BTG2 antiproliferative protein interacts with the human CCR4 complex existing in vivo in three cell-cycle-regulated forms

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Summary

The yeast CCR4-NOT complex exists in two forms (1.0 and 1.9 MDa) that share several common subunits, including yCCR4, yCAF1 and five NOT proteins (NOT1-5). Here, we report that different complexes containing mammalian homologs of CCR4-NOT subunits exist in mammalian cells, with estimated sizes of ~1.9 MDa, ~1 MDa and ~650 kDa, and that BTG2, a member of a protein family with antiproliferative functions, can associate with these complexes. Immunoprecipitation and gel filtration experiments established that BTG2 interacts in vivo with hCCR4 protein via hCAF1 and hPOP2. Moreover, we show that hCCR4, as well as hCAF1 and BTG2, modulate the transcription regulation mediated by ERα. Finally, we demonstrate that the cellular localization of hCAF1 and the cell content in hCAF1-containing complexes change as cells progress from quiescence to S phase. These findings suggest that the different regulatory pathways in which hCAF1 is involved, notably transcription regulation and mRNA turnover, may occur through distinct CCR4 complexes in the course of cell-cycle progression.

Key words: BTG family, hCAF1/POP2, hCCR4, Multiprotein complex, Cell cycle, ERα

Introduction

The BTG/TOB family constitutes an emerging gene family that is involved in cell growth, differentiation and survival. BTG1, BTG2/TIS21/PC3, BTG3/ANA, TOB and TOB2 have been reported to display antiproliferative properties (reviewed by Matsuda et al., 2001; Tirone, 2001). *BTG2*, also known as PC3/TIS21, was previously described as an immediate early gene (Bradbury et al., 1991; Fletcher et al., 1991) involved in the differentiation of various cell types (Berthet et al., 2002; el-Ghissassi et al., 2002; Iacopetti et al., 1999). This gene, regulated by p53, has been found to be involved in DNA-damage-induced G2/M cell-cycle arrest (Rouault et al., 1996). However, the molecular function of this protein family has not yet been completely elucidated. Recently, attention focused on more mechanistic aspects of BTG/TOB family functions, in particular on the nature of the proteins they interact with. We previously demonstrated that BTG1 and BTG2 interact with mCAF1 (CCR4 associated factor 1) (Rouault et al., 1998) and its paralog hPOP2 (or CALIF) (Prévôt et al., 2001), whose yeast ortholog is a component of CCR4-NOT transcriptional complexes (Liu et al., 1998). Following our previous findings, several groups have reported an association between other members of this family and mammalian CAF1 (Bogdan et al., 1998; Ikematsu et al., 1999; Yoshida et al., 2001), suggesting that CAF1 is a crucial partner of the BTG/TOB protein family. We also previously reported that BTG1 and BTG2 are physically and functionally associated with homeoprotein HOXB9, and that they enhance HOXB9-mediated transcription (Prévôt et al., 2000), thus providing the first indication of a link between this protein family and transcription regulation. The involvement of BTG/TOB family proteins in transcriptional regulation is supported by results indicating that PC3 may act as a transcriptional regulator of cyclin D1 (Guardavaccaro et al., 2000), that TOB plays a critical role in BMP2/Smad-regulated gene expression in osteoblasts (Yoshida et al., 2000), and that BTG1 and BTG2 function as coactivators and corepressors of estrogen receptor ERα (Prévôt et al., 2001). In the same study, Prévôt et al., demonstrated that hCAF1 also acts as a modulator and can bind directly to ERα in vitro (Prévôt et al., 2001), suggesting that BTG and CAF1 proteins may modulate ERα-mediated transcription via a CCR4-like complex.

Mouse CAF1 was shown to interact with yCCR4 (Draper et al., 1995), a component of the general transcription multi-subunit complex CCR4/NOT that, in yeast, positively or negatively regulates the expression of genes involved in non fermentative processes, cell-wall integrity, and cell-cycle regulation and progression (Bai et al., 1999; Collart and Struhl, 1994; Liu et al., 1998; Liu et al., 1997). The yeast CCR4-NOT proteins exist in two complexes, of 1.0 and 1.9 MDa, sharing the subunits CCR4, CAF1, and the five NOT proteins (NOT1-5) constituting the core complex. Proteins of the core complex play distinct roles; recent studies have described yCAF1 (Daugeron et al., 2001) and yCCR4 (Chen et al., 2002; Tucker et al., 2002; Tucker et al., 2001) as nucleases involved in mRNA deadenylation.

Several components of the yCCR4-NOT complex (named CNOT, for CCR4-NOT, by the HUGO Gene Nomenclature Committee) have already been identified in humans: two homologs of yCAF1, hCAF1/CNOT7 and hPOP2/ CALIF/ CNOT8 (Albert et al., 2000; Bogdan et al., 1998; Fidler et al., 1999; Rouault et al., 1998), four homologs of NOT proteins (CNOT1, CNOT2, CNOT3, CNOT4) (Albert et al., 2000), and the human homolog of yCCR4 (Dupressoir et al., 2001). Additionally, CNOT4 has been described as a ubiquitin protein ligase (Albert et al., 2002). Use of a tandem affinity purification (TAP) strategy in a large-scale approach permitted to show that human and yeast CCR4–NOT complexes have comparable subunit compositions (Gavin et al., 2002). Despite significant advances in understanding the relationship between yeast and human CCR4 complexes, the characterization of the subunit composition and stoichiometry of human complexes has not yet been achieved. We do not know whether multiple, distinct CCR4-NOT complexes exist in mammals, and which molecular pathways they are involved in. To estimate the number of different complexes containing CCR4-NOT subunits in mammalian cells, HeLa and MRC5 cell lysates were subjected to biochemical fractionation. Protein complexes present in eluted fractions were characterized by immunoblotting using antibodies specific for hCCR4, hCAF1 and hPOP2, whose yeast orthologs are components of the CCR4-NOT complex. We report that these proteins exist in mammalian cells as three distinct complexes, with estimated sizes of ~1.9 MDa, ~1-1.2 MDa and ~650 kDa, which are able to associate with the antiproliferative protein BTG2. Finally, we show that the subcellular localization of hCAF1 is regulated in vivo in a cell cycle-dependent manner: for both G0 and G1 stages we found that hCAF1 concentrated almost exclusively in the nucleus, but by the time cells entered S phase a majority of hCAF1 had become cytoplasmic. Interestingly, we also show that the cellular content in hCAF1-containing complexes changes over the course of the cell cycle, suggesting that this protein, and perhaps also the CCR4-NOT complexes in which it is found, may play different regulatory roles during cell-cycle progression in mammals.

