CRIF1 is barely detectable in adrenal adenoma and papillary thyroid cancer and much lower than in adjacent normal tissue. The results presented here suggest that CRIF1 is a novel nuclear protein that interacts with Gadd45 and may play a role in negative regulation of cell cycle progression and cell growth.

The growth arrest and DNA damage-inducible (Gadd45) family of genes includes Gadd45α (1), MyD118/Gadd45β (2), and CR6/OIG37/Gadd45γ (3–5). Gadd45α was initially identified as a gene whose expression was rapidly induced by UV irradiation of Chinese hamster ovary cells (1). MyD118 is an immediate early response gene induced by interleukin-6 in the murine myeloid cell line M1 (2). CR6/OIG37/Gadd45γ (5) is an activator of MEKK4/MTK1, an interleukin-2-induced immediate early gene (3), and an oncostatin M-inducible gene (OIG37) (4). The Gadd45 family members are highly conserved, with 68 and 70% amino acid sequence similarity between Gadd45γ and Gadd45α, respectively.

Gadd45α is easily induced by agents that cause DNA damage including UV, N-acetoxy-2-acetylaminofluorene, methylmethane sulfonate, and H2O2 (6). DNA damage-induced transcription of the Gadd45 gene is mediated by both p53-dependent (7) and p53-independent mechanisms (8). The phenotype of Gadd45-deficient mice is similar to the phenotype of p53-deficient mice, including genomic instability, sensitivity to radiation-induced carcinogenesis, and a low frequency of exencephaly (9). These findings suggest that Gadd45 is one component of the p53 pathway that maintains genomic stability.

Gadd45 proteins have also been implicated in DNA replication (10), DNA repair (10), G1 progression and G2/M checkpoint control (11, 12), apoptosis, and regulation of signal transduction (5, 13). Gadd45 proteins display a complex array of physical interactions with other proteins involved in these processes. It has been reported that Gadd45 proteins interact with proliferating cell nuclear antigen (10, 14–17, 20) and with cyclin-dependent kinase inhibitor p21 (17–20) in the nucleus. In addition, Gadd45 proteins activate the p38/Jun N-terminal kinase pathway by binding to MKT1/MEKK4 (5) in response to environmental stress. Gadd45 also interacts with Cdc2 and inhibits Cdc2-cyclin B1 kinase activity (21, 22). These findings led to the hypothesis that Gadd45 members play a critical role in negative growth...
control and apoptosis, but the detailed molecular mechanisms by which Gadd45 carries out this role remain to be elucidated.

This study used a yeast-two hybrid screen to identify novel proteins that bind to CR6/Gadd45/H9251, CR6-interacting factor 1 (CRIF1). CRIF1 is a nuclear protein identified in this screen. CRIF1 interacts with Gadd45/H9251, Gadd45/H9252, and Gadd45/H9253, and it is ubiquitously expressed in human tissues. CRIF1 is exclusively localized to the nucleus and colocalizes with Gadd45/H9253. Gadd45 proteins and CRIF1 inhibit Cdc2-cyclin B1 kinase in an additive manner, and CRIF1 inhibits histone H1 kinase activity associated with immunoprecipitated Cdk2-cyclin E in the absence of Gadd45 proteins. Overexpression of CRIF1 inhibits cell cycle progression at G1/S and suppresses growth in serum-stimulated NIH3T3 cells. In addition, adrenal adenoma and thyroid papillary cancer cells express CRIF1 at a very low level, which is lower than in adjacent normal tissue. These findings suggest that CRIF1 is a nuclear protein that modulates cell cycle progression and growth. Altered expression of CRIF1 may also contribute to tumorigenesis in some cell types.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture**—Dulbecco’s modified Eagle’s medium, Trizol, and LipofectAMINE were obtained from Invitrogen. Fetal bovine serum was purchased from Hyclone. Anti-FLAG antibody was purchased from Stratagene. The anti-Myc and anti-Rb (total and phosphospecific) antibodies were purchased from Cell Signaling Technologies, Inc. Horseradish peroxidase-linked anti-rabbit secondary antibody and chemiluminescent detection system were purchased from Cell Signaling Technologies. The Wizard Plus Miniprep DNA purification system and Plasmid Maxi kit were from Qiagen, and a vacuum manifold was purchased from Promega. All other enzymes and chemicals, either molecular biology grade or reagent grade materials, were purchased from Sigma.

