.0 kb *Bam*HI/*Xho*I fragment was cut from pGal4(AD)–hCAF-1 and purified by using low-melt agarose with gelase (Epicentre Technologies, Madison, WI, U.S.A.). This fragment was labelled with [α-^{32}P]CTP by using the Prime-a-Gene labelling system (Promega Corp., Madison, WI), purified through a TE +100 spin column (Clontech Laboratories) and used to probe a HeLa UniZAP™ XR library (Stratagene, La Jolla, CA, U.S.A.).

Genomic Southern blot and Northern blots

A human genomic blot was obtained (Clontech Laboratories). This blot was hybridized with a 700 bp hCAF-1 probe fragment produced by PCR with the primers 5′-GAGCAATGCTGACTATC-3′ and 5′-TTAAGATCTGAGATGG-3′. This fragment was purified from a 6% (w/v) polyacrylamide gel with the crush and soak method [26], labelled with [α-^{32}P]CTP (Dupont–NEN, Boston, MA, U.S.A.) by using the Prime-a-Gene kit (Promega Corp.) and purified through a NucTrap probe purification column (Stratagene).

Human multiple tissue poly(A)$^{+}$ mRNA blots and a cancer cell line blot were obtained (Clontech Laboratories). Total RNA was isolated from RSMCs by using a Total RNA Isolation Kit (Stratagene); 10 μg aliquots were separated by electrophoresis and transferred to Zeta-Probe GT membranes (Bio-Rad, Burlingame, CA, U.S.A.). Northern blots of human tissues and cancer cell lines were hybridized with the *hCAF-1*-specific 1.0 kb *Bam*HI/*Xho*I fragment from pGal4(BD)–hCAF-1 at 68 °C for 2 h in Express-Hyb Hybridization Solution (Clontech Laboratories). The blots were stripped and hybridized with an [α-^{32}P]CTP-labelled full-length BTG1 probe generated by PCR from BTG1 in pRc/CMV. Northern blots of total RNA isolated from RSMCs were hybridized with the *hCAF-1*-specific 1.0 kb *Bam*HI/*Xho*I fragment from pGal4(BD)–HCAF-1 at 65 °C for 18 h in 0.25 M Na_2HPO_4 (pH 7.2)/7% (w/v) SDS. The membrane was washed twice at 65 °C in 20 mM Na_2HPO_4 (pH 7.2)/5% (w/v) SDS for 30 min each. Final washes were at 65 °C in 20 mM Na_2HPO_4 (pH 7.2)/1% (w/v) SDS for 30 min each.

Plasmid construction and gene expression

BTG1-specific cDNA was obtained by reverse transcriptase-mediated PCR of total RNA from a human fibroblast cell line, AG1523. The resulting cDNA was cloned in pRc/CMV (InVitrogen, Carlsbad, CA, U.S.A.). The BTG1Thr^{158}Ala (Thr^{158} → Ala) and BTG1Ser^{159}Ala (Ser^{159} → Ala) mutants were generated in PCR reactions with mutated primers from wild-type (wt) BTG1 cDNA, cloned into pRc/CMV and sequenced to confirm these mutations. The gal4(BD)–BTG1 and gal4(BD)–BTG1 mutant fusion plasmids were constructed in pGBT9 [27]. The DNA species encoding wt BTG1 and the BTG1Thr^{158}Ala and BTG1Ser^{159}Ala mutants were amplified by PCR from the pRc/CMV constructs with the oligonucleotide primers 5′-GGGGAATTCATGCATCCCTTCTACACCCGG-3′ and 5′-GGGGAATTCTTAACCTGATACAGTCATCAT-3′, cut with *Eco*RI and ligated into pGBT9 previously cut with *Eco*RI and dephosphorylated with HK™ phosphatase (Epicentre Technologies). These plasmids were designated pGal4(BD)–BTG1wt, pGal4(BD)–BTG1Thr^{158}Ala and pGal4(BD)–BTG1Ser^{159}Ala.

For the expression of hCAF-1 as a glutathione S-transferase (GST) fusion protein, a 1 kb hCAF-1 *Bam*HI/*Xho*I fragment from pGal4(BD)–hCAF-1 was purified by passage through a TE +100 spin column (Clontech Laboratories) and ligated into the *Bam*HI/*Xho*I site of pGEX-KG. For the expression of BTG1 as a GST fusion protein [28], wt BTG1 and the BTG1Thr^{158}Ala and BTG1Ser^{159}Ala mutants were amplified by PCR from the pRc/CMV clones (see above) and ligated into the *Eco*R1 site of pGEX-KG. These plasmids were transformed into competent *E. coli* BL21. After induction with 1 mM isopropyl β-D-thiogalactoside (0.238 g/l) for 4 h, the GST fusions were purified by using glutathione agarose beads (Pharmacia, Chicago, IL, U.S.A.).

Transcription and translation *in vitro*

The *hCAF-1* coding sequence was amplified by PCR from pGal4(BD)–hCAF-1 with the primers 5′-ATTAAAGCTTCACCATGCCAGCGGCAACTGTAG-3′ and 5′-ATTATCTAGATTAAGATCTGAGATAGGAACGGT-3′, which contain an artificial Kozak consensus site for translation initiation, and *Hin*dIII and *Xba*I restriction sites for directional cloning into pRc/CMV. For expression this construct was transcribed and translated *in vitro* with the TNT rabbit reticulocyte lysate kit (Promega Corp.).

Antibodies

HCAF-1-specific antibody was developed by immunizing rabbits with two N-terminal peptides, N-1 (^{47}VARPIGEFRSNADYQYQLLR^{66}) and N-2 (^{82}MNEQGEYPPGTSTWQFNF^{99}), and a C-terminal peptide, ^{265}GSSYVQNGTGNAYEEEANKQS^{285}, coupled to keyhole-limpet haemocyanin and emulsified in incomplete Freund's adjuvant. BTG1-specific antibody was developed against the C-terminal peptide ^{156}GRTSPSKNYNMM^{167} in the same manner as hCAF-1 antibodies. An antibody against gal4, recognizing both gal4(BD) and gal4(AD) in total yeast extracts, was kindly provided by James Hopper (Hershey Medical Center, Hershey, PA, U.S.A.). Bound antibody was detected in Western blot assays by using goat anti-(rabbit IgG) peroxidase (Gibco–BRL) or the Vectastain Elite rabbit IgG ABC Kit (Vector Laboratories, Burlingame, CA, U.S.A.) and developed with the Renaissance immunodetection system (DuPont–NEN).

Mammalian cell culture

RSMCs were a gift from Dr. Gary K. Owens (University of Virginia, Charlottesville, VA, U.S.A.). In brief, RSMCs in passages 8–12 were cultured in either Dulbecco's modified essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum or serum-free medium as described previously [29]. Cells were harvested under four different growth conditions. Cells at approx. 50% confluence were harvested (subconfluent, 10% serum) or placed in serum-free medium (subconfluent, serum-free medium) for 5 days, then harvested. Cells at confluence were maintained on either 10% (v/v) serum (confluent, 10% serum) or in serum-free medium (confluent, serum-free medium) for an additional 5 days. HeLa, NIH 3T3 and U-2-OS cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum.

Immunohistochemical analysis

RSMCs were grown on eight-well Lab-Tek Chamber Slides and fixed. Slides were blocked in a 1:66 dilution of normal goat serum and 1% (w/v) BSA (albumin, Fraction V; globulin and fatty acid-free; Sigma Chemical Co.). The hCAF-1 C-terminal peptide antibody was diluted 1:1000 and incubated overnight at 4 °C. Preimmune rabbit serum was used as a control for the primary antibody. After being washed with PBS, slides were probed with biotin-conjugated goat anti-(rabbit IgG) followed by development with the Vectastain avidin–biotin ABC reagent (Vector Laboratories) and counterstained with haematoxylin and eosin.

Yeast and mammalian cell extracts

Whole yeast extracts were isolated and analysed in Western blots with gal4 antibody, as described previously [30]. Nuclear and cytoplasmic extracts were prepared from equivalent numbers of RSMCs and HeLa cells [31]. Protein concentrations were determined with the Bioret assay system (Bio-Rad).