Materials and Methods

Cell culture

All cell lines obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Invitrogen Life Technologies, Inc.), in a humidified atmosphere of 5% CO_2 at 37°C.

Cell-extract preparation and chromatography on Superose 6

Subconfluent HeLa cells (5×10^8) were washed and harvested in PBS, then lysed with a manual Dounce B homogenizer in 5 ml of lysis buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 20 mM KCl, 0.2 mM EDTA, 10 μM $ZnCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100) containing a mixture of protease inhibitors (Roche Molecular Biochemicals). The lysate was clarified by centrifugation at 22,000 ***g*** at 4°C for 20 minutes, and then concentrated by centricons (Millipore). Protein concentration was determined by Bradford assay (Bio-Rad). 4 mg of the clear extract in a total volume of 300 μl were directly loaded onto a 24 ml Superose 6 column (HR 10/30, Amersham Pharmacia Biotech) preequilibrated with Tris buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and run in the same buffer. The flow rate was 0.4 ml/minute, and volumes indicated in the figure legends were collected for each fraction. The molecular weight of each fraction was calculated from the elution volumes of the standard molecular weight mixture used for calibrating the Superose 6 column: blue dextran (2000 kDa) at 7.5 ml, thyroglobulin (669 kDa) at 12.3 ml, bovine gammaglobulin (158 kDa) at 15.5 ml, chicken ovalbumin (44 kDa) at 16.9 ml and equine myoglobin (17 kDa) at 18.5 ml.

Purification of recombinant protein and generation of antibodies

Purification of GST–CAF1 was obtained as described previously (Rouault et al., 1998). Rabbit polyclonal antibody was generated by using the corresponding purified recombinant protein as antigen. Rabbit polyclonal antibodies against hPOP2 and hCCR4 proteins were produced using specific peptides (hPOP2, VAQKQNEDVDSAQEK residues 266-280; hCCR4, ETNHKDFKELRYNES residues 431-445).

All rabbit polyclonal antibodies were generated at Agrobio Laboratory (France). The antibodies were purified from immunized-rabbit serum by affinity chromatography using NHS columns (Amersham Pharmacia Biotech) coupled with specific antigens.

Immunoblotting

The Superose 6 column fractions (50 μl) were diluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, then resolved on SDS-PAGE gel as indicated in the figure legends. The gels were electroblotted onto a PVDF membrane, blocked by incubation at room temperature for 1 hour in TBS (150 mM NaCl, 10 mM Tris, pH 7.4) containing 5% non-fat dry milk. The rabbit polyclonal antibodies described above were used to detect hCAF1, hPOP2 and hCCR4. M2 monoclonal antibody (Sigma) was used to detect $CCR4^{FLAG}$, $CCR4^{His\text{-}FLAG}$ and $BTG2^{FLAG}$ fusion proteins. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins or peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako). The proteins were visualized by means of an enhanced chemiluminescence kit (Roche Molecular Biochemicals), following the manufacturer's instructions.

Mammalian expression vectors

GAL4 and VP16 mammalian expression vectors were derived from the SV40 promoter-driven expression vector pSG5 (Stratagene). GAL4 fusion plasmids were obtained by subcloning the appropriate cDNA into the pGal4PolyII plasmid (Green et al., 1988) in-frame with the yeast GAL4 binding domain coding sequence. pGal4-hCCR4 was previously described (Dupressoir et al., 2001), as well as pVP16-hCAF1, pVP16-hPOP2, pVP16-BTG1, pVP16-BTG2, pSG5Foll and pSG5FlagBTG2 constructs (Prévôt et al., 2001; Prévôt et al., 2000). pSG5FlaghCCR4 was obtained by cloning the full-length cDNA of hCCR4 into pSG5Flag plasmid in-frame with the Flag-coding sequence. pSG5HisFlag plasmid was generated by inserting the following *Eco*RI/*Eco*RI double-stranded oligonucleotide, containing the 6×his and the thrombine protease recognition site coding sequence, into the *Eco*RI site of pSG5Flag plasmid in-frame with the Flag coding sequence: 5′AATTCATGAGAGGATCCGCATCA-CCATCACCATCACCTGGTTCCGCGTGGATCTTGG 3′; 3′GTA-CTCTCCTAGGCGTAGTGGTAGTGGTAGTGGTCCAAGGCGCA-CCTAGAACCAATT 5′. pSG5HisFlaghCCR4 plasmid was constructed by inserting the hCCR4 full-length cDNA into pSG5HisFlag plasmid.