The colon carcinoma cell line, RKO, the African green monkey cell line, COS-7, the mouse fibroblasts, NIH3T3, and the colon carcinoma cell line, HCT116, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO2 and 95% air atmosphere at 37 °C.

**Plasmids**—Plasmid DNA was generated using standard cloning procedures and constructs were verified by restriction enzyme analysis and DNA sequencing.

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1 The abbreviations used are: CRIF1, CR6-interacting factor 1; GST, glutathione S-transferase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate.
DNA sequencing. Plasmid pAS-Gadd45α encodes a fusion between the GAL4 DNA binding domain and Gadd45α/CR6. pAS-Gadd45α was constructed by inserting PCR-amplified human Gadd45α cDNA into the pAS2–1 vector (Clontech Laboratories). Constructs for expression of GST fusion proteins were constructed by cloning into pGEX-4T (Amersham Biosciences). Human Gadd45α cDNA (supplied by Dr. A. J. Fornace Jr.) and pCAR-periplasmic mouse Gadd45α/myD118 and human Gadd45α/CR6 cDNA were cloned into pGEX-4T.

For mammalian expression, pFLAG-Gadd45α, pFLAG-Gadd45β, pFLAG-Gadd45γ, and pFLAG-CRIF1 were constructed by inserting cDNA into the EcoRI/XhoI sites of pCMV-Tag2 (Stratagene). pMYC-CRIF1 was constructed by inserting cDNA into the EcoRI/XhoI sites of pCMV-Tag3 (Stratagene).

For mammalian two-hybrid assay, PCR-amplified Gadd45α, Gadd45β/myD118, Gadd45γ/CR6, and CRIF1 cDNA were cloned into pCMV-BD and pCMV-AD (Stratagene).

Yeast Two-hybrid and cDNA Library Screening—Yeast strain Y190 (Clontech Laboratories, Inc.) was transformed with pAS-Gadd45α and a Matchmaker two-hybrid cDNA library from K-562 cells (Clontech Laboratories, Inc.) was transformed with pAS-Gadd45α. Transformants were selected on SD medium lacking histidine, leucine, and tryptophan and containing 45 mM 3-amino-1,2,4-triazole (Sigma). After β-galactosidase filter assay, 72 blue colonies were sequenced and further analyzed. Full-length DNA was rescued from yeast, amplified in PCR and aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was centrifuged at 10,000 g and the supernatant was removed and incubated with primary antibody for 2 h with rocking. Protein A/G beads were added, incubated for 12 h, and centrifuged at 10,000 g. Immunoprecipitates were collected and washed three times with radioimmune precipitation buffer.

For Western blot analysis, cells were lysed at 4 °C for 20 min in radioimmune precipitation lysis buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was centrifuged at 10,000 g for 10 min. The supernatant was incubated with rocking for 2 h with 20 μl of protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and centrifuged for 15 min at 8,000 g. The supernatant was removed and incubated with primary antibody for 2 h with rocking. Protein A/G beads were added, incubated for 12 h, and centrifuged at 10,000 g. Immunoprecipitates were collected and washed three times with radioimmune precipitation buffer.

For Western blot analysis, cells were lysed at 4 °C for 20 min in 10 mM KPO4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 2 mM dithiothreitol, 1% Nonidet P-40, 1 mM Pefabloc (Roche Applied Science), and 10 μg of each aprotinin and leupeptin per milliliter. Total protein lysates were denatured by boiling in Laemmli sam-

For mammalian two-hybrid assay—Plasmids pCB-Gadd45α (for GAL4 DBD-fusion) and pCA-CRIF1 (for NF-κB AD fusion) or pCB-CRIF1 and pCA-Gadd45α (100 ng each) were co-transfected with 100 ng of pFR-Luc reporter (Stratagene) into NIH-3T3 cells and grown in 24-well plates using LipofectAMINE Plus (Invitrogen). Total plasmid DNA was adjusted to 400 ng/well with vector DNA (pCMV-BD or pCMV-AD). 100 ng of pCMV-β-gal DNA (Clontech Laboratories, Inc.) was used as internal control. Cells were lysed 24 h after infection, and luciferase activity was measured by Berthold LB9507 luminometer (Berthold). Luciferase activity was normalized to β-galactosidase activity.