Immunoprecipitations

For immunoprecipitations, proteins were labelled with [α-^{35}S]methionine (DuPont–NEN) by using the TNT Coupled Reticulocyte Lysate System (Promega Corp.). Radiolabelled hCAF-1 was incubated with hCAF-1 antibody overnight at 4 °C in 1 × binding buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/5 mM EDTA/0.5% (v/v) NP40] and then incubated with Pansorbin® (Calbiochem, La Jolla, CA, U.S.A.) at 4 °C for 2 h. The immunoprecipitates were washed, resuspended in sample buffer and resolved by SDS/PAGE on 10–20% (w/v) gradient gels (Bio-Rad) followed by autoradiography on a PhosphorImager (Molecular Dynamics).

CDK reactions

Sf9 Bacculoviral extracts containing the cyclin-dependent enzyme complexes CDK4/cyclin D1, CDK2/cyclin A, CDK2/cyclin E and Cdc2/cyclin B were kindly provided by Lou Lamphere and Jenu Gyurius (Mitotix, Cambridge, MA, U.S.A.). In brief, BTG1–GST fusions were incubated with each of the CDK/cyclin enzymes in kinase buffer [50 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/1 mM dithiothreitol/1 μCi [γ-^{33}P]ATP/20 μm ATP] for 10 min at 30 °C. Glutathione beads were used to isolate the BTG1–GST fusions. The glutathione-purified fractions were resolved by SDS/PAGE and labelled protein was detected on a PhosphorImager. As a control for CDK activity, Rb–GST, kindly provided by Jodi Duke (DuPont Pharmaceuticals Company, Wilmington, DE, U.S.A.) was used.

Co-immunoprecipitations

RSMC nuclear extracts were isolated [31] and incubated with hCAF-1 C-terminal antibody (diluted 1:1000) overnight at 4 °C in 1 × binding buffer. Immunoprecipitates were then subjected to electrophoresis and blotted with the BTG1 C-terminal peptide antibody. Bound antibody was detected with the Vectastain Elite rabbit IgG ABC Kit (Vector Laboratories). As a control for non-specific binding of conjugate, preimmune serum and hCAF-1 antibody alone were incubated without extracts, then immunoprecipitated and probed with the secondary antibodies.

BTG1–GST fusions were labelled in a CDK2/cyclin E reaction (see above) containing either [γ^{33}P]ATP ('labelled' reaction) or unlabelled ATP ('unlabelled' reaction). Purified hCAF-1 obtained by thrombin cleavage of the hCAF-1–GST fusion was added to these reactions and incubated for 4 h followed by incubation overnight with hCAF-1 preimmune or post-immune serum to the N-terminal and C-terminal peptides. The labelled reactions were subjected to electrophoresis and analysed on a PhosphorImager. To determine that the unphosphorylated BTG1Ser^{158}Ala mutant did not bind hCAF-1, the unlabelled reaction was subjected to electrophoresis and Western blotted with mouse anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and developed with the Vectastain Elite mouse IgG ABC Kit (Vector Laboratories).

Northern blots, Western blots and immunoprecipitations were digitized with a UMAX scanner with Magic Scan (version 1.0), formatted in Adobe Photoshop™ (version 2.0) and copied into Microsoft Power Point (version 3.0) for reproduction. Densitometry was performed on scanned images of Northern blots by using IPLab Gel™ version 1.5c (Signal Analytics Corporation, Vienna, VA, U.S.A.).

Transfections and colony formation assay

NIH 3T3 and U-2-OS cells were seeded on 60 mm plates at 1.8×10^5 per plate and maintained on DMEM supplemented with 10 % (v/v) fetal bovine serum and antibiotics; 24 h after seeding, cells were transfected with Promega's Profectin reagents in accordance with kit instructions. Plasmid DNA (12 μg) was used for each transfection; transfections were performed in triplicate. At 18 h after addition of the precipitate, plates were washed twice with growth medium and cultured in fresh medium for an additional 30 h. Each transfection was trypsin-treated, counted and replated in triplicate at a density of 8500 cells per 100 mm plate in growth medium containing 600 μg/ml active G418. Selection was continued for 11 days, with medium being replaced every 3 days. Colonies were stained with 2 % (w/v) Methylene Blue in 50 % (v/v) ethanol and counted. For statistical analysis the colonies from the vector-only group and the experimental groups, hCAF-1 and BTG1, were counted on two or three plates and the means $\pm$ S.D. were calculated. The percentage decrease in colony formation was calculated by subtracting the average number of colonies on the experimental plates (for each treatment) from the average number of colonies on the control plates, dividing by the average number of colonies on the control plates and multiplying by 100. The S.D. in the percentage reduction was calculated by propagating the uncertainty in the average colony counts (as measured by the experimental standard deviations) through the same formula [32].

Table 1 Specific interaction between the hCAF-1 cDNA clones and BTG1 in the yeast 'two-hybrid' system

pGal4(BD) constructs expressing fusions with BTG1, BTG1 mutants and p53 were co-transformed into yeast strains YPB2 (columns 3 and 4) and Y153 (column 3) with pGal4(AD) constructs expressing fusions with hCAF-1, T-antigen or gal4 wt. Plasmids expressing gal4 wt and p53 and T-antigen were used as positive controls. As a negative control, pGal4(BD) and pGal4(BD)–p53 were co-transformed with pGal4(AD)–hCAF-1. Yeast extracts from two independent YPB2 transformants selected by trp^+leu^+ prototrophy were assayed in triplicate for β-galactosidase activity; the S.E.M. was less than 20 %. β-Galactosidase activity is represented in Galacto-light™ units/mg of protein.

gal4(BD) plasmid	gal4(AD) plasmid	Relative β-galactosidase activity	Quantitative β-galactosidase activity (Galacto-light units/mg)
BTG1	Gal4-AD	−	0
Gal4(BD)	gal4 wt	++++	3439
p53	T-antigen	+	37
BTG1	Bbf1 clone 1	++	76
	Bbf1 clone 2	++	120
Gal4(BD)	Bbf1 clone 1	−	0
	Bbf1 clone 2	−	0
p53	Bbf1 clone 1	−	0
	Bbf1 clone 2	−	0
BTG1Ser^{159}Ala	Bbf1 clone 1	−	0
BTG1Thr^{158}Ala	Bbf1 clone 1	−	0

RESULTS

Screening for BTG1-interactive proteins with the yeast 'two-hybrid' system

The yeast 'two-hybrid' system [23] was used to screen a library expressing the gal4(AD) fused to HeLa cell cDNA for proteins that interact with BTG1 [33]. As a 'bait', the plasmid pGal4(BD)–BTG1, which expresses the entire 171 residues of human BTG1 fused in-frame with the gal4(BD) was used. To identify cDNA clones that interact with BTG1, yeast cells harbouring pGal4(BD)–BTG1 were transformed with a HeLa cDNA library fused to the gal4(AD); 20 positive colonies were isolated from 10^6 transformants, three of which demonstrated high levels of β-galactosidase activity.

To determine whether these transformants expressed proteins that specifically interacted with BTG1, the cDNA-containing plasmids were rescued from the positive yeast colonies and co-transformed with either pGal4(BD) or pGal4(BD)–BTG1 into the yeast strains YPB2 and Y153 [27]. As a negative control, these plasmids were also co-transformed with a gal4(BD) plasmid that was fused to an unrelated protein, human p53. Transformants expressing proteins encoded by the positive cDNA species and BTG1 were positive, confirming a specific interaction. Quantitative β-galactosidase assays showed that these transformants had greater levels of activity than yeast cells co-transformed with plasmids expressing both human p53 and T-antigen but lower levels of activity than plasmids expressing gal4(wt) (Table 1).