Transfection, reporter activity and mammalian two-hybrid assay

The plasmids used for transfection were prepared using the

alkaline/PEG/LiCl method. To obtain cells expressing low amounts of $hCCR4^{FLAG}$, $CCR4^{His\text{-}FLAG}$ and $BTG2^{FLAG}$ proteins, HeLa cells were transfected with $hCCR4^{FLAG}$-, $CCR4^{His\text{-}FLAG}$- and $BTG2^{FLAG}$-expressing plasmids and pUC at 1:10 molar ratio to bring the total plasmid DNA to concentrations typically used for protein expression assays. For the two-hybrid assay, HeLa cells were grown in DMEM (Invitrogen Life Technologies) supplemented with 10% fetal calf serum, seeded at 10^4 cells/well in 96-well microtiter plates, then transfected 8 hours later using Exgen 500 (Euromedex, Souffelweyersheim, France). The transfected DNA included 100 ng of pG4-TK-Luc reporter plasmid, together with 50 ng of GAL4 and/or VP16 fusion vectors in the presence or not of pSG5FlaghCAF1 and pSG5FlaghPOP2. For ERα transcription assay, HeLa cells were seeded at 0.8×10^5 cells/well in 24-well microtiter plates, then transfected 8 hours later using 5 μl of Exgen 500/μg DNA. The transfected DNA included various amounts of reporter and expression vectors, as detailed in the figure legends. The amount of transfected SV40 promoter was kept constant by addition of pSG5 to the transfection mixture. pTK-RL vector (Promega) (25 ng) was used as internal control for transfection efficiency. After 24 hours, the cells were washed and, where necessary, treated for 24 hours with a medium containing 10 nM 17β-estradiol. Transfected cells were washed and collected 48 hours after transfection. Luciferase activity was measured in the cell lysates using the Dual Luciferase Kit (Promega), following the manufacturer's instructions. In all experiments, luciferase activity was normalized with reference to the renilla luciferase activity expressed by the pTK-RL vector. Reporter activity was expressed as a ratio of fold induction to the activity of the reporter vector alone. Each set of experiments was performed in quadruplicate and repeated at least three times.

Flag-tagged and Ni^{2+}-NTA agarose columns

Cells were lysed on ice in lysis buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 20 mM KCl, 10 μM $ZnCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100) and a cocktail of protease inhibitors. Lysates were centrifuged in order to separate insoluble proteins. Approximately 0.2 mg of total proteins were incubated with 40 μl of anti-Flag M2 affinity gel (Sigma) or with 40 μl of Ni^{2+}-NTA agarose (Quiagen) at 4°C for 8-12 hours. Beads were then loaded onto a column and washed extensively several times with buffers containing 150 mM NaCl and, for Ni^{2+}-NTA agarose columns, increasing imidazole concentrations (5 to 20 mM). Bound proteins were then eluted with the sample buffer and boiled. Western blots were performed as described previously.

Cell synchronization

Early-passage MRC5 human diploid cells were seeded onto 15 cm plates at a density of 3×10^6 cells per plate. Synchronized G0-G1 MRC5 cells were obtained by cell starvation in 0.1% serum for 72 hours. Populations in early G1, mid-G1 and late G1 stages, at the G1/S transition and in S phase were generated by addition of 10% serum to arrested cells. Cells were collected at the times indicated and processed for immunofluorescence, fluorescence-activated cell sorting (FACS) analysis, or Superose 6 fractionation.

FACS analysis

At the times indicated, cells were detached from the plates by trypsin incubation, rinsed with PBS, and fixed in 70% (v/v) ethanol. They were rehydrated in PBS and incubated with 2 ml of 2N HCl, then with RNase (1 mg/ml) and propidium iodide (Sigma). Cells were analyzed using a flow cytometer (FACScalibur, Becton-Dickinson), and the cell cycle was determined by Cell Quest analysis.

Immunofluorescence microscopy

At the times indicated, MRC5 cells on microscope slides were fixed with 4% paraformaldeyde for 15 minutes, then permeabilized for 5 minutes with 0.1% Triton X-100 in PBS. Non-specific staining was blocked by 30 minutes incubation with 0.2% gelatine in PBS. Anti-hCAF1 purified antibodies were used for immunodetection, followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody. The coverslips containing the stained cells were mounted on microscope slides and immunofluorescence was recorded using a Zeiss Axioplan 2 microscope. HeLa cells were seeded onto microscope slides in 6-well plates at a density of 2×10^5 cells per well. After two days, the cells were fixed and analyzed as described above.

Results

hCCR4, hCAF1 and hPOP2 exist as distinct, large protein complexes in mammalian cells

As a first step to characterize mammalian CCR4-NOT complexes, we produced polyclonal antibodies against the human orthologs of yCCR4-NOT components: CCR4, CAF1 and POP2. Their specificity and sensitivity were tested using HeLa cells transfected or not with $hCCR4^{FLAG}$-, $hCAF1^{FLAG}$- and $hPOP2^{FLAG}$-expressing vectors. Although hCAF1 and hPOP2 exhibit 76% sequence identity, the anti-CAF1 antibody did not crossreact with POP2 protein. Preincubating affinity-purified antibodies with the peptide or recombinant protein used as antigen effectively suppressed immune reactions, and the preimmune sera did not detect any signal (data not shown).

