Interaction of BTG1 and p53-regulated BTG2 Gene Products with mCaf1, the Murine Homolog of a Component of the Yeast CCR4 Transcriptional Regulatory Complex*

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Jean-Pierre Rouault‡‡§§, Déborah Prévôt‡‡§§, Cyril Berthet‡‡, Anne-Marie Biro‡‡, Marc Billaud**, Jean-Pierre Magaud‡‡, and Laura Corbo‡‡§§

From the ‡Unité INSERM U453, Centre Léon Bérard, 69373 Lyon Cedex 08, France, the ‡‡Laboratoire de Cytogénétique Moléculaire, Hôpital Edouard Herriot, 69373 Lyon Cedex 03, France, and the **Laboratoire de Génétique, UMR 5641 CNRS, Domaine Rockefeller, Université Claude Bernard, Lyon 1, France

Both BTG1 and BTG2 are involved in cell-growth control. BTG2 expression is regulated by p53, and its inactivation in embryonic stem cells leads to the disruption of DNA damage-induced G2/M cell-cycle arrest. In order to investigate the mechanism underlying Btg-mediated functions, we looked for possible functional partners of Btg1 and Btg2. Using yeast two-hybrid screening, protein-binding assays, and transient transfection assays in HeLa cells, we demonstrated the physical interaction of both Btg1 and Btg2 with the mouse protein mCaf1 (i.e. mouse CCR4-associated factor 1).

mCaf1 was identified through its interaction with the CCR4 protein, a component of a general transcriptional complex, which, in yeast, regulates the expression of different genes involved in cell-cycle regulation and progression. These data suggest that Btg proteins, through their association with mCaf1, may participate, either directly or indirectly, in the transcriptional regulation of the genes involved in the control of the cell cycle. Finally, we found that box B, one of two conserved domains which define the Btg family, plays a functional role, namely that it is essential to the Btg-mCaf1 interaction.

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§§To whom correspondence should be addressed: Unité INSERM U453, Centre Léon Bérard, 28 rue Laennec, 69373 Lyon Cedex 08, France. Tel.: 33-0-4-78-78-26-91; Fax: 33-0-4-78-78-27-20; E-mail: corbo@lyon.fnclcc.fr.

The abbreviations used are: BTG1, B-cell translocation gene 1; Prmt1, protein-arginine N-methyltransferase; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; PAGE, polyacrylamide gel electrophoresis.

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Experimental Procedures

Yeast Expression Constructs—The Btg1 and Btg2 "bait" for the yeast two-hybrid system was based on the pPC62 yeast expression vector (17). pPC62 was cut with SacI and XhoI and ligated with SacI/XhoI Btg1 and Btg2 human cDNA, obtained by polymerase chain reaction, giving pPC62BTG1 and pPC62 encoding fusion proteins, consisting of the Gal4 DNA-binding domain fused to Btg1 and Btg2. Several Btg1 deletion mutants were also constructed as Gal4 fusions. These are shown in Fig. 1.

Sequences of synthetic oligonucleotides used were: Btg1-1, 5'-agttggagcttgattacaaccttga-3'; Btg1-171, 5'-gettagaTCCAAACAACGTATACT-3'; Btg2-1, 5'-agttggagcttgattacaaccttga-3'; Btg2-179, 5'-gettagaAAGGCCTAGCTGGAGAC-3'; MBTG1-117, 5'-catcatactacATCACTTAAGTTGACGAC-3'; MBTG1-108, 5'-catcatactacAACACACACCAAGGAG-3'; MBTG1-96, 5'-catcatactacGTCAAGCAGCTC-3'; MBTG1-73, 5'-catcataactacGTGATGGCAAGGATACACAAGGT-3'; MBTG1-38, 5'-catcataactacTGCGACGCTGTCACTGCTCG-3'; box B-98, 5'-agttggagcttcccaagttgaaactcata-3'.