Identification of hCAF-1

The three *BTG1*-interactive clones were sequenced to determine whether they represented novel or previously identified genes. Searching GenBank [34,35] revealed that each of the cDNA species showed sequence similarity to a fetal brain cDNA clone [36] HFBCZ09 (accession no. M86044), the yeast transcription factor yCAF-1/POP2 [17] (accession no. D12808) and the murine homologue of yCAF-1, mCAF-1 [27]. These sequences were identical except for 37 bp absent from the 5′ end of clone 2. These clones were designated pGal4(AD)–hCAF-1-1, -2 and -3, and the protein encoded by the cDNA species was designated human CAF-1 (hCAF-1). Clone HFBCZ09 (American Type Culture Collection, Rockville, MD, U.S.A.) was sequenced and found to be identical with the entire sequence of *hCAF-1* clones 1 and 3 and contained an additional 240 bp of 5′ untranslated region.

HCAF-1 clones 1, 2 and 3 contain an uninterrupted open reading frame in-frame with gal4(1–147). The open reading frame is followed by 169 bp of 3′-untranslated region, a polyadenylation site and a poly(A)$^+$ tail. Three possible methionine codons preceded by sequences with similarity to a Kozak consensus sequence for translation start sites [10,37,38] were located 4, 73 and 118 bp downstream of the gal4(AD) fusion junction of clone 1. Thus the mature protein(s) of hCAF-1 could contain 285 residues (molecular mass 32744 Da, pI 4.6), 262 residues (molecular mass 30145 Da, pI 4.71) and 245 residues (molecular mass 28253 Da, pI 4.42). We note that there are a number of acidic residues (126–138, 175–185, 210–230 and 274–285), resembling acidic eukaryotic transcription factors such as gal4 and VP16 [39,40]. HCAF-1 is 64 % similar at the protein level to yCAF1/POP2 (Figure 1) and 99 % similar to mCAF-1 [41].

Southern blots of human, mouse and rat genomic DNA digested with several restriction endonucleases and analysed with an hCAF-1-specific probe revealed several bands in each lane, except for digests with *Bam*HI, which showed a single 14 kb band. Northern analysis of RNA from various human tissues and a series of cancer cell lines revealed a major transcript of 2.5 kb and two minor transcripts of 1.3 and 4.0 kb (results not shown). The *hCAF-1* transcript was present in RNA species of all human tissues and cancer cell lines tested. The highest levels were observed in heart, skeletal muscle, testis, ovary and pancreas, whereas lower levels were observed in liver, kidney, prostate and peripheral blood lymphocytes.

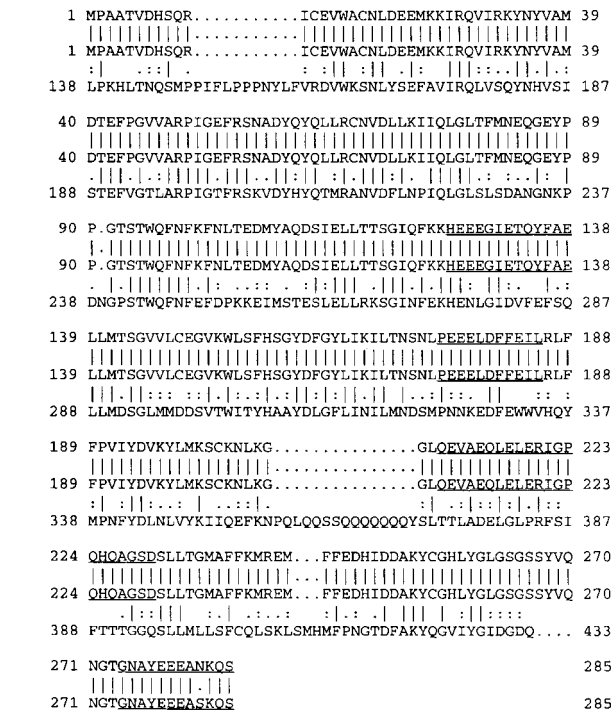

Figure 1 Amino acid sequence alignment of hCAF-1 (top), mCAF-1 (middle) and POP2 (bottom) with the use of the Gap algorithm [36]

Corresponding amino acid residues are numbered at left and right. Vertical lines indicate sequence identity and colons and full points represent conserved amino acid substitutions. Acidic regions of hCAF-1 and mCAF-1 are underlined.

HCAF-1 is concentrated in nuclear extracts of HeLa cells and RSMCs

To examine the expression level of hCAF-1, rabbit antibodies were generated against peptides derived from the N-terminus (N-1, N-2) and C-terminus. An antibody was also generated against a peptide found at the C-terminus of BTG1. The reactivity of the C-terminal antibodies to the hCAF–1-GST and BTG1–GST fusions is shown in Figure 2(A). The ability of hCAF-1 C-terminal antibody to immunoprecipitate hCAF-1 translated *in vitro* is shown in Figure 2(B). Anti-BTG1 antibody weakly immunopreciptated BTG1 translated *in vitro* (results not shown). The antibodies against hCAF-1 N-1 and N-2 showed similar reactivities to the C-terminal hCAF-1 antibody in Western blots of hCAF-1–GST fusions and immunoprecipitations of hCAF-1 translated *in vitro* (results not shown).

To determine whether hCAF-1 and BTG1 were concentrated in a specific cellular compartment of RSMCs, cytoplasmic (Figure 2C, lanes 1–3) and nuclear (Figure 2C, lanes 4–9) extracts were probed with these antibodies. HCAF-1 C-terminal antibody recognized 32 and 45 kDa bands in RSMC nuclear extracts (Figure 2C, lane 5, arrows). These bands could be competed out for binding by preincubation with hCAF-1 C-terminal peptide (Figure 2C, lane 6). The other bands recognized by hCAF-1 C-terminal antibody (Figure 2C, lane 5) were also recognized by preimmune serum (Figure 2C, lane 4) and were not competed for by peptide (Figure 2C, lane 6). The 32 kDa form of hCAF-1 was not detected in RSMC cytoplasmic extracts, whereas the 45 kDa band was detected (Figure 2C, lane 2, arrow). The cytoplasmic 45 kDa band was also competed out for binding by the hCAF-1 C-terminal peptide (Figure 2C, lane 3). HCAF-1 N-1 antibody detected the 32 kDa band in RSMC nuclear extracts but not the 45 kDa band (Figure 2C, lane 8). Similarly, this reactivity could also be competed out by preincubation with hCAF-1 N-1 peptide (Figure 2C, lane 9). Antibody against the HCAF-1 C-terminus also detected 30 and 32 kDa bands in Western blots of HeLa cells and a 30 kDa form in NIH 3T3 nuclear extracts (Figure 2D, lanes 2 and 4). These bands were competed out by the addition of the C-terminal peptide (results not shown).

BTG1 antibody recognized a 20 kDa band in Western blots of RSMC nuclear extracts (prepared from RSMCs grown to confluence in the presence of serum) and in HeLa nuclear extracts (Figure 2E, lanes 2 and 4 respectively). BTG1 was not detected in cytoplasmic extracts of RSMCs or HeLa cells (results not shown). Preimmune serum showed no reactivity to nuclear or cytoplasmic fractions (Figure 2E, lanes 1 and 3). Immunohistochemical analysis of RSMCs with C-terminal hCAF-1 antibody detected hCAF-1 concentrated in the nucleus as dense circular foci (Figure 3A). Preimmune serum showed no staining (Figure 3B). Cells stained only with haemotoxylin and eosin are shown in Figure 3(C).

Effect of the proliferative state on the expression of hCAF-1 in primary RSMCs

In contrast with the multiple bands observed in Northern analysis of human RNA, *rCAF-1* RNA is observed predominantly as a single 2.5 kb transcript in RSMCs (Figure 4A). Expression studies with a primary culture of RSMCs show that the level of this transcript increases as cells become confluent (C) (Figure 4A, lanes 3 and 4). The *rCAF-1* message was barely detected in non-confluent (NC) cells (Figure 4A, lanes 1 and 2).

