Interaction of CR6 (GADD45γ) with Proliferating Cell Nuclear Antigen Impedes Negative Growth Control*

Received for publication, June 27, 2000, and in revised form, September 14, 2000
Published, JBC Papers in Press, October 5, 2000, DOI 10.1074/jbc.M005626200

Naiyer Azam, Mariappan Vairapandi, Wei Zhang, Barbara Hoffman‡, and Dan A. Liebermann‡

From the Fels Institute for Cancer Research and Molecular Biology and the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

GADD45, MyD118, and CR6 (also termed GADD45α, β, and γ) comprise a family of genes that encode for related proteins playing important roles in negative growth control, including growth suppression. Data accumulated suggest that MyD118/GADD45/CR6 serve similar but not identical functions along different apoptotic and growth suppressive pathways. It is also apparent that individual members of the MyD118/GADD45/CR6 family are differentially induced by a variety of genetic and environmental stress agents. The MyD118, CR6, and GADD45 proteins were shown to predominantly localize within the cell nucleus. Recently, we have shown that both MyD118 and GADD45 interact with proliferating cell nuclear antigen (PCNA), a protein that plays a central role in DNA replication, DNA repair, and cell cycle progression, as well as with the universal cyclin-dependent kinase inhibitor p21. Moreover, it is shown that CR6 interacts with PCNA via a domain that also mediates interaction of both GADD45 and MyD118 with PCNA. Importantly, evidence has been obtained that interaction of CR6 with PCNA impedes the function of this protein in negative growth control, similar to observations reported for MyD118 and GADD45 (1).

The maintenance of normal cellular homeostasis requires that cells accurately decipher signals for cell cycle progression, growth, terminal differentiation, and apoptosis. Also, in recent years it has become increasingly evident that both the molecular basis for the initial increase in the susceptibility of malignant cells to anti-cancer agents and the development of treatment resistance originate from genetic lesions that alter the function of genes that play a role in normal cell homeostasis, notably in determining cell cycle progression and the apoptotic set point (2, 3). Thus, understanding the molecular genetic pathways that mediate negative growth control is of high priority from both a basic science and cancer therapeutic point of view.

To this end, cDNA clones of myeloid differentiation primary response (MyD)† genes, activated in M1 myeloblastic leukemia cells, in the absence of de novo protein synthesis, upon induction of terminal differentiation associated with growth arrest and apoptosis were isolated and characterized (4–6). In the course of this work several novel MyD genes were isolated including MyD118 (7). The MyD118 gene product was found to be remarkably similar to protein encoded by GADD45, a growth arrest- and DNA damage-induced gene, regulated in part by the p53 tumor suppressor gene (8, 9). A third member of the MyD118/GADD45 family, CR6 (cytokine response gene 6), was originally identified as an immediate early response gene in T cells stimulated by interleukin-2 (10, 11). Recently, the full-length sequences of murine and human CR6 cDNAs have been determined (12, 13). It has become evident that the MyD118/CR6/GADD45 family encodes for small (18 kDa) evolutionarily conserved proteins that are highly homologous to each other (55–58% overall identity at the amino acid level) (13), are highly acidic (pI ~4.0–4.2), and are primarily localized within the cell nucleus.

Data have accumulated to indicate that MyD118/CR6/GADD45 serve similar but not identical functions along different apoptotic and growth suppressive pathways. For example, it was observed that MyD118, but not GADD45, is activated upon transforming growth factor-β1-induced apoptosis. On the other hand, GADD45, but not MyD118 or CR6, was identified as target for p53 function (14–16). All three genes appear to be induced with different expression kinetics during terminal hematopoietic differentiation, which is associated with growth arrest and apoptosis (12). Also, distinct expression patterns for these genes were observed in a variety of murine tissues (12).

Importantly, it became evident that individual members of the MyD118/CR6/GADD45 family are differentially induced by a variety of genetic and environmental stress agents, suggesting that each gene is optimally induced by a certain subset of environmental stresses (13, 17). Short term transfection assays have revealed that MyD118/CR6/GADD45 can synergize in suppression of colony formation by several different human tumor cell lines (12, 18).

MyD118, CR6, and GADD45 were found to interact with several cellular proteins. All three proteins interact with and activate the stress-responsive MTK1/MEKK4, which is an upstream activator of the p38/JNK kinase pathways (19, 20). Evidence also has been obtained that MyD118 and GADD45 interact with PCNA (18, 21, 22), a normal component of multiple quaternary complexes, including the cycling CDKs and the CDK inhibitor p21 (23–25), which plays a central role in DNA repair and DNA replication (26–28). MyD118 and GADD45 also were found to interact with p21 (18).