Library Screening—To identify genes encoding proteins that interact with Btg1, we used the two-hybrid system to detect interactions via the reconstitution of a functional transcription activator in yeast, with, as a recipient, the yeast strain (18) which was transformed with pPC62BTG1. The Btg1-expressing yeast cells were subsequently transformed with the Gal4 transactivation domain-tagged 14.5-day-old mouse embryo cDNA library (17), following the protocol described by Durfee et al. (19), the only specific modification being the use of salmon sperm DNA as a carrier. After 4 days at 30°C on L/W/H amino acid-depleted SC medium, 100 grew. Two of these transformants were tested for β-galactosidase activity using a yeast colony filter assay. Positive (blue) colonies were grown for 2 days to recover the prey plasmid. The electromax tosidoase activity using a yeast colony filter assay. Positive (blue) colonies were grown for 2 days to recover the prey plasmid.

Bacterial Expression Constructs—mCAF1 Open Reading Frame—The cDNA encompassing the entire mCAF1 open reading frame was cloned by reverse transcriptase-polymerase chain reaction. One μg of NIH 3T3 total RNA was reverse-transcribed in the presence of 10 mM Tris-HCl, pH 8, 1 mM MgCl2, 50 mM KCl, 0.5 mM dNTPs, 1 μg of random primers, following the manufacturer's instructions. The transfected DNA included 0.5 μg of the pG4-TK-CAT reporter plasmid, 200 ng of the GAL4, and/or VP16 fusion vectors, 50 ng of the pCMV-LACZ control plasmid in 5 μl of LipofectAMINE (Life Technologies, Inc.). The amount of yeast vector transfected was kept constant, where necessary, by the addition of pSG5 to the transfection mixture. The transfected cells were washed and collected 48 h after transfection.

CAT Enzyme-linked Immunosorbent Assay—CAT enzyme-linked immunosorbent assays were performed using the Boehringer Mannheim CAT enzyme-linked immunosorbent assay kit, following the manufacturer’s instructions. The transfected cells were lysed in 150 μl of lysis buffer. The supernatants were assayed for CAT protein production and β-galactosidase activity. All transfection data were normalized by β-galactosidase activity, which was quantified by 0-nitrophenyl-β-D-galactopyranoside assay using a standard linear curve. Reporter activity was expressed as the ratio of fold induction to the activity of the reporter vector alone. Each set of experiments was repeated at least three times, and similar results were obtained.

Immunoblot Analysis—For protein expression assays, 50 μl of lysate of HeLa cells transfected as described was subjected to electrophoresis on a 12% polyacrylamide–SDS gel. The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. Equal amounts of protein were loaded into each lane, as measured by the Bradford assay and confirmed by red Ponceau staining of transferred membranes. Gal4 fusion proteins were detected with an anti-GAL4 DNA-binding domain mouse monoclonal antibody (RKS1, Santa Cruz Biotechnology, Inc.). The M2 monoclonal antibody (IBI Flag system, Kodak) was used to detect the VP16 fusion proteins. The membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins. The proteins were visualized by means of the enhanced Amershams chemiluminescence kit following the manufacturer’s instructions.

RESULTS

Interaction of mCaf1 with Btg1 and Btg2—In this study, we used the two-hybrid interaction system developed by Fields and Song (23). To screen for cDNA encoding proteins able to interact with Btg1, the Y190 pPC62BTG1 yeast strain was transformed with a 14.5-day-old mouse embryo cDNA library cloned in pPC51 (17). Out of 5 × 108 transformants plated on an L/W/H amino acid-depleted SC medium, 100 grew. Two of
sites, upstream from GAL1 (UAS G). The resulting yeast cells were then transformed with a GAL4-tagged activation domain (introduced into the Y190 yeast strain which harbors two reporter genes, HIS3 and LACZ, under the control of promoters containing Gal4-binding sites, upstream of GAL1 (UAS_L)). The resulting yeast cells were then transformed with a GAL4-tagged activation domain (TAD) cDNA expression library (see “Experimental Procedures”). B, the Y190 yeast strain was transformed with BTG1 or mutated BTG1 cDNA cloned in-frame with the GAL4 BD of the pPC62 vector along with the 7.1 clone carrying the mCAF1 cDNA cloned in-frame with the GAL4 TAD of the pPC51 vector. The co-transformed Y190 yeast strain was grown in a L/W/H amino acid-depleted SC medium. A yeast colony filter assay was used to determine the β-galactosidase activity of the clones which came out positive in the preceding selection. Results are given for each individual transformation assay (−, no colony and thus no interaction; +, colonies which were further tested for β-galactosidase activity).