Northern Blot Analysis—The human multiple tissue mRNA blots (Clontech Laboratories, Inc.) containing 2 μg of poly(A)+ RNA per lane were hybridized using QuickHyb solution (Stratagene) under stringent conditions as recommended by the manufacturer. CRIF1 cDNA or DECAprobe glyceraldehyde-3-phosphate dehydrogenase template (Ambion) was labeled with [α-32P]dATP with the Strip-EZTM DNA labeling kit (Ambion).

Immunoprecipitation and Western Blot Analysis—The following immunoprecipitation procedures were carried out at 4 °C using COS-7 cells coexpressing tagged Gadd45α, p, and γ with/without pFLAG-CRIF1 transfection. Cells were grown on 100-mm dishes, washed with phosphate-buffered saline twice, and incubated for 30 min with radioimmune precipitation lysis buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The cell lysate was centrifuged at 10,000 × g for 10 min. The supernatant was incubated with rocking for 2 h with 20 μl of protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and centrifuged for 15 min at 8,000 × g. The supernatant was removed and incubated with primary antibody for 2 h with rocking. Protein A/G beads were added, incubated for 12 h, and centrifuged at 10,000 × g. Immunoprecipitates were collected and washed three times with radioimmune precipitation buffer.

For Western blot analysis, cells were lysed at 4 °C for 20 min in 10 mM KPO4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2. 50 mM β-glycerophosphate, 2 mM dithiothreitol, 1% Nonidet P-40, 1 mM Pefabloc (Roche Applied Science), and 10 μg of each aprotinin and leupeptin per milliliter. Total protein lysates were denatured by boiling in Laemmli sam-

**Fig. 2. Expression and cellular localization of CRIF1.** A, the full-length CRIF1 coding sequence was synthesized by PCR with specific primers and inserted downstream of the T7 promoter into the pSP72 vector. In vitro transcription and translation were performed using a TNT-T7-coupled reticulocyte lysate system (Promega) in the presence of [35S]methionine. A plasmid containing the luciferase gene was used as a control. The synthesized proteins were analyzed by 15% SDS-PAGE in the absence or presence of β-mercaptoethanol. B, the human CRIF1 full-length sequence was subcloned into the pCMV-Tag2 mammalian expression vector (Stratagene) downstream of the human cytomegalovirus promoter to generate pFLAG-CRIF1. pFLAG-CRIF1 or control pCMV-Tag2 was transfected into COS-7 cells by LipofectAMINE Plus. Proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis and Western blot with anti-FLAG antibody. C, the human mRNA blots were from Clontech Laboratories and included 2 μg of poly(A)+ RNA lane. CRIF1 cDNA or DECAprobe glyceraldehyde-3-phosphate dehydrogenase template (Ambion) was labeled with [α-32P]dATP with the Strip-EZTM DNA labeling kit (Ambion). D, Western blot with antiserum from New Zealand White rabbits immunized with CRIF1 peptide. Cell lysates from mouse, rat, and human cell lines were separated by 15% SDS-PAGE. E, COS-7 cells, transfected with pFLAG-CR6 and pMyc-CRIF1, grown on uncoated glass coverslips (Bellco) were incubated with anti-FLAG antibody and anti-c-Myc antibodies. Cells were washed five times with PBS+ and incubated with secondary antibody conjugated to TRITC or fluorescein isothiocyanate for 60 min at room temperature. Cells were washed five times in PBS+ before mounting. Fluorescence was visualized with a Nikon Eclipse E400 microscope equipped with epifluorescence optics and a digital camera; images were captured as Adobe Photoshop files.
buffer (20 mM Tris-HCl, pH 7.8, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM leupeptin, 1 mM pepstatin) were washed extensively with binding buffer, and bound proteins were eluted in 25 μl of Laemmli sample buffer, boiled for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis (10%) followed by autoradiography. B, pCB-Gadd45γ (for GAL4 DBD-fusion) and pCA-CRIF1 (for NF-κB DBD fusion) or pCB-CRIF1 and pCA-Gadd45γ (100 ng each) were co-transfected with 100 ng of pFR-Luc reporter (Stratagene) into NIH-3T3 cells and grown in 24-well plates using LipofectAMINE Plus (Life Technologies). Total plasmid DNA was adjusted to 400 ng/well with pCMV-β-gal or pCMV-AD. 100 ng of pCMV-β-gal DNA (Clontech Laboratories) was used as internal control. 24 h after transfection, cells were lysed, and luciferase activity was measured by a Berthold LB9507 luminometer. Luciferase activity was normalized to β-galactosidase activity or protein. C and D, in vivo interactions between CRIF1 and Gadd45 proteins. COS-7 cells were transfected with tagged Gadd45α, -β, and -γ expression plasmids and CRIF1 expression plasmid. Immunoprecipitates containing CRIF1 were prepared with anti-FLAG and anti-Myc antibodies, and the immunoprecipitates were applied to a 15% polyacrylamide gel. Anti-FLAG (for GAL4 IP), anti-Myc (for CRIF1 IP), and anti-FLAG (for CRIF1 IP) antibodies were used for immunoblot analysis. E, COS-7 cells were transfected with pFLAG-Gadd45α, -β, and -γ expression plasmids, and immunoprecipitates were prepared by anti-FLAG antibody. Anti-CRIF1 antibody was used to detect coimmunoprecipitated endogenous CRIF1. IB, immunoblot; IP, immunoprecipitation.