Given the central role assigned for PCNA in cell proliferation and its identification as a cellular partner for MyD118 and...
GADD45, in this work we sought to determine whether CR6 interacts with PCNA, and if so, to dissect CR6/PCNA interacting domains and analyze their relevance for negative growth control. We show that PCNA domains, which previously were identified to mediate interaction with MyD118 and GADD45, also mediate interaction with CR6. PCNA interacting domains within CR6 were localized to the C terminus of the protein, similar to what was observed with MyD118 and GADD45 (1). CR6, like MyD118 and GADD45, also is shown to interact with p21. Importantly, it is shown that interaction of CR6 with PCNA, like interactions of MyD118 and GADD45 (1), impedes its function in negative growth control.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transient Transfection, and Cytokine Treatment—**H1299 and 293T cells were obtained from American Type Culture Collection and were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Cellgro) with 10% fetal bovine serum in a humidified atmosphere of 10% CO2 (293T were cultured at 5% CO2). H1299 and 293T cells were cultured to give 60–80% confluency at the time of transfection. One day before transfection H1299 cells were plated at 1.2 million cells/100-mm dish, and 293T cells were plated at 3 million cells/100-mm dish, with mid-log phase M1 cells cultured in RPMI medium supplemented with 10% heat-inactivated horse serum (Life Technologies, Inc.). The cells were cultured in a humidified atmosphere with 10% CO2 at 37 °C. One day before purification human rIL-6 (100 ng/ml) (Amgen Inc) treatment, 0.1 million M1 cells were plated per 150-mm dish.

**Yeast Two-hybrid Analysis—**Manipulation of Escherichia coli and DNA was performed according to standard methods (29). Yeast two-hybrid analysis was performed with the CLONTECH Matchmaker Two-Hybrid System-2, essentially as described in the CLONTECH protocol. Full-length murine CR6 cDNA in pBluescript (12) and human PCNA in p3038-ThTPCNA (30) were used to generate all the deletion constructs of CR6/PCNA. Deletion mutants were constructed by taking advantage of unique restriction enzyme sites within the coding sequence of these cDNAs. Full-length cDNAs and truncated cDNAs encoding for deletion peptides were cloned in-frame into pAct2 (GAL4 activation domain: "trap") as well as into the modified pAS2.1 (GAL4 DNA-binding domain: "bait") vector. All of the constructs were sequenced to verify in-frame cloning. Transformation into yeast strain Y187 was performed according to CLONTECH protocol. Yeast strain Y187 was used for testing interaction of the β-Gal gene (blue selection). Yeast strains CG1945 or Y190 were used to test interaction by activation of the histidine gene. Interaction assays were conducted only with pAct2/pAS2.1 constructs that tested negative for self-activation. Interactions either positive (+) or negative (−) were ascertained by both β-Gal expression (blue selection) and histidine expression (histidine selection). Quantitation of relative binding affinities of protein and deletion peptides was determined in yeast strain Y187. Three positive double transfectants were selected on minimal medium plates for each transfection and grown overnight in liquid selection minimal medium. The expression of β-Gal was quantified, according to the CLONTECH protocol, with O-nitrophenyl β-d-galacto- pyranoside as the substrate, and the intensity of yellow color development was measured colorimetrically at 420 nm.

**In Vitro Interaction Assays Using Coupled Transcription/Translation—**For in vitro association assays, full-length PCNA and Bax cDNA coding sequences were cloned into pET14b (18). Full-length p21 was cloned into pCDNA3. HA-tagged full-length CR6 and C-terminal peptide coding sequences were cloned into HApDCNA3.1 (−) kindly provided by Dr. Dhanasekaran. Full-length CR6 and the C-terminal peptide sequences were cloned into the unique EcoRI site of HApDCNA3.1 (−). N-terminal peptide encoding cDNAs were generated by truncating and linearizing full-length CR6 cDNA in HApDCNA3.1 (−) with unique restriction enzymes at the required sites, and were then used in the transcription/translation reaction (TNT; Promega). PCNA 87–261 aa (EcoRV-NdeI1 Fragment) was cloned into the pAS2.1 vector. Subsequently, PCNA 87–127 aa (NdeI-EcoRI fragment from pAS2.1) and PCNA 87–165 aa (NdeI-LgII1 fragment from pAS2.1) were excised and cloned in-frame into pET23b (Novagen), which was used in the TNT reaction. Constructs were purified by CsCl gradient centrifugation and suspended in DNase and RNase free H2O. The T7 based coupled transcription/translation system, with rabbit reticulocyte lysate (TNT; Promega) and Easy Tag L1 (“S”methionine (PerkinElmer Life Sciences), was used to produce radiolabeled proteins and peptides. In vitro interaction experiments were done as described (18). Briefly, equal amounts of 35S-labeled PCNA, Bax, and CR6 protein/peptides were mixed together (102 cpm each) in 200 μl of interaction buffer A (20 mM Tris, pH 8.3, 150 mM NaCl, 1.0% Nonidet F-40, 0.1% Tween 20, and 1.0 mg/ml bovine serum albumin). 35S-Labeled Bax was added to all reactions as a negative control for interaction. Each interaction reaction was carried out, in triplicate, for 30 min on ice. Subsequently each reaction mixture was nonspecifically immunoprecipitated with rabbit IgG or mouse IgG for 1 h. Following pulling down of nonspecific immune complexes with protein A/G-agarose beads (Oncogene Sciences), supernatants were subjected to co-immunoprecipitation with specific primary antibodies: PCNA (PC10; Santa Cruz), p21 (Ab-5; Calbiochem), Bax (N20; Santa Cruz), and HA (Y11; Santa Cruz). Immune complexes were pulled down with protein A/G-agarose beads. Beads were washed four times with interaction buffer A (without bovine serum albumin), suspended in Laemmli protein loading buffer, heated at 75 °C for 10 min, and loaded on 15% SDS-PAGE gel for analysis. Following electrophoresis the gels were fixed with 25% isopropanol in 10% acetic acid, subjected to fluorography with Amplify (Amersham Pharmacia Biotech), and vacuum dried before exposure to x-ray film.