A schematic representation of the two-hybrid system used to isolate the Btg1-associated protein mCAF1. BTG1 cDNA was cloned in-frame with the GAL4 DNA-binding domain (BD) of the pPC62 vector. This construction was introduced into the Y190 yeast strain which harbors two reporter genes, HIS3 and LACZ, under the control of promoters containing Gal4-binding sites, upstream of GAL1 (UAS_L). The resulting yeast cells were then transformed with a GAL4-tagged activation domain (TAD) cDNA expression library (see “Experimental Procedures”). B, the Y190 yeast strain was transformed with BTG1 or mutated BTG1 cDNA cloned in-frame with the GAL4 BD of the pPC62 vector along with the 7.1 clone carrying the mCAF1 cDNA cloned in-frame with the GAL4 TAD of the pPC51 vector. The co-transformed Y190 yeast strain was grown in a L/W/H amino acid-depleted SC medium. A yeast colony filter assay was used to determine the β-galactosidase activity of the clones which came out positive in the preceding selection. Results are given for each individual transformation assay (−, no colony and thus no interaction; +, colonies which were further tested for β-galactosidase activity).

Fig. 1. Detection of Btg1/mCAF1 interaction in yeast. A, schematic representation of the two-hybrid system used to isolate the Btg1-associated protein mCAF1. BTG1 cDNA was cloned in-frame with the GAL4 DNA-binding domain (BD) of the pPC62 vector. This construction was introduced into the Y190 yeast strain which harbors two reporter genes, HIS3 and LACZ, under the control of promoters containing Gal4-binding sites, upstream of GAL1 (UAS_L). The resulting yeast cells were then transformed with a GAL4-tagged activation domain (TAD) cDNA expression library (see “Experimental Procedures”). B, the Y190 yeast strain was transformed with BTG1 or mutated BTG1 cDNA cloned in-frame with the GAL4 BD of the pPC62 vector along with the 7.1 clone carrying the mCAF1 cDNA cloned in-frame with the GAL4 TAD of the pPC51 vector. The co-transformed Y190 yeast strain was grown in a L/W/H amino acid-depleted SC medium. A yeast colony filter assay was used to determine the β-galactosidase activity of the clones which came out positive in the preceding selection. Results are given for each individual transformation assay (−, no colony and thus no interaction; +, colonies which were further tested for β-galactosidase activity).

These clones produced β-galactosidase, and they were further analyzed. One of them was found to encode a protein with a sequence identical to that of a homoeotic protein (which will be described elsewhere). The second gene (clone 7.1) was found to be identical to the mCAF1 mouse gene. mCAF1 is the mouse homolog of the yCAF/POP2 yeast gene, which is involved in a number of transcription processes.

According to Draper (24), the 7.1 clone identified in the yeast two-hybrid system begins at nucleotide 71 (EMBL/GenBank accession number number U21855). The cDNA encompassing the entire mCAF1 open reading frame was cloned by reverse transcriptase-polymerase chain reaction using NIH 3T3 total RNA.

When tested using the two-hybrid system, the 7.1 clone product also interacted with the Btg2 fusion protein. Given that the Btg protein family is characterized by the presence of two conserved boxes separated by a spacer of relatively constant length, we looked at whether these boxes play a role in the association between the Btg proteins and mCAF1. Using a series of deletion mutants, encompassing or not the two conserved boxes, the region of the Btg1/mCAF1 association was mapped to box B. The results of this assay are summarized in Fig. 1B. All the pPC62-BTG1 derivative constructs containing box B interacted with the 7.1 clone product. Further deletions, including that of box B, completely abolished the association Btg1/mCAF1. In addition, when box B was fused to Gal4 DNA-binding domain, an interaction with the mCAF1 protein took place (Fig. 1B). These results suggest that the Btg-mCAF1 interaction is mediated by box B, one of the two conserved domains defining the Btg family, and that this domain has some functional significance.