Mouse CAF1 has been identified as interacting with, and being a component of, the yeast general transcriptional complex CCR4-NOT, which is likely to play fundamental roles in gene regulation (Bai et al., 1999; Collart and Struhl, 1994; Liu et al., 1998; Liu et al., 1997). We previously showed that CCR4/CAF1 interaction is evolutionarily conserved, and that hCCR4 can bind directly to hCAF1 and hPOP2 both in vivo and in vitro (Dupressoir et al., 2001). Biochemical fractionation was performed in order to determine whether single or multiple complexes containing CCR4-NOT subunits exist in mammals. HeLa cells were solubilized in non-denaturing buffer, and then extracted proteins were directly fractionated by size on a gel filtration column with low-salt buffer. This procedure permitted to avoid ion-exchange chromatography and exposure to high salt concentrations that might have caused subunits to break off from large, multiprotein complexes. HeLa cellular extracts were fractionated by gel filtration chromatography using a Superose 6 column. Using polyclonal antibodies directed against hCCR4, hCAF1 and hPOP2, we examined the relative migration profiles of endogenous proteins. As shown in Fig. 1A, two major peaks were observed at ~1.9 MDa (fractions 6-9) and ~1-1.2 MDa (fractions 16-20) for hCCR4, hCAF1 and hPOP2, although the elution patterns of hCAF1 and hCCR4 covered a wider range of fractions than hPOP2. In addition, a smaller peak was observed at ~650 kDa (fractions 25-26) that contained only hCCR4 and hCAF1. The relative broad elution profiles of hCCR4 and hCAF1 suggest that they may form a greater variety of complexes than hPOP2. No significant monomeric forms of hCAF1, hCCR4 and hPOP2 were found in the cell lysates, suggesting either that the formation kinetics of the complexes strongly favors the sequestration of the proteins in the complexes, or that the monomeric forms of the proteins are unstable. We obtained similar results using cellular extracts treated with DNAse and RNAse to remove high-molecular-mass nucleic acids (data not shown). In summary,

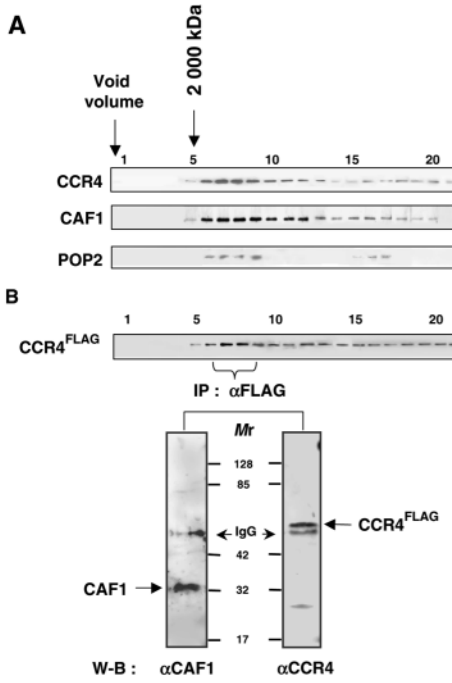

Fig. 1. hCCR4, hCAF1 and hPOP2 form common complexes in mammalian cells. (A) HeLa cell lysate was analyzed by gel filtration chromatography using a Superose 6 (HR10/30) column. Protein extracts were pre-cleared by centrifugation at 22,000 ***g*** for 20 minutes, then 300 μl of the sample (4 mg) were loaded onto the column. The flow rate was 0.4 ml/minute, and 250 μl were collected in each fraction, from which 50 μl were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot using anti-hCCR4, -hCAF1 and -hPOP2 antibodies. The numbers indicate fractions and the control lines represent extracts from HeLa cells transfected respectively with $hCCR4^{FLAG}$-, $hCAF1^{FLAG}$- and $hPOP2^{FLAG}$-expressing plasmids. (B) hCAF1 was associated with hCCR4 in high molecular weight complex. Cellular extracts from HeLa-expressing Flag-tagged CCR4 were fractionated by gel filtration chromatography using a Superose 6 column as described before. Fractions 6-9 were pooled and incubated with anti-Flag M2 affinity gel at 4°C for 8-12 hours. Bound proteins were eluted with the sample buffer and boiled. Western blots were performed using anti-hCCR4 and anti-hCAF1 antibodies. Molecular size markers are given in kilodaltons.

this analysis revealed the presence of multiple large CCR4-containing complexes in HeLa cells ranging in size from 1.9 MDa to 650 kDa.

The co-elution of hCAF1, hPOP2 and hCCR4 was not conclusive evidence that these proteins exist in the same multiprotein complex in vivo. To address this point**,** we used HeLa cells expressing Flag-tagged-CCR4 by transfection, since previous experiments had indicated that endogenous hCCR4, when associated in multiprotein complexes, was inaccessible to anti-hCCR4 antibody (data not shown). Transfection conditions permitting to obtain a very low expression of exogenous proteins were used to prevent spurious associations upon overexpression. Cellular extracts from transfected cells were fractionated as described before, and subjected to immunoblot analysis using anti-Flag antibody. As shown in Fig. 1B, $CCR4^{FLAG}$ was eluted from the column at the same position as the endogenous protein. Fractions 6-9 **(**Fig. 1B) were pooled then subjected to immunoprecipitation using anti-Flag antibody that co-precipitated the endogenous hCAF1 protein, as revealed by anti-hCAF1 (Fig. 1B). We also tested the immunoprecipitate for the presence of hPOP2; a very weak signal could be detected after a long exposition (data not shown). These results indicate that hCCR4 associates with hCAF1 in the same 1.9 MDa complex in vivo. However, we could not conclude whether hCAF1 and hPOP2 occur in the same or in different CCR4-containing complexes. Further studies will be necessary to clarify this point.