As a control for mRNA expression, the blot was analysed for the presence of the cellular housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Figure 4B). Densitometry showed that the level of GAPDH was equal in cells grown in the presence (+) of serum (Figure 4B, lanes 1 and 3) and equal, but lower, in cells grown in the absence (−) of serum (Figure 4B, lanes 2 and 4). In addition, staining of a duplicate gel with ethidium bromide showed the presence of equal amounts of 28 S and 18 S ribosomal RNA in each sample (results not shown). Densitometry analysis, internally standardized to the levels of GAPDH within each lane, showed a 3-fold increase in *rCAF-1* message levels in RSMCs grown in the presence (+) of serum (Figure 4B, lanes 1 and 3) and a 10-fold increase in the absence (−) of serum (Figure 4B, lanes 2 and 4). A similar pattern of expression is observed for *BTG1* message (M. Corjay, unpublished work).

To determine whether hCAF-1 protein levels were correlated with the increasing mRNA levels, nuclear extracts isolated from RSMCs in non-confluent (NC) and confluent (C) states were probed with hCAF-1 C-terminal antibody (Figure 4C, lanes 1–4) and BTG1 antibody (Figure 4D, lanes 1–4). Preimmune serum did not recognize the 32 and 45 kDa bands (Figure 4C, lanes 5–8). This analysis demonstrated that both rCAF-1 and BTG1 protein levels patterned their message levels, increasing most when cells were confluent (Figures 4C and 4D, lane 4).

The rCAF1/BTG1 complex is present in RSMCs under contact-inhibited growth conditions

To determine whether the rCAF-1/BTG1 complex was present in nuclear extracts of dense cultures of RSMCs, co-immunoprecipitation experiments with antibodies against hCAF-1 and BTG1 were performed (Figure 4E). Antibody against the C-terminus of hCAF-1 co-immunoprecipitated BTG1 from RSMC

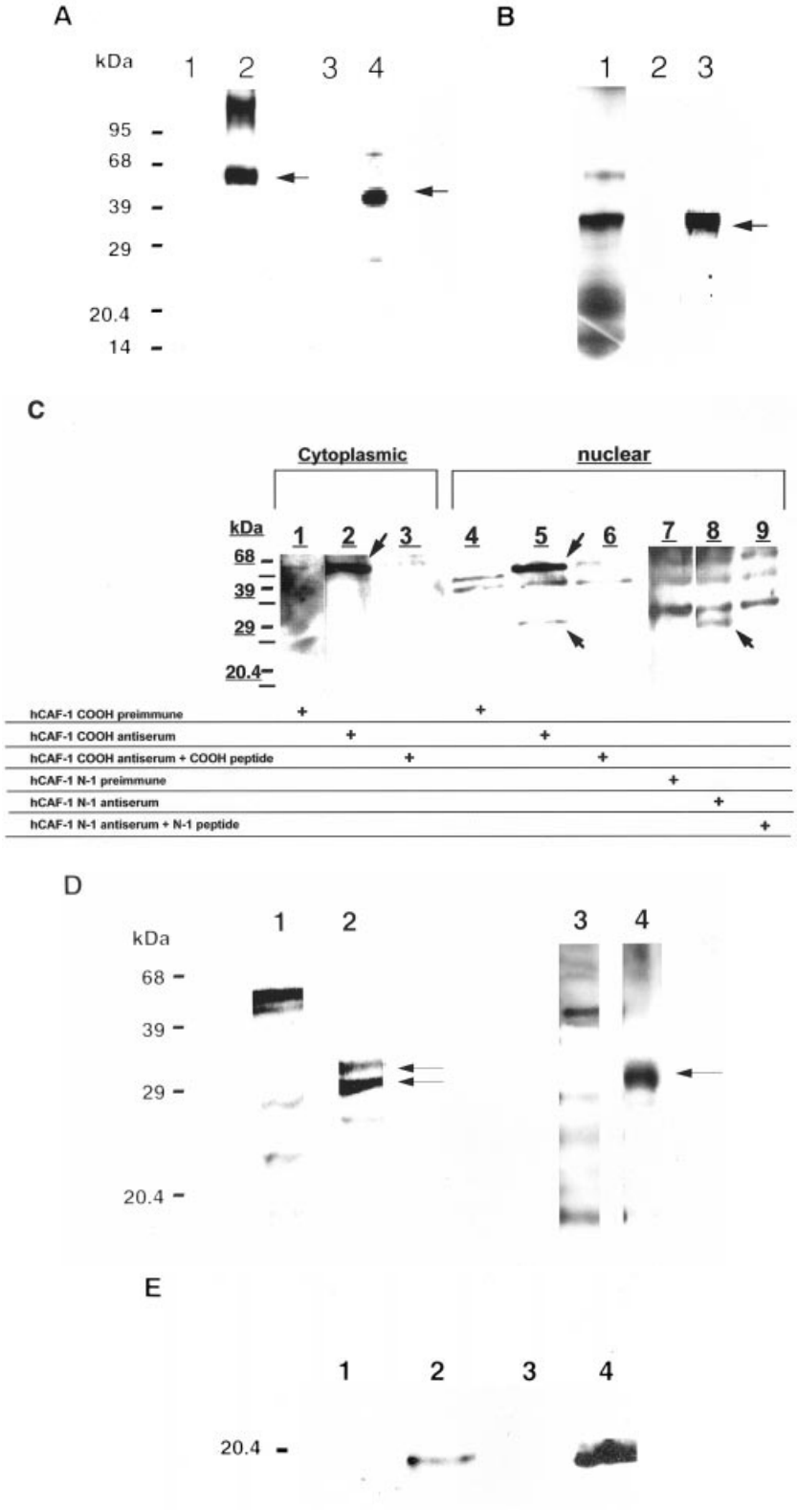

Figure 2 Detection of recombinant and cellular forms of CAF-1 and BTG1

(**A**) Western blot of GST (lane 1) and hCAF-1–GST fusion (lane 2) probed with hCAF-1 antibody (arrow in lane 2 indicates hCAF-1 fusion). GST (lane 3) and BTG1–GST fusion (lane 4) were probed with BTG1 antibody (arrow in lane 4 indicates BTG1 fusion). (**B**) [α-^{35}S]Methionine-labelled hCAF-1 translated *in vitro* (lane 1) and immunoprecipitation of *in vitro* translated hCAF-1 with preimmune serum (lane 2) and C-terminal hCAF-1 antibody (lane 3). (**C**) Cytoplasmic extracts of RSMCs (lanes 1, 2 and 3) and nuclear extracts of RSMCs (lanes 4–9) immunoblotted with either HCAF-1 C-terminal preimmune serum (lanes 1 and 4), hCAF-1 C-terminal antibody (lanes 2 and 5), N-1 hCAF-1 preimmune serum (lane 7) and N-1 hCAF-1 antibody (lanes 8 and 9). HCAF-1 C-terminal peptide was used to compete for binding of hCAF-1 C-terminal antibody in lanes 3 and 6. N-1 hCAF-1 peptide was used to compete for binding of N-1 hCAF-1 antibody in lane 9 (arrows indicate forms of hCAF-1). (**D**) Nuclear extracts of HeLa cells (lanes 1 and 2) and NIH 3T3 cells (lanes 3 and 4) probed with preimmune serum (lanes 1 and 3) or hCAF-1 C-terminal antibody (lanes 2 and 4). (**E**) Nuclear extract of RSMCs (lanes 1 and 2) and HeLa cells (lanes 3 and 4) probed with preimmune serum (lanes 1 and 3) or BTG1 C-terminal antibody (lanes 2 and 4).