Flow Cytometry Analysis—Samples were prepared for flow cytometry essentially as described previously (24). Briefly, cells were washed with 1× phosphate-buffered saline and then fixed with ice-cold 70% ethanol. Samples were washed with 1× phosphate-buffered saline and then stained with propidium iodide 60 μg/ml (Sigma) for 30 min at 37 °C. Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer and Cell Quest version 1.2 software (Becton Dickinson Immunocytometry Systems, San Jose, CA). At least 10,000 cells were analyzed per sample. Cell cycle distribution was quantified using the ModFit LT version 1.01 software (Verity Software House Inc., Topsham, ME).

Small Interfering RNA (siRNA) Experiments—The 21-nucleotide siRNA were synthesized and purified using a Silencer siRNA construction kit (Ambion Ltd.). The siRNA sequence targeting the human CRIF1 (GenBank™ accession number AF479749) corresponded to the coding region, as showed in Fig. 6A. The desalted DNA of the sense and antisense oligonucleotides targeting four different regions on the human CRIF1 (Fig. 6A) were synthesized, and the eight nucleotides at the 3'-end of both oligonucleotides had the following sequence: 5'-GGATCCTC-3', which is complementary to the T7 promoter. In order to produce an efficient transcription template, the sense and antisense siRNA transcripts need to be converted to double-stranded RNA with a T7 promoter in 37 °C. The sense and antisense siRNA transcripts were transcribed for 2 h in separate reactions with T7 RNA polymerase. The reactions were then mixed, and the combined reaction was incubated overnight at 37 °C for double-stranded RNA. A single-strand specific ribonuclease and DNase digestion were used to eliminate the 5'-overhanging leader sequence and the DNA template.
CRIF1 Interacts with Gadd45

respectively. The resulting siRNA was recovered from the mixture of nucleotides, enzymes, short oligomers, and salts in the reaction by column purification. The cells were transiently transfected with the CRIF1-siRNA duplex and the expression plasmids using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s instructions. Briefly, the plasmids were mixed with the Plus reagent and then incubated with LipofectAMINE. The LipofectAMINE Plus DNA complex was added to the cells and further incubated at 37 °C for 3 h. The control cells received the LipofectAMINE Plus alone. The cell viability was detected using a trypan blue dye exclusion test. After incubation, the medium was removed, and the cells were given fresh medium and maintained for an additional 24 h. The transfection efficiency was evaluated with pCMV-SPORT-β-gal (500 ng) and pEGFP-CRIF1 (500 ng) in a six-well culture plate.

Transfected with pFLAG-Gadd45

(CRIF1). Full-length human CRIF1 cDNA was isolated from a positive in this screen and called CR6-interacting factor 1 (CRIF1). Human CRIF1 cDNA cloned into pCMV-Tag2 (Stratagene). Cell

Immunofluorescence and Immunohistochemistry—COS-7 cells were transfected with pFLAG-Gadd45 and pMyc-CRIF1 and grown on uncoated glass coverslips (Bellco Glass, Inc.). Cells were washed three times in PBS+ (PBS with 1 mM calcium and 1 mM magnesium). Cells were fixed with 3.7% formaldehyde for 15 min at room temperature, washed briefly in PBS+, and quenched with 50 mM NH4Cl in PBS for 5 min. Cells were washed twice in PBS+ and incubated in blocking solution containing 3% bovine serum albumin in PBS+. For permeabilization, blocking and all subsequent incubation steps included 0.1% Triton X-100. Anti-FLAG antibody (Stratagene) and anti-c-Myc antibody (New England Biolabs, Inc., Beverly, MA) were incubated with the cells overnight at 4 °C. Cells were washed five times with PBS+ and incubated with secondary antibody conjugated to TRITC or fluorescein isothiocyanate for 30 min at room temperature. The cells were washed five times in PBS+, and fluorescence was visualized using a Nikon Eclipse E400 microscope equipped with epifluorescence optics and a digital camera; images were captured as Adobe Photoshop files.