BTG2 may form complexes with hCCR4, hCAF1 and hPOP2 in mammalian cells

We previously reported that BTG1 and BTG2 proteins directly interact with mammalian CAF1 and POP2 (Prévôt et al., 2001; Rouault et al., 1998). Physical interactions between BTG2 and components of CCR4-NOT complexes were examined in a number of assays. Following transient transfection of $CCR4^{His\text{-}FLAG}$ and $BTG2^{FLAG}$ expression constructs, cellular extracts were bound with Ni^{2+}NTA agarose. After several washes with buffers containing increasing imidazole concentrations, bound proteins were eluted and analyzed by western blotting using anti-Flag and anti-hCAF1 antibodies. As shown in Fig. 2A, Ni^{+2}-NTA agarose retained $CCR4^{His\text{-}FLAG}$ and $BTG2^{FLAG}$ detected by anti-Flag antibodies and hCAF1 detected by anti-hCAF1 antibodies. These results indicate that hCCR4, hCAF1 and BTG2 form a complex in vivo.

To better define the physical relationship between these proteins, two-hybrid assays were performed in HeLa cells. As shown in Fig. 2B, GAL4hCCR4 strongly interacts with both VP16hCAF1 and VP16hPOP2, as already reported (Dupressoir et al., 2001), but not with VP16BTG2, indicating that the binding of BTG2 to hCCR4 does not result from direct interactions. Knowing that BTG2 interacts with hCAF1 and hPOP2, we tested whether these proteins are required for the interaction with hCCR4. The coexpression of hCAF1 or hPOP2 with fusion proteins GAL4hCCR4 and VP16BTG2 increased the expression of the pG4-TK-LUC reporter, indicating that the interaction of BTG2 with hCCR4 is dependent on the presence of hCAF1 or hPOP2, acting as bridges. The cotransfection of pSG5FlagFOLL, which encodes an unrelated protein that was used as a control, failed to bridge BTG2 and CCR4 (Fig. 2B). When hCAF1 or hPOP2 are expressed, either alone or in combination with GAL4hCCR4, the basal promoter activity is not increased (Fig. 2B). Together, these results strongly indicate that BTG2 forms complexes with hCCR4, hCAF1 and hPOP2 in vivo. We obtained comparable results using BTG1 protein (data not shown).

BTG2 cofractionates with CCR4-containing complexes

We thus examined the possible occurrence of BTG2 protein in high-molecular-mass CCR4 complexes. Cellular extracts from

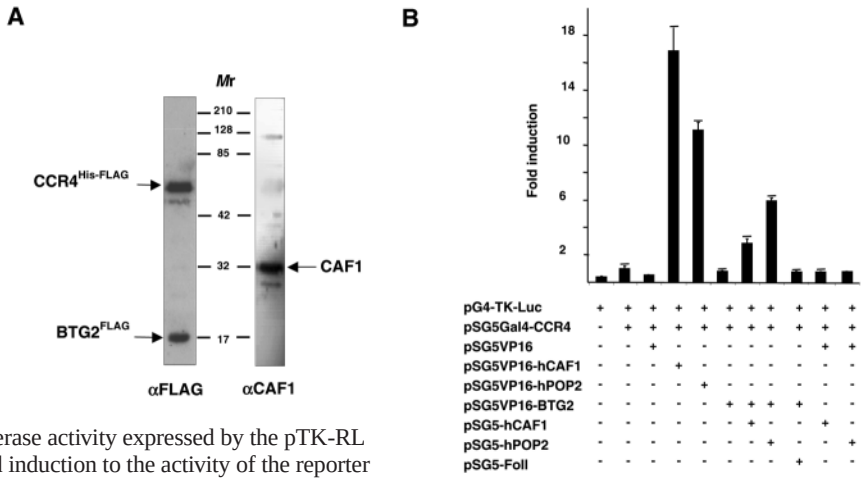

Fig. 2. hCAF1 and hPOP2 act as bridges between hCCR4 and BTG2. (A) Ni^{2+}-NTA agarose column. A cellular extract from HeLa cells transfected with $CCR4^{His\text{-}FLAG}$ and $BTG2^{FLAG}$ expression constructs was bound with Ni^{2+}NTA agarose. After intensive washes, bound proteins were eluted and analyzed by western blotting using anti-Flag and anti-hCAF1 antibodies. Molecular size markers are given in kilodaltons. (B) Mammalian two-hybrid assay. HeLa cells were seeded at 10^4 cells/well in 96-well microtiter plates, then transfected after 8 hours using Exgen 500. The transfected DNA included 100 ng of pG4-TK-Luc reporter plasmid together with 50 ng of GAL4 and/or VP16 fusion vectors in the presence or absence of 50 ng of pSG5FlagCAF1, pSG5FlagPOP2 or pSG5FoII. In all experiments, luciferase activity was normalized with the renilla luciferase activity expressed by the pTK-RL vector. Reporter activity was expressed as a ratio of fold induction to the activity of the reporter vector alone.

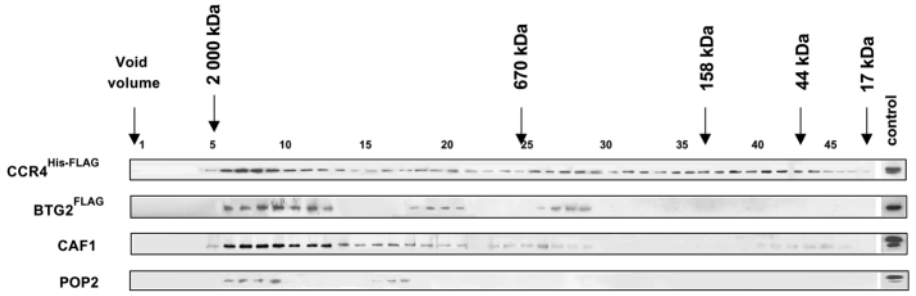

Fig. 3. Co-elution of BTG2 protein and CCR4 complexes. Cellular extracts from HeLa cells transfected at low efficacy with $CCR4^{His\text{-}FLAG}$- and $BTG2^{FLAG}$-expressing plasmids were fractionated by gel filtration chromatography using a Superose 6 column, and subjected to immunoblot analysis as described in Fig. 1 using anti-Flag, anti-hCAF1 and anti-hPOP2 antibodies.