**In Vivo Interaction Assays Using Transient Transfection into 293T or H1299 Cells—**For the in vivo interaction studies, the HA tag epitope sequences in the mammalian expression vector HApDCNA3.1 (−) were used. 10 μg of the pCDNA constructs encoding for Bax, p21, or HA-PCNA were co-transfected by the LipofectAMINE method with 0.5 μg of pTK-hyg. After 12 h, transfected cells were trypsinized and washed twice with PBS, and lysed in 0.4 ml of buffer containing protease inhibitors, as described previously. Triplet 1.0-ml aliquots of diluted cell lysates (200 μg) were subjected to co-immunoprecipitation as described above for in vitro interaction studies, using specific primary antibodies, or nonspecific rabbit or mouse IgG. Immune complexes were separated on 15% SDS-PAGE gel. The protein bands were transferred to nitrocellulose membrane (Hybond-EC; Amersham Pharmacia Biotech), Western probed with HA antibodies, and visualized by ECL (Amersham Pharmacia Biotech). Membranes were reprobed with PCNA antibodies after stripping the blot according to the manufacturer’s protocol.

**In Vivo Interaction Assays in IL-6-treated M1 Cells—**At 48 h after IL-6 treatment, M1 cells were harvested, washed twice with PBS, and lysed in 0.4 ml of buffer containing protease inhibitors, as described previously. Triplet 1.0-ml aliquots of diluted cell lysates (200 μg) were subjected to co-immunoprecipitation as described above for in vitro interaction assays, using specific primary antibodies, or nonspecific rabbit or mouse IgG. Immune complexes were separated on 15% SDS-PAGE gel. The protein bands were transferred to nitrocellulose membrane, Western probed with p21, CR6, PCNA antibodies, and visualized by ECL.

**Colony Suppression Analysis—**A short term transfection assay was used to assess the ability of full-length CR6 protein and N- or C-terminally truncated peptides to suppress colony formation in H1299 cells (18, 31). pTK-hyg was obtained from CLONTECH, Inc. Briefly, H1299 cells were co-transfected with the LipofectAMINE method with 0.5 μg of pTK-hyg together with 10 μg of empty HApDCNA3.1 (−) construct (negative control) or HApDCNA3.1 (−) encoding for HA-tagged full-length CR6 protein or N/C-terminal peptides. pTK-hyg was used for hygromycin selection and as an internal normalization control for transfection efficiency. Co-transfections were performed in 60-mm tissue culture dishes. One day following transfection, the cells were washed with PBS and trypsinized. The cells were replated in duplicates with hygromycin-containing medium (200 μg/ml) and incubated for 14 days to allow colonies to develop. Then the medium was removed, and colonies were washed once with PBS and fixed with 75% methanol in 25% acetic acid for 5 min, and the plates were dried. Colonies were stained with Lillie’s crystal violet (2 g of crystal violet and 0.8 g of ammonium oxalate in 80% ethanol) for 5 min and subsequently washed with deionized water to remove excess stain. Stained colonies containing more than 10 cells were scored and counted. The percentage of colony formation was normalized to colonies formed following transfection with empty HApDCNA3.1 (−). pCDNA3 encoding p53 or p21 were co-transfected with pTK-hyg as positive controls for anti-sense CR6 was used as an additional negative control.