Interaction of mCAF1 with Btg1 and Btg2 in Vitro—To verify that mCAF1 can interact directly with Btg1 and Btg2, we performed in vitro association assays with purified recombinant glutathione S-transferase fusion proteins. GST-Btg1, GST-Btg2, and GST alone, used as a control, were coupled to glutathione-Sepharose beads and incubated with [35S]methionine-labeled mCAF1. As shown in Fig. 2B, the specific retention of mCAF1 was observed with the GST-Btg1 and GST-Btg2 beads, but not with the control GST beads. And the incubation of GST-Btg1 and GST-Btg2 with [35S]methionine-labeled luciferase, used as a control, failed to show any specific interaction (Fig. 2B). These results point to a direct physical interaction of mCAF1 with both Btg1 and Btg2. It can be seen, however, that the interaction of mCAF1 with Btg1 was significantly weaker than with Btg2 (Fig. 2B).

Interaction of mCAF1 with Btg1 and Btg2 in Mammalian Cells—We next studied the interactions between mCAF1 and the Btg proteins in mammalian cells. Two-hybrid protein-protein interaction assays were performed in mammalian culture cells, with a reporter plasmid (pG4-TK-CAT) containing six GAL4 consensus elements upstream from the thymidine kinase (TK) promoter region fused to the CAT gene. Vectors were
constructed to express both Btg1 and Btg2 fused to the DNA-binding domain (amino acids 1-147) of the yeast Gal4 transcription factor. A vector expressing mCaf1 fused to the VP16 activation domain (amino acids 413-490) was also produced. HeLa cells were transfected with the reporter plasmid alone or with chimeras constructs so as to analyze the ability of Btg1 and Btg2 to associate with mCaf1.

Only the coexpression of either GalBtg1 or GalBtg2 with the VpCaf construct elicited a significant increase in the expression of the pG4-TK-CAT reporter (Fig. 3B), which indicates a strong association between Caf1 and both Btg1 and Btg2. The interactions between Caf1 and the Btg proteins were also analyzed in reciprocal combination: mCaf1 fused to a GAL4 DNA-binding domain (pGALCAF) and BtgS fused to the VP16 activation domain (pVPBTG). As expected (Fig. 3B), the concomitant expression of the GalCaf1 and VpBtg proteins produced a strong increase in the activity of the CAT reporter. The expression of fusion proteins in transfected cells were determined by Western blot analysis, using specific antibodies (Fig. 3C). None of the hybrid proteins, on their own, activated the expression of the CAT reporter gene (Fig. 3B). And indeed, mCaf1 and Btg proteins did not seem to be capable of stimulating transcription when tethered to multimerized DNA sites through a Gal4-binding domain in HeLa cells. In contrast, LexACaf1 fusion can activate transcription from a LexA operator-controlled reporter gene in yeast (data not shown), as was also found by Draper et al. (24). Experiments aimed at elucidating this discrepancy are being currently carried out.

A Btg1 deletion mutant, Btg1(1–96) (see Fig. 1B), lacking box B, failed to produce a specific interaction with mCaf1 (Fig. 3B), in agreement with our yeast two-hybrid assays. These results indicate that Btg1 and Btg2 are indeed able to interact with mCaf1 in mammalian cells, and that box B is necessary to this interaction. However, in contrast with the results obtained with yeast, we did not find any evidence of an interaction between box B alone and mCaf1 in HeLa cells (data not shown).

When GalCaf was coexpressed with VpCaf, basal promoter activity did not increase (data not shown), which suggests that the mCaf1 protein cannot form dimeric complexes. Likewise, the coexpression of GalBtg and VpBtg did not enhance the activity of the reporter gene, which indicates that the Btg proteins do not interact either in homo- and in heterodimeric complexes in HeLa cells (data not shown). This finding is in keeping with the results obtained in yeast by Lin et al. (15).