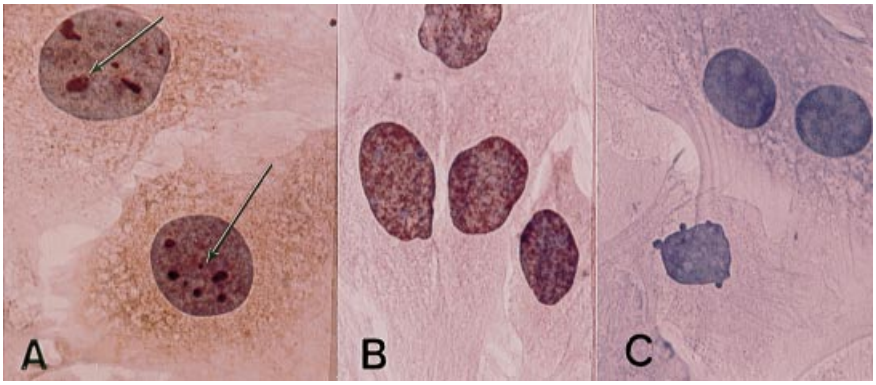

Figure 3 Immunohistochemical analysis of hCAF-1 expression in RSMCs

Confluent RSMCs stained with hCAF-1 C-terminal peptide antibody (**A**), preimmune rabbit serum (**B**) or haematoxylin and eosin (**C**). Arrows denote the localization of hCAF-1 in the nucleus. Magnification 75 ×.

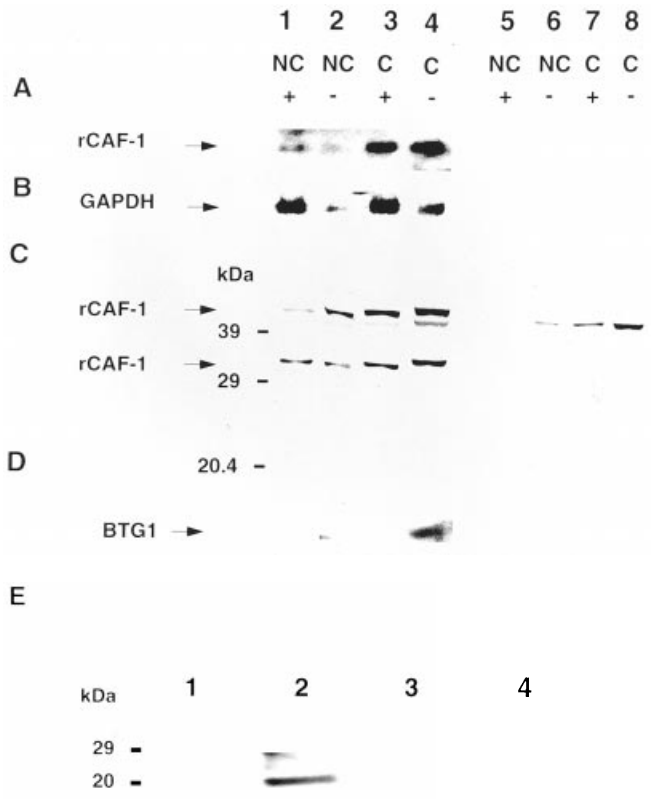

Figure 4 Detection of *rCAF-1* mRNA and rCAF-1 and BTG1 expression levels and complex formation in RSMCs

RSMCs were grown in the presence (+) (lanes 1, 3, 5 and 7) and in the absence (−) (lanes 2, 4, 6 and 8) of serum to non-confluent (NC) and confluent (C) growth states respectively. Arrows show the positions of the *rCAF-1* message, *GAPDH* message, and rCAF-1 and BTG1 proteins. Northern blots of RSMC RNA were analysed with (**A**) an hCAF-1-specific probe and (**B**) a rat GAPDH-specific probe. (**C**) Western blots of the same nuclear extracts were probed with hCAF-1 C-terminal antibody (lanes 1–4) and with preimmune serum (lanes 5–8). (**D**) Western blot of the same extracts probed with anti-BTG1 antibody. (**E**) RSMC nuclear extracts immunoprecipitated with either preimmune (lane 1) or post-immune (lane 2) serum followed by Western analysis with BTG1 C-terminal antibody. As controls for non-specific binding of conjugate to antibody in the immunoprecipitates, preimmune serum (lane 3) and hCAF-1 antibody (lane 4) incubated in the absence of extracts were subjected to electrophoresis and probed in a Western blot with anti-BTG1 antibody.

nuclear extracts (Figure 4E, lane 2) in contrast with preimmune sera (Figure 4E, lane 1). As additional controls for non-specific binding of conjugate to BTG1-immunoprecipitating antibody, anti-(rabbit IgG) peroxidase conjugate did not itself cross-react in the absence of extracts (Figure 4E, lanes 3 and 4).

Presence of an intact p34^{cdc2} kinase site in BTG1 is necessary for interaction with hCAF-1 in yeast

Phosphorylation at CDK sites mediates many important biological interactions in cells [42]. Initial attempts at demonstrating the hCAF-1/BTG1 interaction by using hCAF-1/BTG1–GST fusions and radiolabelled forms of BTG1 and hCAF-1 translated *in vitro* were unsuccessful (results not shown). These observations suggested that post-translational modifications of these proteins might be important for their association. Because BTG1 contains a putative p34^{cdc2} kinase site at Ser-159 [43] that might be phosphorylated in yeast cells by p34$^{cdc28/cdc2}$ kinase [44], this site was mutated and tested for the interaction of BTG1 and hCAF-1 in the yeast 'two-hybrid' system.

Point mutations were made by PCR-mediated site-directed mutagenesis in pGal4(BD)–BTG1 that changed either Ser^{159}, the putative p34^{cdc2} kinase site, or Thr^{158}, an adjacent residue, to alanine. These plasmids, expressing either gal4(BD)–BTG1Thr^{158}Ala or gal4(BD)–BTG1Ser^{159}Ala fusions, were co-transformed into yeast strain YPB2 with pGal4(AD)–hCAF-1. The resulting transformants were *lac*Z-negative and *HIS*3-negative, indicating that neither BTG1 mutant (Ser^{159}Ala or Thr^{158}Ala) interacted with hCAF-1 (Table 1). To show that this was not a result of aberrant protein expression, Western blots of yeast extracts were probed with antibody against gal4. Each co-transformant expressed both the 45 kDa gal4(AD)–hCAF-1 fusion and the 35 kDa gal4(BD)–BTG1 fusion (results not shown), whereas untransformed yeast showed no cross-reactive proteins.

Phosphorylation of BTG1 *in vitro* requires Ser^{159}

To determine whether BTG1 was a substrate for human CDKs, BTG1wt–GST, BTG1Thr^{158}Ala–GST and BTG1Ser^{159}Ala–GST fusions were expressed and purified. Silver staining of the purified BTG1-GST fusions indicated single bands. The fusions were recognized by both BTG1 and GST antibodies (results not shown). These BTG1–GST fusions were phosphorylated with