Anti-CRIF1 antisera was obtained from New Zealand White rabbits immunized with conjugated synthetic peptide AEEQKRREREQHI-ASC correspond- ing to the midregion of CRIF1. The sera were titrated against the GST-CRIF1 fusion protein by enzyme-linked immunosorbent assay, and the serum exhibiting the highest titer (1:25,600) was used in subsequent experiments. Thyroid gland and adrenal gland tissues were obtained from surgery patients. Tissue specimens were fixed with 3.7% formaldehyde, embedded in paraffin, and sectioned and then stained with hematoxylin and eosin to evaluate histologic type. Samples used in this study were confirmed histologically as benign or malignant.

CRIF1 expression was analyzed by immunohistochemistry in two adrenal gland and seven thyroid cancer tissues. Samples were microwaved for 10 min, and slides were incubated with anti-CRIF1 anti-serum (1:800) at room temperature for 1 h. Freeimmune sera did not stain tissues at a 1:800 dilution. Slides were washed in phosphate-buffered saline and incubated with a linking solution containing biotinylated goat anti-rabbit IgG (Dako LSAB kit; Dako Laboratories) at room temperature for 30 min. Slides were sequentially incubated with streptavidin peroxidase at room temperature for 30 min and 3,3’-diaminobenzidine chromogen substrate solution (Dako Laboratories), counterstained with Mayer’s hematoxylin and mounted. Three indepen- dent assayers evaluated the slides. Two independent assayers graded the slides in a blinded fashion. The slides were graded with respect to staining intensity using the following scale: 0 (no staining), +1 (weak), +2 (moderate), and +3 (high).

Other Assays—Protein concentration was determined using the Bradford method (Bio-Rad) with recrystallized bovine serum albumin as a standard.

Statistical Significance—All experiments were repeated at least three times with different batches of cells. Values shown are the mean ± S.E. of three or more experiments. Significance between experimental values was determined by two-way analysis of variance.

RESULTS

Isolation, Expression, and Subcellular Localization of CRIF1—A yeast two-hybrid screen (25) was carried out to identify human proteins that interact with Gadd45. The screen used a Matchmaker two-hybrid cDNA library from K-562 cells (pACT2; Clontech Laboratories, Inc.) and a con- struct expressing a fusion between the GAL4 DNA binding domain and Gadd45. A partial cDNA was identified as a positive in this screen and called CR6-interacting factor 1 (CRIF1). Full-length human CRIF1 cDNA was isolated from a human placenta cDNA library (Stratagene) by hybridization (23).

The CRIF1 cDNA had little 5’-untranslated sequence and a 669-bp open reading frame encoding a predicted polypeptide of 222 amino acids with a predicted molecular mass of 25 kDa (Fig. 1A). A polyadenylation signal (AAATA) is located 27 bp downstream of the poly(A) tail. A BLAST (26) search revealed that this cDNA encodes a novel protein with no homology to any proteins in the data base. The CRIF1 gene is located on chromosome 19 at 19p13.2. The CRIF1 gene has one exon of 351 bp and a second exon of 315 bp. Homology searches using BLAST identified the human and mouse genomic clones as BC004944 (similar to RIKEN cDNA 2310040G17) and NM026320 (mouse RIKEN cDNA 2310040G17), respectively. The amino acid se- quence deduced from the CRIF1 cDNA isolated by two-hybrid screen is identical to BC004944 and has 90% homology with the mouse CRIF1 homologue NM026320 (Fig. 1B).