Expression of CAF1—The in vitro translation of mCaf1 mRNA derived from full-length cDNA produced a doublet with an apparent molecular mass of 31 kDa (see Fig. 2B, mCaf1 imput).

The subcellular localization of mCaf1 was examined by indirect immunofluorescence of HeLa cells transiently transfected with a plasmid expressing a flag epitope-tagged version of mCaf1. mCaf1 was detected in both the nucleus and the cytoplasm (Fig. 4, Ca).

Finally, the expression pattern of the CAF gene in various tissue types was studied. Northern blot analysis in Lovo- and NIH 3T3-derived total RNA revealed a 2.5-kilobase message, as well as one of 1.2 kilobases (Fig. 4A), although their structure remains unclear at present. Furthermore, a multiple human tissue dot blot analysis (CLONTECH) (Fig. 4B) did not reveal any CAF mRNA in nervous system-derived tissues, but showed variable levels of expression among the positive tissues. High levels of expression were found in the case of the stomach (C8), salivary gland (D7), thyroid gland (D6), kidney (E1), lung (F2), fetal thymus (G6), and fetal lung (G7). The same procedure revealed similar patterns of BTG1 and BTG2 expression (data not shown).

**DISCUSSION**

The ability of normal cycling cells to exit from the cell cycle in response to changes in environmental conditions (e.g., nutrient deprivation, growth-inhibiting factors, or high cell density) points to the existence of pathways that inhibit growth. The Btg family of proteins seems to be involved in the cell-cycle regulation network. In order to investigate the mechanism underlying the Btg-mediated functions, we looked for possible functional partners of Btg1 and Btg2. We identified a mouse protein, mCaf1, which binds to both Btg1 and Btg2. The physical interaction of Btg1 and Btg2 with mCaf1 was confirmed by GST pull-down experiments and a reciprocal set of experiments was carried out, using the Gal4 DNA-binding domain and the VP16 activation domain to demonstrate the functional and physical associations of mCaf1 with both Btg1 and Btg2 in transfected recipient HeLa cells. Interestingly, the specific interaction of Btg1 and Btg2 with mCaf1 occurs through a conserved domain of the Btg protein family, box B. And in fact Btg3, another member of the Btg family, also displayed a two-hybrid interaction with mCaf1 (data not shown). It may even be the case that all the members of the Btg family may be able to interact with mCaf1. The functional specificity of complexes formed by mCaf1 and the individual members of the Btg family could perhaps be achieved through the use of different downstream cellular targets.

A two-hybrid assay demonstrated that mCaf1, which is an evolutionarily conserved protein, interacts with the general transcriptional regulator CCR4 (24). mCAF1 is a homolog of yeast yCAF1/POP2, whose protein is a component of the CCR4 complex (24). Genetic analysis in yeast suggests that the CCR4 complex may perform multiple functions in transcription regulation. It also appears to be a key regulator in a numbers of cellular processes. It is required, for example, for the full derepression of ADH2 and other non-fermentative genes under glucose-derepressed conditions (25, 26). ccr4 and ycaf1 mutations affect the expression of genes involved in maintaining chromatin structure (27, 28) and in methionine biosynthesis (29). The CCR4 function occurs downstream from SPT6 and SPT10, as a post-chromatin remodeling event (30, 31). And yCAF1 disruption display phenotypes and transcriptional defects very similar to those of CCR4. Thus CAF1/POP2 and CCR4 appear to operate along the same pathway in the yeast model.

Moreover, yCaf1 associates with a yeast cell-cycle-regulated protein kinase, Dbf2, which is also a component of the CCR4 complex (32). These results provided a possible link between the CCR4 complex and the regulation of the cell cycle. Other proteins, components of the NOT complex, have recently been identified as being part of the CCR4 complex, which is thought to affect transcription both positively and negatively (33). Because of the evolutionary conservation of Caf among eukaryotes, it seems likely that this complex is conserved between yeast and higher eukaryotes, and that the Btg proteins are also among its components. Btg proteins may play a role in modulating transcriptional activity within a multiprotein complex, or in regulating the DNA binding properties of this complex, possibly operating as a kind of molecular glue. Or they may act in a transient way to facilitate interactions between multiple proteins and DNA, in which case they would not be a structural part of the transcriptionally active multiprotein complex. Experiments are currently being carried out to determine whether (and if so, how) Btg1 and Btg2 exist as multimeric complexes, and to investigate their potential role in transcription regulation.