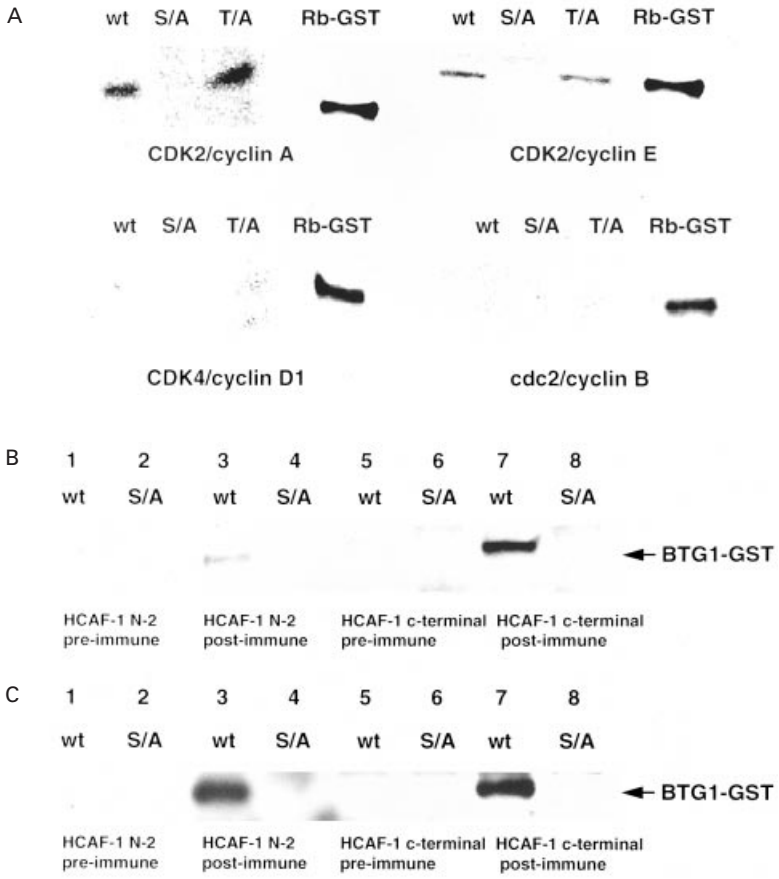

Figure 5 Mapping of the hCAF-1/BTG1 interaction to a p33^{cdk2} site on BTG1

(**A**) Phosphorylation of BTG1–GST fusions with different CDK/cyclin enzyme complexes. BTG1–GST fusions are depicted as follows: BTG1–GST (wt), BTG1Ser^{159}Ala–GST (S/A) and BTG1Thr^{158}Ala–GST (T/A). As a control for CDK activity, a retinoblastoma–GST (Rb-GST) fusion was phosphorylated by each enzyme. CDK/cyclin complexes used in each phosphorylation assay are indicated. (**B**) Co-immunoprecipitation of BTG1/hCAF-1 complex using labelled P33γ-ATP detected by PhosphorImager analysis. (**C**) Co-immunoprecipitation of BTG1/hCAF-1 complex with unlabelled ATP detected by Western analysis with anti-GST antibody. Input BTG–GST fusions in CDK2/cyclin E enzyme reactions are depicted at the top of each lane and immunoprecipitating antibody is shown at the bottom of each lane. BTG–GST fusions (25 μg) were placed in kinase reactions followed by 100 ng of purified hCAF-1. The complexes were immunoprecipitated with a 1:100 dilution of preimmune serum or anti-(hCAF-1) antibody.

various combinations of CDK/cyclin complexes. BTG1wt–GST and BTG1Thr^{158}Ala–GST fusions were phosphorylated by CDK/cyclin E and cyclin A (Figure 5A) but not by CDK4/cyclin D1 and cdc2/cyclin B. However, the BTG1Ser^{159}Ala–GST fusion was not phosphorylated by any of the CDK/cyclin complexes (Figure 5A). Retinoblastoma–GST fusion was used as a positive control for kinase activity.

Phosphorylation of BTG1 Ser^{159} mediates its association with hCAF-1 *in vitro*

To determine whether the interaction of hCAF-1 was due to the phosphorylation of BTG1 by CDK2/cyclin E, phosphorylation reactions *in vitro* containing either BTG1wt–GST fusion or BTG1Ser^{159}Ala–GST fusion were incubated with hCAF-1 and immunoprecipitated with antibody against hCAF-1. The BTG1wt–GST fusion was detected when hCAF-1 antibody was used to immunoprecipitate the complex (Figure 5B, lanes 3 and 7). Preimmune serum did not immunoprecipitate the complex (Figure 5C, lanes 1 and 5). When the reactions contained unlabelled ATP, Western blotting of the immunoprecipitates with GST-specific antibody detected the BTG1wt–GST fusion (Figure 5C, lanes 3 and 7) but did not detect BTG1Ser^{158}Ala–GST fusion in the immunoprecipitates (Figure 5C, lanes 4 and 8). Thus the interaction of BTG1 with hCAF1 was mediated *in vitro* by the phosphorylation of Ser^{159}, consistent with the observation that an intact Ser^{159} site is required for interaction in yeast. Interestingly, anti-BTG1 antisera, raised against the Ser^{159} region, failed to co-immunoprecipitate the hCAF-1/BTG1 complex, suggesting that this region of BTG1 might be masked by association with hCAF-1 (results not shown).

Overexpression of hCAF-1 and BTG1Ser^{159}Ala and BTG1Ser^{158}Ala are anti-proliferative

HCAF-1 displayed anti-proliferative activity in colony formation assays when transfected into NIH 3T3 cells and the human U2-OS cell line (Figure 6). In NIH 3T3 cells, a 67% decrease in colonies was observed, coupled with a 50% decrease in colony size compared with transfection with vector alone (Figure 6, top panel). In the U2-OS cell line, a 90% decrease in colonies was observed, coupled with a 50% decrease in colony size compared with transfection with vector alone (Figure 6, middle panel). BTG1 caused similar growth inhibition to hCAF-1 in both NIH 3T3 and U-2-OS cells. No additional decrease in colony formation was observed when both plasmids encoding hCAF-1 and

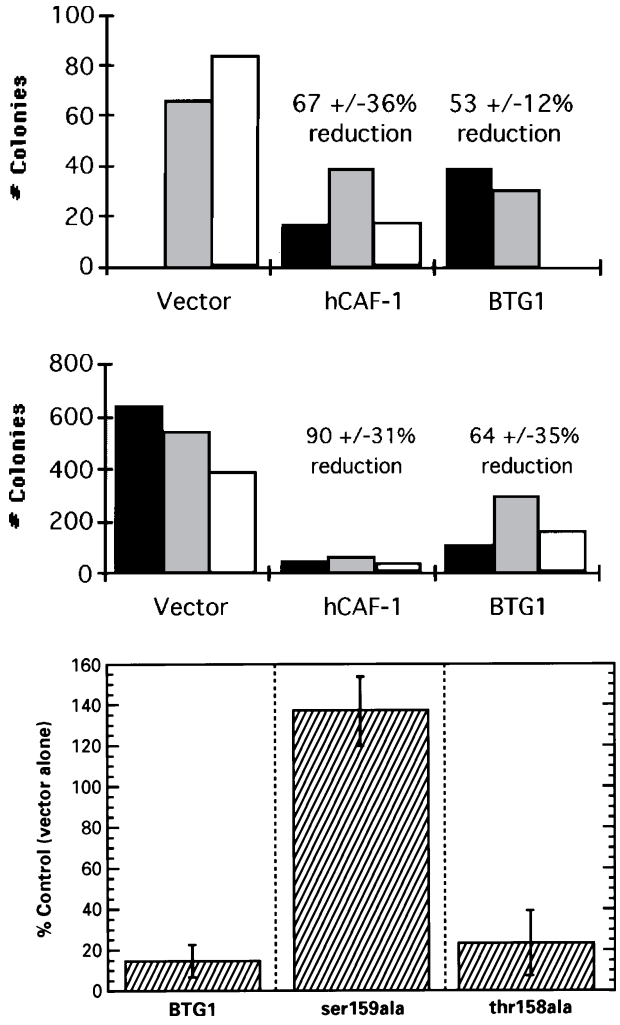

Figure 6 Effect of forced overexpression of hCAF-1, BTG1 and BTG1 mutants

Cells were transfected as described in the Materials and methods section. Colonies were stained and counted after 11 days. Each bar represents the average of each transfection plated in triplicate, with the corresponding percentage inhibition. Top panel, NIH 3T3 cells; middle panel, osteosarcoma cell line U2-OS; bottom panel, BTG1 and BTG1 mutants in NIH 3T3 cells. The bottom panel is an average of six independent transfections, with each transfection done in triplicate.

BTG1 were co-transfected into NIH 3T3 cells (results not shown). In NIH 3T3 cells, BTG1Ser^{159}Ala was devoid of anti-proliferative activity, whereas BTG1Ser^{158}Ala was like BTG1wt (Figure 6, bottom panel).

DISCUSSION

In this paper we have identified and characterized a cDNA encoding the human CAF-1 protein and showed that it binds to the anti-proliferative protein BTG1 as a result of the phosphorylation of BTG1 Ser^{159}. This conclusion is supported by the observations that an intact BTG1 Ser^{159} site is required for (1) phosphorylation of recombinant BTG1 *in vitro*, (2) binding of BTG1 with hCAF-1 in the yeast two-hybrid system and (3) binding with hCAF-1 *in vitro*. Additionally, an antibody raised against the Ser^{159} region of BTG1 (which recognizes recombinant BTG1) did not co-immunoprecipitate the hCAF-1/BTG1 complex *in vitro*, whereas an antibody raised against hCAF-1 did so.