**Apoptosis Analysis—**H1299 cells growing on coverslips in 35-mm tissue culture dishes were co-transfected with 2 μg of CR6 construct (in HApDCNA3.1 (−)) and 0.2 μg of β-Gal expression vector (pSV-β-Gal; Promega) using the LipofectAMINE method. β-Gal expression and apoptosis was analyzed 72 h following transfection. The cells were
washed once with PBS and fixed for 10 min with 0.05% glutaraldehyde in PBS. Following three washes with PBS to remove the fixative, X-gal solution (20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, 1 mM magnesium sulfate in PBS; X-gal is added to a final concentration of 1 mg/ml just before use, from a 20 mg/ml stock solution in N,N-dimethyl formamide) was spread over the dishes, and the dishes were incubated overnight at room temperature. The next day, the X-gal solution was removed, and cell nuclei were stained for 3 min with 0.1 μg/ml Hoechst No.33342 (Sigma) and then washed three times with PBS. The coverslips were mounted in Vectashield mounting medium H-1000 (Vector Labs inc. CA 94010) and analyzed under a Leitz fluorescent microscope. The percentage of apoptotic cells were determined by dividing the number of β-Gal expressing blue cells that exhibit apoptotic nuclear morphology (condensed/fragmented nucleus) by the total number of blue cells. At least 200 cells from five randomly chosen fields were analyzed for each experiment.

RESULTS

CR6 Interacts with PCNA and p21—Previously we have demonstrated that both MyD118 and GADD45 interact with PCNA and p21. It was, therefore, of interest to determine whether CR6, the third member in this related gene family, interacts with PCNA and/or p21. To this end the yeast two-hybrid system (YTHS) was employed (“Experimental Procedures”). Full-length CR6 was cloned into the Gal4 DNA-binding domain of pAS2.1 YTHS vector. The CR6 construct was co-transfected into yeast with pAct2 YTHS constructs encoding for either PCNA or p21 and fused to the Gal4 activation domain. Interactions, either positive (+) or negative (−) were ascertained by both β-Gal expression (blue color) and histidine expression (histidine selection) (“Experimental Procedures”). Empty pAct2 vector was used as the negative control in the YTHS for both qualitative and quantitative interaction with the full-length CR6 in pAS2.1 vector. As shown in Fig. 1 (A and B), full-length CR6 interacted with PCNA. Full-length CR6 interacted also with p21. The relative binding affinities of CR6 interactions with PCNA or p21 were quantified using the YTH liquid culture assay (“Experimental Procedures”). In this assay, the level of β-Gal expression in yeast under control of the interacting partner proteins encoded by pAct2 and pAS2.1 was quantified by measuring the intensity of yellow color development using O-nitrophenyl β-D-galactopyranoside as substrate. As shown in Fig. 1A, CR6 interacted equally strongly with PCNA and p21. To determine whether the interactions of CR6 with PCNA and p21, identified by the YTH approach, reflect direct association between these proteins, in vitro binding assays were performed with 35S-labeled PCNA or p21 and full-length HA-tagged CR6 protein that were generated by coupled transcription/translation. The murine Bax protein (pI 4.0) (32), was included as an internal control to monitor the specificity of the interactions. Equal amounts of 35S-labeled proteins were mixed, and following incubation, the protein mixtures were immunoprecipitated with antibodies specific to PCNA, p21, HA epitope, or Bax. As shown in Fig. 1B, PCNA or p21 were contained within HA-CR6 immune complexes, and CR6 was contained in PCNA or p21 immune complexes (Fig. 1B). The specificity of these interactions was inferred by the observation that Bax was not contained within PCNA, p21, or HA-CR6 immune complexes.

To further establish that these interactions are biologically relevant, M1 cells were treated with IL-6, and the cell lysates were obtained from untreated cells at 0 and 48 h after addition of IL-6. The Western blot analysis of cell lysates shows the abundance of PCNA protein at both 0 and 48 h (Fig. 1C). CR6 and p21 proteins were absent in untreated cells but were significantly induced at 48 h, as shown in Fig. 1C. As shown in Fig. 1D, the p21 immune complex obtained from cell lysate after 48 h of IL-6 treatment contained CR6. Reciprocally, the CR6 immune complex contained p21. It can also be seen that the PCNA immune complex, obtained from M1 cell extracts contained CR6. Reciprocally, the CR6 immune complex contained PCNA. Taken together these results demonstrate in vivo association of endogenous CR6 with endogenous p21 and PCNA.
Identification of CR6 and PCNA Interacting Domains Using the Yeast Two-hybrid System—Recently, we have observed that similar domains within PCNA, MyD118, and GADD45 mediate interactions between these proteins. Thus, it was of interest to determine whether amino acid domains, which mediate interactions between PCNA and CR6, are similar or different from those found to mediate interactions between PCNA, MyD118, and GADD45.