HeLa cells transfected at low efficacy with $BTG2^{FLAG}$ and $CCR4^{His\text{-}FLAG}$ expressing plasmids were fractionated by gel filtration chromatography using a Superose 6 column, and subjected to immunoblot analysis. As shown in Fig. 3, BTG2 migrated through the Superose 6 column as high-molecular-mass complexes and appeared to co-elute with hCCR4, hCAF1 and hPOP2 in fractions containing the 1.9 MDa and 1-1.2 complexes (Fig. 3, fractions 6-10 and 16-20) and with hCCR4 and hCAF1 in fractions containing the ~650 kDa complex (fractions 26-28). It is important to note that the monomeric forms of both transfected proteins were weakly detected, indicating that they are expressed at physiological concentration. The overlap between the elution patterns of BTG2, hCAF1, hPOP2 and hCCR4 suggests that BTG2 protein interacts with the native CCR4 complexes. We obtained similar results using BTG1 protein (data not shown).

CCR4 enhances the transcriptional activity of ERα in mammalian cells

The above results revealed that BTG2, hCAF1 and hCCR4 are present in the same complexes, suggesting that these proteins may be involved in common regulation pathways. Our previous observations that BTG proteins and hCAF1, probably through a CCR4-like complex, regulate the transcriptional activity of nuclear receptors, notably Estrogen Receptorα (ERα), incited us to study the capacity of hCCR4 to regulate ERα transcription. HeLa cells, that lack endogenous ERα, were transfected with a vector expressing ERα and a Luciferase reporter gene linked to multimer palindromic ERE sequence, pERE-Luc, along with either a control plasmid or vectors expressing hCCR4, BTG2 and hCAF1 in the presence of 17β-estradiol. As shown in Fig. 4, hCCR4, as BTG2 and hCAF1, significantly enhanced the ERα-mediated activation of the Luciferase reporter gene. No effect of hCCR4 on reporter-gene activity was observed in the absence of ERα (data not shown). Besides, cotransfection with pSG5FlagFOLL, which encodes an unrelated protein used as a control, had no effect on reporter gene activation (Prévôt et al., 2001). These results reveal that mammalian CCR4, CAF1 and BTG2 regulate the transcription mediated by ERα, suggesting that mammalian CCR4 complexes may participate in this regulation pathway.

The cell-cycle-dependent localization of hCAF1

Although a substantial body of evidence supports the conclusion that both yCAF1 and yCCR4 are components of transcriptional complexes, recent work has shown that they exhibit poly(A)-specific 3′ to 5′ exonuclease activity in yeast (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). In addition, Tucker et al. have shown that both proteins are localized primarily in the cytoplasm of yeast cells, which is consistent with their role in cytoplasmic deadenylation but conflicts with their role in transcription regulation. Thus we analyzed the subcellular localization of hCAF1 on asynchronously growing HeLa cells by indirect immunofluorescence using the purified antibody described before. hCAF1 staining was either predominant in the nucleus, or present in both the cytoplasm and the nucleus (Fig. 5A). This dual staining of asynchronous cells suggests that the localization of hCAF1 changes over the course of the cell cycle. To test this idea, we examined the localization of hCAF1

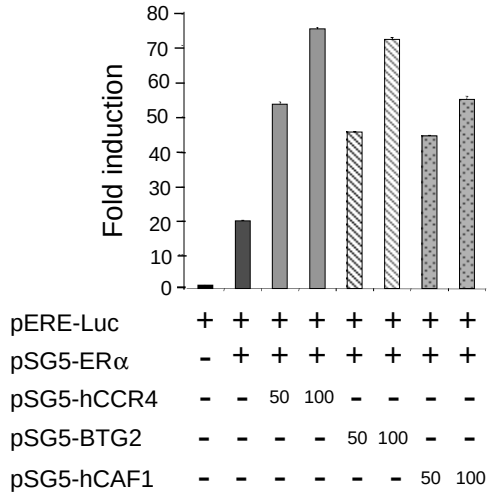

Fig. 4. BTG2, hCAF1 and hCCR4 act as ERα coactivators. HeLa cells were transiently transfected with 50 ng of pERE-Luc plasmid in the presence of 10 ng of pSG5ERα and indicated amounts (ng) of BTG2-, hCAF1- or hCCR4-expressing vectors or control plasmids (empty pSG5). After 24 hours, the cells were washed, and then treated for 24 hours with a medium containing 10 nM 17β-estradiol. Transfected cells were washed and collected 48 hours after transfection, then assayed for Luciferase activity. Normalized values are expressed as in Fig. 2B.

in primary human diploid fibroblast cells at different stages of the cell cycle. Human cell lines are notoriously difficult to synchronize by methods other than drug blocking, so we chose to use normal human diploid fibroblasts, MRC5 that could be efficiently synchronized by serum deprivation. MRC5 cells have the same growth-arrest mechanisms as normal cells, including density-mediated growth inhibition and the induction of quiescence in response to serum deprivation. They are arrested in G0 by 3-day culture in 0.1% serum. As shown in Fig. 5B1, serum starvation results in the accumulation of a quiescent population that synchronously progresses through G1 and S phase after serum restimulation, allowing for the isolation of populations in mid-G1 (after 12 hours), at G1/S transition (17 hours), and in S phase (24 hours) (henceforth, all cell-cycle phase designations will refer to these time points). We compared the localization of hCAF1 at different cell-cycle stages by indirect immunofluorescence (Fig. 5B2). In G0 or G1, almost all the hCAF1 present in cells was detected in the nucleus; by the time the cells had entered S phase, most of the hCAF1 had become cytoplasmic. These observations indicate that the sub-cellular localization of hCAF1 is dependent on cell-cycle progression, and that this might influence its biological activity.