However, there remains the possibility that CCR4 is not involved in the mCaf1-Btg interaction. And in fact Draper et al.
Fig. 3. Interaction of mCaf1 with Btg1 and Btg2 in the mammalian two-hybrid system. 200 ng of the indicated pGAL4 and pVP16 fusion expression plasmids were transiently co-transfected into HeLa cells with 0.5 μg of a reporter gene containing six GAL4-binding sites upstream from a minimal TK promoter fused to the CAT gene. Total DNA was kept constant at 1 μg. Cells were co-transfected as described under “Experimental Procedures.” Reporter activity was normalized with β-galactosidase activity, and the data expressed as the ratio of fold induction to the activity of the reporter vector alone. Each set of experiments was repeated at least three times, and similar results were obtained. Bars indicate standard deviation of the mean of at least three independent transfections. A, schematic representation of the reporter and effector vectors. The indicated regions of the BTGs and the mCAF1 cDNA were cloned into a vector containing the GAL4 DNA-binding domain. Similarly, overlapping regions were inserted into a vector containing the VP16 activation domain. B, interaction between GalBtgs and VpCaf, and between GalCaf and VPBtgs, in the mammalian two-hybrid system. As a positive control, we used the pGALVP plasmid which encodes a protein consisting of the GAL4 DNA-binding domain fused to the VP16 activation domain. C, expression of the Gal4 and VP16 fusion proteins expressed in HeLa cells transfected as described above. Lysates from transfected HeLa cells were subjected to Western blot analysis after SDS-PAGE, and then revealed by the indicated antibodies.
have observed that yCaf1 may recruit the transcriptional machinery by an independent mechanism that does not involve CCR4. In both models Btg1 and Btg2 play a role in the regulation of transcription, which is consistent with the fact that, in the two-hybrid assays, both Btg1 and Btg2 were found to interact with a homeodomain containing transcription factor (data not shown).

In addition to affecting the cell cycle similarly to the dbf2 and ccr4 mutations, the caf mutation suppresses the defect of rad 52-20, an allele of RAD52, a gene involved in UV sensitivity and DNA repair (24). Of particular interest in this regard is a recent sequence analysis study which indicates the presence of a proofreading exonuclease domain in the mCaf1 protein (35). And it is interesting to speculate that Btg2, whose expression is rapidly induced after genotoxic stress, and which is transcriptionally activated by p53, may contact a cellular protein involved in the control of cell cycle progression and DNA repair. With this in mind, along with the observation that BTG2 inactivation in embryonic stem cells leads to the disruption of DNA damage-induced G2/M arrest (3), it is reasonable to conjecture that Btg2 might participate, in association with mCaf1, in DNA repair processes, including replication-associated DNA repair.

Lin et al. (15) have recently shown that both Btg1 and Btg2 interact with a protein-arginine N-methyltransferase (Prmt1), and modulate its activity positively (15). And the importance of this result has been increased by the fact that Prmt1 has also been found to participate in a molecular association with the Ifnar1 chain of the interferon type 1 receptor (16). Given that Prmt1/Ifnar1 association is necessary for interferon-mediated growth arrest (16), it is likely that Btg1 and -2 can be involved in this process.

These observations indicate that Btg proteins may associate with other proteins at different stages in the cell cycle, and may play a variety of roles as intermediaries between signal transduction pathways and the final transcriptionally competent initiation complex. The study of the Btg-associated proteins, and their regulation, could bring to light new mechanisms which influence the cell cycle. The complete elucidation of these pathways is vital to the understanding of oncogenesis and tumor progression.

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