CRIF1 cDNA was in vitro translated, and the protein product had an apparent size of 28 kDa by SDS-PAGE (Fig. 2A). CRIF1 was also expressed transiently in COS-7 cells transfected with human CRIF1 cDNA cloned into pCMV-Tag2 (Stratagene). Cell
CRIF1 Interacts with Gadd45

Gadd45 proteins are localized predominantly in the nucleus (5, 10, 17). CRIF1 has a putative nuclear localization signal (28) in its C-terminal region (Fig. 1A), suggesting that it might colocalize with Gadd45. The subcellular location of CRIF1 and Gadd45 was studied using COS-7 cells transfected with pMyc-CRIF1 and pFLAG-CR6. Myc and FLAG epitope tags were visualized by immunofluorescent staining (Fig. 2E). The results show that FLAG-CR6 protein and Myc-CRIF1 colocalize in the nucleus (Fig. 2E).

Interaction between CRIF1 and Gadd45 Proteins in Vitro and in Vivo—The interactions between CRIF1 and Gadd45 proteins were examined in vitro using recombinant GST-Gadd45α, Gadd45β, and Gadd45γ immobilized on glutathione-agarose beads and [35S]methionine-labeled in vitro translated CRIF1. In vitro translated CRIF1 bound to GST-Gadd45γ but did not bind to GST (Fig. 3A, lane 5 versus lane 2). CRIF1 also binds to GST-Gadd45α and GST-Gadd45β (Fig. 3A, lanes 3 and 4). In vitro translated luciferase protein did not interact with GST or GST-Gadd45 proteins. As previously described (10, 14, 16, 17, 27), in vitro translated proliferating cell nuclear antigen and p21 bound to GST-Gadd45, Gadd45β, and Gadd45γ (data not shown).

CRIF1 interactions with Gadd45 proteins were also detected in mammalian cells using a mammalian cell two-hybrid assay (Fig. 3B). Gadd45α, Gadd45β, and Gadd45γ were cloned into pCMV-BD (Stratagene, La Jolla, CA) to create fusions with the DNA binding domain. CRIF1 was cloned into pCMV-AD (Stratagene) to fuse with the NF-κB activation domain. The luciferase reporter gene was activated when the CRIF1 fusion protein was co-expressed with Gadd45α, Gadd45β, or Gadd45γ fusion proteins (Fig. 3B). Similar results were obtained when CRIF1 was fused with the DNA binding domain and Gadd45 proteins were fused with the activation domain (data not shown). Co-expression of Gadd45α, Gadd45β, and Gadd45γ fusion constructs with vector pCMV-AD did not activate luciferase expression. This result indicates that CRIF1 interacts specifically with Gadd45 proteins.

In vivo interactions between CRIF1 and Gadd45 family proteins were examined using COS-7 cells co-expressing FLAG-tagged Gadd45α, -β, and -γ. CRIF1 was immunoprecipitated with anti-Myc and anti-FLAG antibodies and the precipitates were blotted with anti-FLAG or anti-Myc antibodies to detect tagged Gadd45 family proteins. As shown in Fig. 3, C–E, tagged Gadd45 family proteins were present in the CRIF1 immunoprecipitates. In addition, lysates from COS-7 cells expressing FLAG-tagged Gadd45α, -β, and -γ were immunoprecipitated with anti-FLAG antibody. CRIF1 was detected in the anti-FLAG immunoprecipitates (Fig. 3F). These results suggest that Gadd45 family proteins bind CRIF1 in vitro and in vivo.

Inhibition of Cdc2 and Cdk2 Kinase and Regulation of Cell Cycle by CRIF1—Gadd45 proteins regulate G1/S and G2/M checkpoints in several cell types (11, 18, 21, 22). Gadd45 proteins bind Cdc2 kinase, which inhibits cellular proliferation (11). Because these findings suggest that CRIF1 interacts with Gadd45 proteins in vitro and in vivo, Cdc2 kinase activity was examined in the presence or absence of Gadd45 proteins and CRIF1. As shown in Fig. 4, recombinant Gadd45α and Gadd45γ reduced histone H1 kinase activity in Cdc2-cyclin B1 immunoprecipitates to ~60% of the control (the presence of Cdc2-cyclin B1 was demonstrated by immunoblot; data not shown). The addition of recombinant CRIF1 resulted in a progressive inhibition of the enzymatic activity of the immunoprecipitated Cdc2-cyclin B1 complex. The simultaneous addition of recombinant Gadd45α or Gadd45γ and CRIF1 inhibited histone H1 kinase activity of Cdc2-cyclin B1 in an additive manner (Fig. 4A). In contrast, Gadd45 proteins do not inhibit Cdk2-cyclin E