Distinct steady-state of hCAF1-containing complexes during cell-cycle progression

The above results demonstrate that hCAF1 localizes in different cellular compartments as the cell cycle progresses, raising the possibility that changes in the localization of hCAF1 strictly mirror those of hCAF1-containing complexes. Thus, we examined the occurrence of hCAF1-containing complexes in MRC5 cells during cell-cycle progression and followed the migration profile of hCAF1. Lysates of MRC5 cells arrested in G0 by serum starvation, or in G1-S 17 hours after serum re-stimulation (see above), were fractionated on Superose 6 columns. The elution profiles of endogenous hCAF1 were analyzed by Western analysis using anti-hCAF1 specific antibody. The analysis revealed that the hCAF1 found in extracts from G1-S-enriched cells occurred in complexes identical to those described for exponentially growing HeLa cells (compare Fig. 5C with Fig. 1). Remarkably, when cells were blocked in G0, hCAF1 was undetectable in fractions containing complexes of ~1-1.2 MDa and 650 kDa (Fig. 5C). In addition, in these particular extracts, the 1.9 MDa complex seems to be reduced to ~1.8-1.7 MDa, as indicated by the detection of a hCAF1 peak in fraction 8 (Fig. 5C). These data suggest that, in vivo, hCAF1 is present in different CCR4-NOT complexes, depending on the cell-cycle stage.

Discussion

The yeast CCR4-NOT complex consists of at least two complexes of 1.0 and 1.9 MDa, respectively, as determined by gel filtration. Several human orthologs of components of the yCCR4-NOT complexes have been identified (Albert et al., 2000; Bogdan et al., 1998; Dupressoir et al., 2001; Fidler et al., 1999; Rouault et al., 1998) and it has been shown that human and yeast CCR4–NOT complexes have comparable subunit compositions (Gavin et al., 2002). Our data provide direct evidence of the existence of different classes of CCR4-like complexes in vivo in mammals: two major peaks at ~1.9 MDa and ~1-1.2 MDa include hCCR4, hCAF1 and its paralog hPOP2, and a smaller peak at ~650 kDa contains only hCCR4 and hCAF1. It is however unclear whether hCAF1 and hPOP2 occur in the same or in different CCR4-containing complexes. The fact that hPOP2, but not hCAF1, is able to interact with hNOT2 and hNOT3 (Albert et al., 2000) suggests that the two proteins are functionally distinct, and that they could participate in the formation of different complexes in mammals. In addition, since hCAF1 and hPOP2 were found to be expressed in every cell type tested (data not shown), functional specificity and selectivity are likely to be achieved by interactions with different partners.

One important implication of the work presented here is that the antiproliferative protein BTG2 can participate in the formation of mammalian CCR4-like complexes. We do not yet know whether BTG2 is a stable constituent of the ‘core’ CCR4-NOT complex or whether its interaction with the CCR4-NOT complex is transient and dynamic. We found that hCAF1, hPOP2, hCCR4, and BTG2 have similar fractionation profiles (Fig. 3), and we confirmed on an affinity column that BTG2 forms complexes with hCCR4 and hCAF1 (Fig. 2A). In addition, by a modification of the mammalian two-hybrid assay, we were able to provide evidence that hCCR4 interacts in vivo with BTG2 via CAF1 and POP2 (Fig. 2B). All described components of human CCR4–NOT complexes have yeast orthologs, indicating that the biological functions of these complexes are conserved through eukaryotic evolution. In contrast, proteins homologous to BTG/TOB have been detected in all animals (Chen et al., 2000) but not in the yeast S. cerevisiae. The presence of BTG2 in CCR4–NOT complexes might thus indicate the metazoan evolution of regulatory or signal domains of signal transduction. We can speculate that