To this end, deletion constructs of PCNA cDNA, encoding for N-terminal, middle, or C-terminal peptides of PCNA were cloned into the Gal4 DNA-binding domain of the pAS2.1 YTHS vector. Each of these constructs were co-transfected into yeast with pAct2 YTHS constructs encoding CR6, fused to the Gal4 activation domain. As mentioned earlier, interactions were ascertained by both β-Gal expression (blue color selection) and histidine expression (histidine selection), as described under “Experimental Procedures.”

The relative binding affinities of different PCNA domains with CR6 were quantified using the YTH liquid culture assay (“Experimental Procedures”). As shown in Fig. 2, the interaction between full-length PCNA and full-length CR6 was weaker than the interaction when using PCNA/1-46 or PCNA/87-127 peptides. This observation is consistent with the notion that full-length PCNA in addition to CR6 interacting domain contains domains that hinder interaction.

Having mapped domains within PCNA that interact with CR6, next we mapped domains within CR6 that mediate interaction with PCNA cDNA restriction enzyme sites used to generate truncated cDNAs encoding for the deletion peptides are shown at the bottom of the figure. Numbers in parentheses indicate relative binding affinities and standard deviations, determined by using the yeast two-hybrid liquid culture assays, as indicated under “Experimental Procedures.”

Identification of CR6 and PCNA Interacting Domains Using the Yeast Two-hybrid System—Recently, we have observed that similar domains within PCNA, MyD118, and GADD45 mediate interactions between these proteins. Thus, it was of interest to determine whether amino acid domains, which mediate interactions between PCNA and CR6, are similar or different from those found to mediate interactions between PCNA, MyD118, and GADD45.

To this end, deletion constructs of PCNA cDNA, encoding for N-terminal, middle, or C-terminal peptides of PCNA were cloned into the Gal4 DNA-binding domain of the pAS2.1 YTHS vector. Each of these constructs were co-transfected into yeast with pAct2 YTHS constructs encoding CR6, fused to the Gal4 activation domain. As mentioned earlier, interactions were ascertained by both β-Gal expression (blue color selection) and histidine expression (histidine selection), as described under “Experimental Procedures.”

The relative binding affinities of different PCNA domains with CR6 were quantified using the YTH liquid culture assay (“Experimental Procedures”). As shown in Fig. 2, the interaction between full-length PCNA and full-length CR6 was weaker than the interaction when using PCNA/1-46 or PCNA/87-127 peptides. This observation is consistent with the notion that full-length PCNA in addition to CR6 interacting domain contains domains that hinder interaction.

Having mapped domains within PCNA that interact with CR6, next we mapped domains within CR6 that mediate inter-
action with PCNA. The YTH vector pAS2.1 was used to construct yeast expression vectors that encode for N- and C-terminal deletion peptides of CR6. These deletion peptides were tested for interaction with PCNA cloned in the YTH vector pAct2 (Fig. 3, and see legend). It can be seen that CR6/76–159 interacted with PCNA as well as full-length CR6, whereas CR6/1–135 failed to interact. This indicated that the C-terminal (76–159 aa) region of CR6 harbors a domain that is required for interaction with PCNA.

The relative binding affinities of full-length and C-terminal peptides of CR6 with PCNA were quantified using β-Gal expression in yeast and are shown in Fig. 3. Notably, the binding affinity to PCNA of the CR6/76–159 C-terminal peptide was higher than the full-length CR6. Taken together these findings indicate that amino acid domains, which mediate interactions between CR6 and PCNA, are similar to the domains that were observed to mediate interaction between MyD118, GADD45, and PCNA (see Ref. 1 and “Discussion”).

Analysis of CR6 and PCNA Interactions in Vitro—To determine whether the interacting domains identified in CR6 and PCNA by the YTH approach are capable of associating directly in vitro, binding assays were performed with 35S-labeled PCNA, HA-tagged CR6 full-length protein and deletion peptides, generated by coupled transcription/translation. The murnine Bax protein was included as an internal control to monitor the specificity of the binding assay. Equal amounts of 35S-labeled proteins were mixed, and following incubation, the protein mixtures were immunoprecipitated with antibodies specific to PCNA, HA, or Bax. Because the PCNA monoclonal antibody (PC10) used was specific for amino acids 111–120 of PCNA, it could be used only to test for interaction of the encoded CR6 protein with p21 or HApcDNA3.1(−) expression vector encoding either for Bax (as negative control), or p21, or HApcDNA3.1(−) expression vectors encoding for full-length CR6 and deletion peptides, all with an HA tag, were transiently transfected into 293T cells, and 24 h later cell extracts were tested for interaction of the encoded CR6 protein with p21 or endogenous PCNA by co-immunoprecipitation with either p21, PCNA, or HA antibody.