We have also shown that hCAF-1 expression, like BTG1, is up-regulated by cell–cell contact, and overexpression by transfection exerts an anti-proliferative effect similar to that of BTG1. We also find that in RSMC cultures with cell–cell contact, anti-hCAF1 antibody co-immunoprecipitates BTG1. These data suggest that hCAF-1 and its family members might represent a new family of anti-proliferative proteins that associate and function with BTG1 and its family members.

At the protein level, hCAF-1 is 99 % similar to murine CAF-1 and similar to CAF-1 proteins from *S. cerevisiae*, *Caenorhabditis elegans* and *Arabidopsis thaliana* [41]. The hCAF-1 protein migrated at the predicted molecular mass of 32 kDa after transcription and translation *in vitro* (Figure 2B). Similar-sized forms were detected in Western blots of nuclear extracts from HeLa cells, RSMCs and NIH 3T3 cells. We also observed a 30 kDa form in HeLa cell nuclear extracts, suggesting that two of the three proposed translation start sites might be used.

In addition to the presence of the 32 kDa band (rCAF-1) in nuclear extracts of RSMC, a 45 kDa band was observed in cytoplasmic and nuclear extracts by using an antibody against C-terminal hCAF-1 (Figure 2C). This result was unexpected because the 45 kDa form was not observed in either HeLa or NIH 3T3 cells (Figure 2D). An antibody against N-terminal hCAF-1 did not detect this band in RSMCs, suggesting that the 45 kDa species differs from the 32 kDa species at the N-terminus. This larger species could arise from an alternatively spliced transcript because the 2.5 kb rCAF-1 transcript observed in Northern blots of RSMCs could potentially give rise to a 45 kDa protein. Alternatively, this could be a related protein found in certain cell types. For example, a recent report has identified a BTG1 homologue, Tob, that contains significant sequence similarity to the N-terminus of BTG1 but contains an additional C-terminal region [16,19].

Both rCAF-1 forms increased proportionally with the 2.5 kb message observed in cell synchrony experiments, suggesting that their expressions are regulated in a similar manner (Figures 4A and 4C; compare lane 4 with lane 2, and lane 3 with lane 1). The 3′ untranslated region of the *hCAF-1* message is short and does not seem to contain any motifs conferring RNA stability [45], suggesting hCAF-1 levels might be influenced by transcript half-life. Alternatively the amount of the hCAF-1 might be regulated at the level of translation, a commonly observed trait of proto-oncogenes [38]. Because the 45 kDa form seems immunologically related to the 32 kDa form at the C-terminus, it might also associate with BTG1. Other CAF-1 family members might exist that contain regions similar to the C-terminus but differ at the N-terminus. Therefore these potential family members might be capable of binding to BTG1 or related proteins in different contexts.

RCAF-1 is concentrated in nuclear extracts (Figures 2C and 2D) and is distributed in a punctate pattern within the nucleus of RSMCs as revealed by immunohistochemical staining (Figure 3), resembling the staining of proteins found in the nucleolus [46] and that of BTG1 in the nucleus of QM7 avian fibroblasts [47]. Western analysis shows equal amounts of the 45 kDa form in both nuclear and cytoplasmic compartments (Figure 2C). However, relatively weak immunohistochemical staining of the cytoplasm suggests that the abundance and intensity of the nuclear staining are predominantly due to the 32 kDa form.

The hCAF-1 protein sequence does not contain any obvious DNA-binding consensus sequences [48]; however, hCAF-1 contains a potential basic nuclear localization motif beginning with the sequence Met-Lys-Lys at its N-terminus (Figure 1). This

sequence resembles the SV40 T-Ag nuclear localization signal sequence [49]. Interestingly, an N-terminal truncation mutant of hCAF-1, which lacks this region, was not anti-proliferative on transfection into NIH 3T3 cells (results not shown), perhaps indicating a lack of nuclear localization. However, in some instances nuclear targeting sequences are not required for nuclear localization. For example, a mutant of $p21^{Cip1}$ that lacks its nuclear localization motif is nevertheless found in the nucleus, suggesting that other transport mechanisms such as complex formation might be involved [50]. It is possible that the association of hCAF-1 and BTG1 might direct this localization. In support of this, an anti-peptide antibody raised against the Ser^{159} region of BTG1 detected BTG1 in Western blots of RSMC nuclear extracts (reduced and denatured) but not by immunohistochemical staining. Because the co-immunoprecipitation of the BTG1/rCAF-1 complex by anti-(hCAF-1) antisera showed that these proteins associate in the nucleus of G_0/G_1-arrested RSMCs (Figure 4E), it is possible that the rCAF-1/BTG1 complex masks the epitope recognized by the anti-BTG1 antibody.

Experiments with primary RSMCs show that *rCAF-1* and *BTG1* message and protein levels are up-regulated at confluence in the absence of serum containing growth factors (Figure 4). This suggests a possible mechanism in which cell–cell contact might influence the expression and interaction of rCAF-1 and BTG1. The growth suppressive function of many cell cycle control proteins (retinoblastoma, CDKs and cyclins) is dependent on either phosphorylation or dephosphorylation at critical serine, threonine and tyrosine residues [42,51]. Transient overexpression of hCAF-1 and BTG1 is anti-proliferative in NIH 3T3 cells and U-2-OS cells (Figure 6). Importantly, the anti-proliferative activity of BTG1 in NIH 3T3 cells and in RSMCs [18] is dependent on the same intact Ser^{159} site on BTG1 [43], which these studies indicate is required to drive hCAF1/BTG1 association. We have demonstrated that this site is phosphorylated *in vitro* by the CDK2/cyclin E and CDK2/cyclin A enzymes and that this phosphorylation event mediates its association with hCAF-1 (Figure 5). With respect to the results in the 'two-hybrid' system, we believe a cdc2-like phosphorylation event is mediating their association in yeast. This is plausible because human cdc2 kinase can substitute for its homologue in the fission yeast *Schizosaccharomyces pombe* [52], which is an analogue of cdc28 kinase in *S. cerevisiae* [40,44]. Taken together, these results suggest that the hCAF-1/BTG1 interaction is regulated by contact-inhibition and by CDK2 or a CDK2-like kinase-directed phosphorylation of BTG1 Ser^{159} at the G_1/S transition.

The mechanism by which hCAF-1 and BTG1 mediate their anti-proliferative effects needs further exploration. A recent report indicates that mCAF-1 and yCAF-1/POP2 bind to the yeast nuclear protein CCR4 [41]. Of possible relevance is another report that the Dsb kinase has also been identified as being physically bound to the CCR4 transcriptional machinery [53] and that the disruption of *dsb* resulted in phenotypes and transcriptional defects similar to those found in strains lacking CCR4 or yCAF1. The homology of hCAF-1 to mCAF-1 and yCAF-1 [17,41] suggests that hCAF-1 or the hCAF-1/BTG1 complex might associate with related transcription complexes in mammalian cells. In view of this, it is interesting that the BTG1-related protein, Tob [16], is an inhibitor *in vitro* of the CDK2 phosphorylation of Rb, and that the BTG2 protein [19], a p53-regulated homologue of BTG1, contains the same CDK2-like phosphorylation site as BTG1 (Ser^{159}). Thus it will be important to determine whether hCAF-1 associates and functions with either Tob or BTG2.

Finally we note that hCAF-1, mCAF-1 and yCAF1 contain regions of similarity to the CDK inhibitory domains of $p21^{Cip1}$ [50,54,55], $p27^{Kip1}$ [56,57] and $p57^{Kip2}$ [58,59]. Future studies will address whether hCAF-1 is an inhibitor of CDK kinase activity, and will consider the regulation of hCAF-1 and BTG1 expression and complexation *in vivo*.