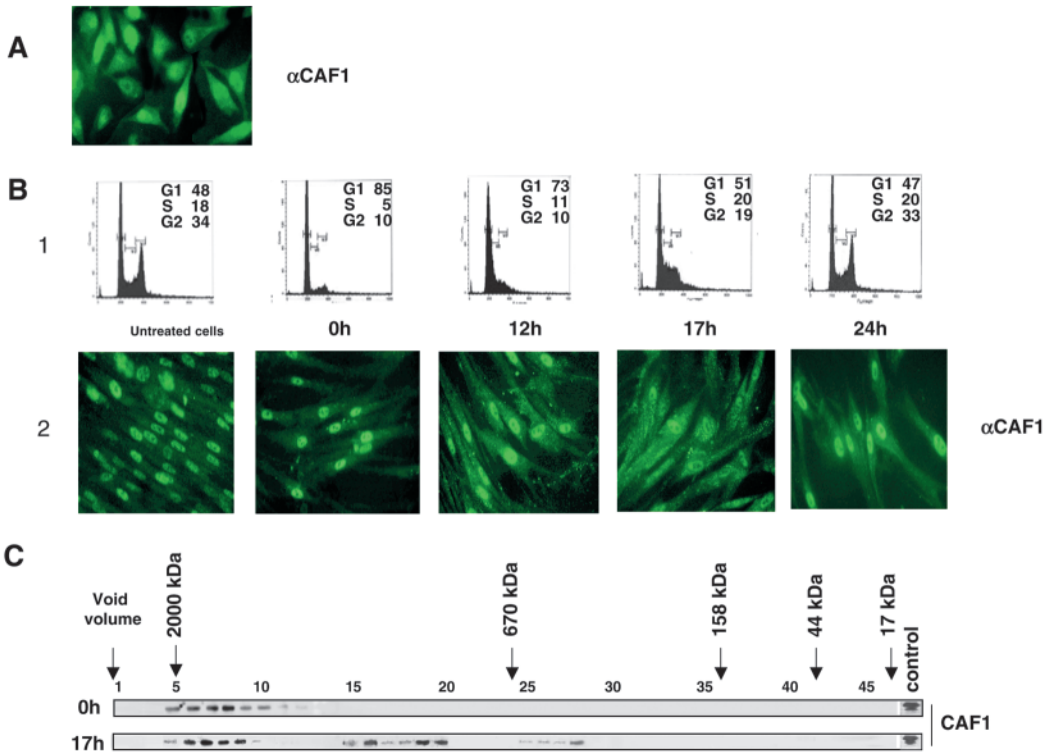

Fig. 5. Localization and fractionation profile of endogenous hCAF1 over the course of the cell cycle. (A) Subcellular localization of endogenous hCAF1. HeLa cells were immunostained with anti-CAF1 polyclonal antibody. (B) MRC5 cells were synchronized by serum starvation (t0). After re-addition of serum, cells were analyzed at indicated time points for either immunofluorescence or fluorescence-activated cell sorting (FACS) analysis. (B1) Cell-cycle distribution, as determined by FACS analysis. The cells were fixed in 70% ethanol and treated with RNase. The DNA was stained with propidium iodide. (B2) Immunofluorescence of endogenous hCAF1. MRC5 cells were fixed in 4% PAF at the times indicated and stained for detection of endogenous hCAF1 with polyclonal antibody anti-hCAF1, followed by fluoresceine isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody. (C) Distinct steady-state fractionation profiles of hCAF1 during the course of the cell cycle. Lysates of MRC5 cells arrested in G0 by serum starvation or in G1-S 17 hours after serum restimulation were fractionated on a Superose 6 column (cell lysates and Superose 6 column preparations are described in Fig. 1A). The elution profiles of endogenous hCAF1 were analyzed by western analysis using anti-hCAF1-specific antibody.

these complexes have acquired new specificities through the recruitment of metazoa-specific proteins, such as proteins of the BTG/TOB family.

However, understanding the biological functions of these complexes in mammals is limited due to the lack of knowledge of their target genes and pathways. To begin to address these questions we investigated whether hCCR4 is involved in the transcriptional regulation of estrogen nuclear receptor ERα, as was demonstrated for BTG proteins and hCAF1. Our results indicate that hCCR4 can function as a coactivator of ERα (Fig. 4), thus opening the attractive possibility of a link between mammalian CCR4-NOT complexes and nuclear receptor regulation pathways.

Although both yCAF1 and yCCR4 have been described as components of nuclear transcriptional complexes, recent studies indicated that they are also involved in cytoplasmic mRNA degradation (Chen et al., 2002; Daugeron et al., 2001; Tucker et al., 2002; Tucker et al., 2001). In addition, Chen et al., and our unpublished data have shown that hCCR4 and both hCAF1 and hPOP2 also exhibit poly(A)-specific 3′ to 5′ exonuclease activity in vitro. We further analyzed the cellular localization of hCAF1 and of hCAF1-containing complexes during cell-cycle progression. To this end, MRC5 cells were made quiescent by serum starvation, which resulted in the arrest of a majority of cells, as determined by FACS analysis (Fig. 5B1). The cells stimulated to re-enter the cell cycle by addition of 10% serum entered S phase between 16 and 20 hours after restimulation (Fig. 5B1). We found that hCAF1 was concentrated almost exclusively in the nucleus of both G0 and G1 cells, but by the time the cells entered S phase, a majority of hCAF1 had become cytoplasmic (Fig. 5B2). We have also examined the occurrence of hCAF1-containing complexes as a function of the cell cycle. Using whole cell lysates from MRC5 cells blocked at G0, when hCAF1 was localized exclusively in the nucleus, we detected hCAF1 only in the 1.9 MDa complex (Fig. 5C). When we analyzed extracts of MRC5 cells in G1-to-S transition (17 hours after serum restimulation, see Fig. 5B1,B2) hCAF1 was present in all complexes described for exponentially growing HeLa cells (compare Fig. 5C with Fig. 1). We can speculate that, during G0, the hCAF1 associated to the 1.9 MDa complex is localized to the nucleus where it is involved in the transcriptional regulation of CAF1-responsive genes. By the time re-stimulated cells have entered S phase, most hCAF1 is present in the cytoplasm where it associates with complexes regulating cytoplasmic pathways, mRNA turnover for instance. We are currently investigating the localization and the biological functions of hCAF1-containing complexes in the course of cell-cycle progression.

Collectively, these results provide the first indication of a link between the antiproliferative protein BTG2 and mammalian CCR4 complexes. In addition, we show that different classes of CCR4-containing complexes exist in vivo, and that their composition and cellular compartmentalization are regulated during the mammalian cell cycle. Furthermore, both the variable subunit composition and stoichiometry of the complexes are consistent with multiple functional roles in a variety of regulatory contexts.

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