The results of the in vitro association assays are shown in Fig. 5. As shown in Fig. 5A, p21 immune complex contained CR6. Reciprocally, CR6 immune complex contained p21, which confirms the in vitro association of CR6 with p21. The specificity of the interactions in 293T cells were further demonstrated by the observation that neither CR6 nor p21 immune complexes contained Bax. It can also be seen that PCNA immune complex, obtained from 293T cell extracts transfected with HA-CR6/pcDNA3.1(−), contained HA-CR6. Reciprocally, HA tag immune complex obtained from the same cells contained
PCNA Impedes CR6 Negative Growth Control

To accomplish this, a hygromycin selection plasmid (pTK-hyg) together with HApcDNA3.1(−), encoding for HA-CR6 deletion peptides, or empty HApcDNA3.1(−) control plasmid, at 1:20 ratio, were transfected into H1299 cells. Following 2 weeks of selection in hygromycin-containing medium, surviving colonies were fixed, stained, and scored. In all the experiments empty vector and antisense-CR6 in pcDNA3 were used as negative control, and p53 and its target gene p21 were used as positive controls. The results of the short term colony suppression assays are shown in Fig. 6. Representative tissue culture plates that were fixed, stained, and scored are shown in Fig. 6A. Results of all experiments are summarized in Fig. 6C, where colony formation using empty HApcDNA3.1(−) vector is the standard for no colony suppression and is, therefore, set at 100%. Data used for calculating colony suppression have been derived only from experiments where the initial expression of the various proteins and peptides did not vary significantly, as shown in Fig. 6B.

As shown in Fig. 6, p53 suppressed colony formation by about 90%, whereas p21 and CR6 inhibited colony formation by about 75 and 60%, respectively. Interestingly, the C-terminal CR6 peptide, which harbors the PCNA interacting domain, suppressed colony formation significantly less efficiently than the full-length protein (30 and 60%, respectively), whereas the non-PCNA interacting N-terminal CR6 peptide suppressed colony formation more efficiently than the full-length protein (80 and 60%, respectively). Taken together, these findings indicate that interaction of CR6 with PCNA impedes the function of this protein in negative growth control.

Interaction with PCNA Impedes CR6-mediated Apoptotic Cell Death—To further examine the role of CR6 interaction with PCNA in H1299 colony suppression and determine whether it was due to apoptotic cell death, H1299 cells growing on coverslips were co-transfected with limiting amount (0.2 μg) of β-Gal expression plasmid together with excess (2.0 μg) pcDNA3.1(−) encoding for full-length HA-CR6 or deletion peptides. Following 72 h the transfected cells were analyzed for β-Gal expression (blue color), and nuclei of the transfected cells were stained with Hoechst to detect apoptotic morphology characterized by nuclear shrinkage and chromatin condensation (Fig. 7). Percentage of apoptotic cells was determined as the number of β-Gal expressing blue cells that exhibited apoptotic nuclear morphology divided by the total number of β-Gal expressing blue cells.

As shown in Fig. 7, ectopic expression of CR6 induced apoptosis in H1299 cells. Furthermore, as observed for H1299 colony suppression, C-terminal CR6 peptide, which harbors the PCNA interacting domain, induced apoptosis appreciably less efficiently than the full-length proteins. In contrast, induction of apoptosis by N-terminal CR6 peptide, which did not interact with PCNA, was significantly more efficient than what was observed with the full-length CR6 (Fig. 7).

These findings suggest that apoptotic cell death is a major factor, which contributes to the apparent ability of CR6 to suppress colony formation of tumor cells. These findings also extend the notion that interaction of CR6 with PCNA impacts negatively on the ability of this protein to induce cellular death.

DISCUSSION

In this work we have provided evidence that CR6, like MyD118 and GADD45, interacts both with PCNA and the cyclin-dependent kinase inhibitor p21. The physiological significance of CR6/MyD118/GADD45 interaction with p21 for cell cycle arrest, DNA repair, and/or DNA replication remains to be explored. Although the endogenous CR6 is undetectable in
untreated leukemic M1 cells, CR6 protein expression is induced after IL-6 treatment. The endogenous CR6 is shown to interact both with endogenous PCNA and p21 proteins. Upon completion of this work, and consistent with our findings, interaction of CR6 (termed OIG37) with PCNA and p21 has been reported by another group of investigators (33).

PCNA is a normal component of multiple quaternary complexes, which include the cycling CDKs and the CDK inhibitor p21WAF1/CIP1 (34–36), that play a pivotal role in cell cycle regulation (23, 24, 34), DNA replication (28), and repair of damaged DNA (37). Given the central role of PCNA in cell proliferation and that similar domains within PCNA were observed to interact with similar amino acid regions within MyD118 and GADD45, clearly it was of interest to dissect domains that mediate interaction between CR6 and PCNA, to determine whether these are same or different from those observed to mediate interactions between PCNA, MyD118, and GADD45, and also to analyze the significance of CR6-PCNA interaction for negative growth control. To unequivocally identify amino acid regions that mediate interactions between CR6 and PCNA, several complementary in vivo and in vitro methods were used, including the yeast two-hybrid system, in vitro transcription/translation and transient expression in various cells.