We thank Julie C. Bunville for DNA sequencing, Karen L. Krakowski for synthesis of oligonucleotides, Rose Wilk for synthesis of hCAF-1 peptides, Charles W. Plaugher for preparation of hCAF-1 antibody, Allen Bernstein for help in making plasmid constructs, James Hopper for providing gal4 antibody, Robert Leipold for statistical analysis, Lou Lamphere and Jenu Gyurius for CDK/cyclin complexes, Jodi Duke for Rb-GST, and Paul Bartel and Stanley Fields for 'two-hybrid' system reagents, technical advice and helpful discussions. This work was supported in part by the DuPont Merck Post-Doctoral Fellowship Program.

REFERENCES

1 Elledge, S. J. and Harper, J. W. (1994) Curr. Opin. Cell Biol. **6**, 847–852
2 Bishop, J. M. (1991) Cell **64**, 235–248
3 Hunter, T. and Pines, J. (1994) Cell **79**, 573–582
4 Peter, M. and Herskowitz, I. (1994) Cell **79**, 181–184
5 Fisher, D. E. (1994) Cell **78**, 539–542
6 Rimokh, R., Rouault, J. P., Wahbi, K., Gadoux, M., Lafage, M., Achimbaud, E., Charrin, C., Gentilhomme, O., Germain, D. and Samurat, J. (1991) Genes Chromosomes Cancer **3**, 24–36
7 Rouault, J. P., Rimokh, R., Tessa, C., Paranhis, G. G., French, M., Duret, L., Garaccio, M., Germain, D., Samarut, J. and Magaud, J. P. (1993) EMBO J. **11**, 1663–1670
8 Rouault, J.-P., Samurut, C., Duret, L., Tessa, C., Samarut, J. and Magaud, J.-P. (1993) Gene **129**, 303–306
9 Bradbury, A., Possenti, R., Shooter, E. M. and Tirone, F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3353–3357
10 Kozak, M. (1991) J. Cell Biol. **115**, 887–903
11 Rogers, S., Wells, R. and Rechsteiner, M. (1986) Science **234**, 364–368
12 Bai, C., Richman, R. and Elledge, S. J. (1994) EMBO J. **13**, 6087–6098
13 Salama, S., Hendricks, K. B. and Thorner, J. (1994) Mol. Cell. Biol. **14**, 7953–7966
14 Ernst, M. (1995) Mol. Cell. Biol. **15**, 872–882
15 Moreno, S., Labib, K., Correa, J. and Nurse, P. (1994) J. Cell Sci. Suppl. **18**, 63–68
16 Matsuda, S., Kawamura-Tsuzuku, J., Ohsugi, M., Yoshida, M., Emi, M., Nakamura, Y., Onda, M., Yoshida, Y., Nishiyama, A. and Yamamoto, T. (1996) Oncogene **12**, 705–713
17 Rouault, J.-P., Falette, N., Guehennex, F., Guillot, C., Rimokh, R., Wang, Q., Berthet, C., Moyret-Lalle, C., Savatier, P., Pain, B. et al. (1996) Nature Genet. **14**, 482–486
18 Wilcox, J. N., Scott, N. A., Subramanian, R. R., Ross, C. E., Adams-Burton, C. R., Stoltenborg, J. K. and Corjay, M. H. (1995) Circulation **92**, I34–I35
19 Matsuda, S., Kawamura-Tsuzuku, J., Kihara, A., Onda, M. and Akiyama, T. (1996) Abstracts, Twelth Annual Meeting on Oncogenes, abstr. 209
20 Raburn, D. J., Hamil, K. G., Tsuruta, J. K., O'Brien, D. A. and Hall, S. H. (1995) Endocrinology (Baltimore) **136**, 5769–5777
21 Sakai, A., Chibazakura, T., Shimizu, T. and Hishinuma, F. (1992) Nucleic Acids Res. **20**, 6227–6233
22 Schiestle, R. H. and Gietz, R. D. (1989) Curr. Genet. **16**, 339–346
23 Chien, C., Bartel, P., Sternglanz, R. and Fields, S. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 9578–9582
24 Hoffman, C. S. and Winston, F. (1987) Gene **57**, 267–279
25 Maniatis, T., Fritsch, E. F. and Sambrook, J. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26 Ausebel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (1993) Current Protocols in Molecular Biology, 2nd edn., pp. 6.1.1–6.3.5, Wiley, New York
27 Bartel, P., Chien, C., Sternglanz, R. and Fields, S. (1993) Biotechniques **14**, 920–924
28 Guan, K. and Dixon, J. E. (1991) Anal. Biochem. **192**, 262–267
29 Blank, R. S., Thompson, M. M. and Owens, G. K. (1988) J. Cell Biol. **107**, 299–306
30 Clark, K. L., Dignard, D., Thomas, D. Y. and Whiteway, M. (1993) Mol. Cell. Biol. **13**, 1–8
31 Andrews, N. C. and Faller, D. V. (1991) Nucleic Acids Res. **19**, 2499
32 Bevington, P. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, Chapter 4, McGraw-Hill, New York
33 Fields, S. and Song, O. (1989) Nature (London) **340**, 245–246
34 Lipman, D. J. and Pearson, W. R. (1985) Science **227**, 1435–1441
35 Pearson, W. R. and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2444–2448

36 Adams, M. D., Dubnick, M., Kerlavage, A. R., Moreno, R., Kelley, J. M., Utterback, T. R., Nagle, J. W., Fields, C. and Venter, J. C. (1992) Nature (London) **355**, 632–634
37 Cavener, D. R. and Ray, S. C. (1991) Nucleic Acids Res. **19**, 3185–3192
38 Kozak, M. (1991) J. Biol. Chem. **266**, 19867–19870
39 Hahn, S. (1993) Cell **72**, 481–483
40 Ma, J. and Ptashne, M. (1987) Cell **51**, 113–119
41 Draper, M. P., Salvadore, C. and Denis, C. L. (1995) Mol. Cell. Biol. **15**, 3487–3495
42 Fagan, R., Flint, K. J. and Jones, N. (1994) Cell **78**, 799–811
43 Pearson, R. B. and Kemp, B. E. (1991) Methods Enzymol. **200**, 62–68
44 Lorincz, A. T. and Reed, S. I. (1984) Nature (London) **307**, 183–185
45 Shaw, G. and Kamen, R. (1986) Cell **46**, 659–667
46 Pavel, H., Pascal, R. and Hernandez-Verdun, D. (1992) J. Histochem. Cytochem. **40**, 1089–1096
47 Marchal, S., Casser-Malek, I., Magaud, J. P., Rouault, J. P., Wrutnick, C. and Cabello, G. (1995) Exp. Cell Res. **220**, 1–10
48 Silver, P. A. (1991) Cell **64**, 489–497
49 Dingwall, C. and Laskey, R. A. (1991) Trends Biochem. Sci. **16**, 478–481
50 Nakanishi, M., Robetorye, R. S., Adami, G. R., Pereira-Smith, O. M. and Smith, J. R. (1995) EMBO J. **14**, 555–563
51 Coleman, T. R. and Dunphy, W. G. (1994) Curr. Opin. Cell Biol. **6**, 877–892
52 Lee, M. G. and Nurse, P. (1987) Nature (London) **327**, 31–35
53 Liu, H. Y., Toyn, J. H., Chiang, Y. C., Draer, M. P., Johnston, L. H. and Denis, C. (1997) EMBO J., **16**, 5289–5298
54 Chen, J., Jackson, P. K., Kirschner, M. W. and Dutta, A. (1995) Nature (London) **374**, 386–388
55 Harper, J. W., Adami, G. R., Wei, N., Keyomasi, K. and Elledge, S. J. (1993) Cell **75**, 805–816
56 Polyak, K., Kato, J., Solomon, M. J., Sherr, C. J., Massague, J., Roburst, J. M. and Koff, A. (1994) Genes Dev. **8**, 9–22
57 Toyoshima, H. and Hunter, T. (1994) Cell **78**, 67–74
58 Lee, M. H., Reynisdottir, I. and Massagué, J. (1995) Genes Dev. **9**, 639–649
59 Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. and Elledge, S. J. (1995) Genes Dev. **9**, 650–652

Received 20 February 1998/16 July 1998; accepted 17 September 1998