The YTH approach has led to the identification of three CR6 interacting domains within PCNA that have been localized to the N-terminal (1–46 aa), middle (87–127 aa), and C-terminal (224–261 aa) regions of PCNA. Evidence also was obtained for regions within PCNA, i.e. 47–86 and 150–223 aa, that compromised the binding of these PCNA domains to CR6 (Fig. 2). It is possible that such regions may play a physiological role in modulating PCNA interactions with CR6 in response to or following exposure of cells to genotoxic stimuli. Only the N-terminal and the middle domains of PCNA were found to associate with CR6 in vitro. Thus, the apparent interaction of CR6 with C-terminal PCNA in yeast may reflect indirect interaction. The C terminus of PCNA contains the PCNA/PCNA interacting domain (38), and it is possible that following co-transfection of YTH vectors into yeast, the C-terminal region of PCNA encoded by one YTH vector interacts with endogenous PCNA that is associated with CR6 encoded by the other YTH vector. Another possibility is that, unlike in vitro conditions, under in vivo conditions the C-terminal region of PCNA may assume a tertiary structure and/or may be post-translationally modified to facilitate interaction with CR6.

Initial information regarding the strength of interactions between PCNA and CR6 was obtained using the yeast two-hybrid approach in a semi-quantitative assay. Based on this assay, evidence was obtained that the N-terminal (1–46 aa)
CDK1 (cdc2) and inhibits the kinase activity of the CDK1 interact with and activate the stress-responsive MTK1/MEKK4 B complex, implicated in GADD45 induction of a G2/M cell cycle addition, it was documented that GADD45 can recognize dam-check point in response to certain genotoxic stresses (52, 53). In
summary, we have observed that the expression kinetics of CR6 differed from the expression of MyD118 and GADD45 in murine tissues, in hematopoietic cell development, and in the response of cells to varying genotoxic agents (12, 13, 33). CR6, however, like MyD118 and GADD45 was found to inhibit col-
ony formation and to interact with and activate MTK1/MEKK4 kinase, an upstream regulator of the stress-induced p38/JNK kinase pathways (13). Thus, to further dissect the role CR6, MyD118, and GADD45 proteins play in negative growth control and to determine what role the intricate network of homo-
and hetero-interactions and interactions with other cellular proteins play in cell cycle arrest, apoptosis, and DNA repair, a battery of CR6/MyD118/GADD45 deletion mutants (12 aa in length each) are being genetically engineered. This battery of mutants should provide valuable tools to further elucidate the physiological role that this family of acidic proteins and their myriad of interactions with other cellular proteins play in negative growth control and DNA repair.

REFERENCES

1. Vairapandi, M., Azam, N., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2000) J. Biol. Chem. 275, 16810–16819
2. Muschel, R. J., Soto, D. E., McKenna, W. G., and Bernhard, E. J. (1998) Oncogene 17, 3359–3363
3. Fischer, D. E. (1994) Cell 73, 539–542
4. Liebermann, D. A., and Hoffman, B. (1994) Stem Cells 12, 352–369
5. Liebermann, D. A., Hoffman, B., and Steinman, R. A. (1995) Oncogene 11, 199–210
6. Lord, K. A., Abdollahi, A., Hoffman-Liebermann, B., and Liebermann, D. A. (1990) Cell Growth Differ. 1, 657–665
7. Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) Oncogene 6, 165–167
8. Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994) Mol. Cell. Biol. 14, 2352–2360
9. Guilloif, C., Grana, X., Selvakumaran, M., De Luca, A., Giordano, A., Hoffman, B., and Liebermann, D. A. (1995) Blood 85, 2601–2608
10. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) Cell 71, 587–597
11. Wang, X., Gorospe, M., and Holbrook, N. J. (1999) J. Biol. Chem. 274, 29599–29602
12. Vairapandi, M., Balliet, A. G., Furnace, A. J., Jr., Hoffman, B., and Liebermann, D. A. (1999) Oncogene 18, 4899–4907
13. Takekawa, M., and Saito, H. (1998) Cell 95, 521–530
14. Selvakumaran, M., Lin, H. K., Sjin, R. T., Reed, J. C., Liebermann, D. A., and Hoffman, B. (1994) Mol. Cell. Biol. 14, 2352–2360
15. Guilloif, C., Grana, X., Selvakumaran, M., De Luca, A., Giordano, A., Hoffman, B., and Liebermann, D. A. (1995) Blood 85, 2601–2608
16. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) Cell 71, 587–597
17. Wang, X., Gorospe, M., and Holbrook, N. J. (1999) J. Biol. Chem. 274, 29599–29602
18. Vairapandi, M., Balliet, A. G., Furnace, A. J., Jr., Hoffman, B., and Liebermann, D. A. (1999) Oncogene 18, 4899–4907
19. Takekawa, M., Posas, F., and Saito, H. (1997) EMBO J. 16, 4973–4982
20. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 8288–8295
21. Smith, M. L., Chen, I. T., Zhan, Q., Bae, I., Chen, C. Y., Gilmer, T. M., Kastan, M. B., O’Connor, P. M., and Furnace, A. J., Jr. (1994) Science 266, 1376–1380
22. Hall, P. A., Kearsey, J. M., Coates, P. J., Norman, D. G., Warbrick, E., and Cox, L. S. (1995) Oncogene 10, 2427–2433
23. Grana, X., and Reddy, E. P. (1995) Oncogene 11, 211–219
24. Sherr, C. J. (1994) Cell 78, 651–555
25. Zhang, H., Xiong, Y., and Beach, D. (1993) Mol. Cell. Biol. 13, 450–459
26. Henderson, D. S., Banga, S. S., Grigliatti, T. A., and Boyd, J. F. (1994) Mol. Cell. Biol. 14, 1450–1459
27. Wilcock, D., and Lane, D. P. (1991) Nature 349, 429–431
28. Prelich, G., Tan, K. C., Kostura, M., Mathews, B. M., So, A. G., Downey, K. M., and Stillman, B. (1987) Nature 326, 517–520
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Fien, K., and Stillman, B. (1992) Mol. Cell. Biol. 12, 155–163
31. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Furnace, A. J., Jr. (1994) Mol. Cell. Biol. 14, 2361–2371
32. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 74, 609–619
33. Nakayama, K., Hara, T., Hibi, M., Hirano, T., and Miyajima, A. (1999) J. Biol. Chem. 274, 24766–24772
34. Hunter, T., and Pines, J. (1994) Cell 79, 573–582
35. Xiong, Y., Zhang, H., and Beach, D. (1992) Cell 71, 505–514
36. Xiong, Y., Zhang, H., and Beach, D. (1993) Genes Dev. 7, 1572–1583
37. Shi, J. K., Kenny, M. K., and Wood, R. D. (1992) Cell 70, 367–374

2 N. Azam, B. Hoffman, and D. A. Liebermann, unpublished results.
PCNA Impedes CR6 Negative Growth Control

38. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Cell 79, 1233–1243
39. Jonsson, Z. O., and Hubscher, U. (1997) Bioessays 19, 967–975
40. Sancar, A. (1994) Science 266, 1954–1956
41. Kelman, Z., and Hurwitz, J. (1998) Trends Biochem Sci 23, 236–238
42. Dynlacht, B. D. (1997) Nature 389, 149–152
43. Nigg, E. A. (1996)Curr. Opin Cell Biol 8, 312–317
44. Reardon, J. T., Ge, H., Gibbs, E., Sancar, A., Hurwitz, J., and Pan, Z. Q. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6482–6487
45. Zhang, P., Sun, Y., Hsu, H., Zhang, L., Zhang, Y., and Lee, M. Y. (1998) J. Biol. Chem. 273, 713–719
46. Gary, R., Ludwig, D. L., Cornelius, H. L., MaInnes, M. A., and Park, M. S. (1997) J. Biol. Chem. 272, 24522–24529
47. Warbrick, E., Lane, D. P., Glover, D. M., and Cox, L. S. (1997) Oncogene 14, 2313–2321
48. Levin, D. S., Bai, W., Yao, N., O'Donnell, M., and Tomkinson, A. E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12863–12868
49. Warbrick, E., Lane, D. P., Glover, D. M., and Cox, L. S. (1995) Curr. Biol. 5, 275–282
50. Chen, J., Peters, R., Saha, P., Lee, P., Theodoras, A., Papano, M., Wagner, G., and Dutta, A. (1996) Nucleic Acids Res. 24, 1727–1733
51. Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996) Cell 87, 297–306
52. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3706–3711
53. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999) Oncogene 18, 2892–2890
54. Carrier, F., Georgel, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Gariboldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace, A. J., Jr. (1999) Mol. Cell. Biol. 19, 1673–1685
Interaction of CR6 (GADD45γ) with Proliferating Cell Nuclear Antigen Impedes Negative Growth Control
Naiyer Azam, Mariappan Vairapandi, Wei Zhang, Barbara Hoffman and Dan A. Liebermann

J. Biol. Chem. 2001, 276:2766-2774. doi: 10.1074/jbc.M005626200 originally published online October 5, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005626200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 53 references, 21 of which can be accessed free at http://www.jbc.org/content/276/4/2766.full.html#ref-list-1