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**Fig. 5. Effects of CRIF1 on cell cycle progression and cellular proliferation.** A, transfected cells were prepared as described under “Experimental Procedures.” Cell cycle analysis was performed using a Becton Dickinson fluorescence-activated cell analyzer and Cell Quest version 1.2 software (Becton Dickinson Immunocytometry Systems, Becton Dickinson fluorescence-activated cell analyzer and Cell Quest software). At least 10,000 cells were analyzed per sample, and cell cycle distribution was quantified using the ModFit LT version 1.01 software (Verity Software House Inc., Topsham, ME). B, NIH 3T3 cells were cotransfected with 5 μg of the indicated constructs and 1 μg of pCMV-puro and cultured in a puromycin-containing medium for 5 days. All assays were carried out in triplicate. A representative growth curve from the first set of clones is presented. Closed circle, control; diamond, clone A; square, clone B.

Lysates were analyzed by Western blot using anti-FLAG antibody (Stratagene), and an immunoreactive band was detected of the expected size (Fig. 2B).

CRIF1 mRNA was examined by Northern blot using mRNA from cultured cells and human tissues (Clontech Laboratories). A CRIF1 mRNA of ~0.7 kb was detected in human cultured cells (data not shown). CRIF1 was highly expressed in heart, thyroid, lymph node, trachea, and adrenal tissue and was expressed at a low level in liver, skeletal muscle, kidney, pancreas, testis, ovary, and stomach (Fig. 2C). CRIF1 mRNA expression was similar to Gadd45γ (data not shown). Gadd45γ is induced by DNA-damaging agents including methylethylsulfone and UV (5). However, CRIF1 mRNA was not induced by ionizing radiation, methylethylsulfone, or UV in SW620 or MCF7 cells (data not shown).

Native CRIF1 was detected in human cells by Western blot analysis using anti-CRIF1 antiserum (Fig. 2D). The anti-CRIF1 antiserum specifically detects 28-kDa CRIF1 in anaplastic thyroid cancer cell lines NPA, FRO, and ARO and primary cultured smooth muscle cells and endothelial cells from umbilical vein (Fig. 2D). Immunoreactive CRIF1 protein was not detected in either the rat or mouse cells, such as the rat FRTL-5 thyroid cells and mouse NIH 3T3 cells (Fig. 2D).
histone H1 kinase activity, and Gadd45 proteins have no effect on CRIF1-dependent inhibition of Cdk2-cyclin E histone kinase activity (Fig. 4B).

Because CRIF1 interacts with Gadd45 and inhibits the activity of Cdc2-cyclin B1 and Cdk2-cyclin E complexes, it is possible that CRIF1 could also influence cell cycle progression. This idea was tested by overexpressing CRIF1 in NIH 3T3 cells and monitoring cell cycle progression by flow cytometry. LipoBfectAMINE Plus was used to reproducibly transfect NIH3T3 cells with pFLAG-CRIF1 with an efficiency of 30–50% (Fig. 5A), eliminating the need for cotransfection with a selectable marker. Cell cycle progression was analyzed within 1–2 days after transfection. pFLAG-CRIF1 and pCMV-Tag2 (vector control) were transfected into NIH 3T3 cells, and CRIF1 expression was measured by immunoblotting with mouse anti-FLAG antibody. Cells transfected with pFLAG-CRIF1 expressed significantly more CRIF1 than pCMV-Tag2-transfected control cells (data not shown). Cells overexpressing CRIF1 (transfected with 4 μg of pFLAG-CRIF1) were more likely to be in G1 and less likely to be in S phase than control cells (Fig. 5A).

The growth rate was also examined in cells overexpressing pFLAG-CRIF1. NIH3T3 cells were cotransfected with pCMV-Tag2 or pFLAG-CRIF1 and pCMV-puro and grown for 5 days; pCMV-puro encodes a gene that confers puromycin resistance for selection purposes during cell growth. Cells transfected with pFLAG-CRIF1 grew more slowly than control cells transfected with pCMV-Tag2 (Fig. 5B). After 5 days of selection, the number of cells overexpressing pFLAG-CRIF1 was about half the number of control cells.

Effects of CRIF1-siRNA Duplexes on Rb Phosphorylation and Regulation of Cell Cycle—In order to examine the role of endogenous CRIF1 in the regulation of cell proliferation and the cell cycle, 21-nucleotide duplexes of siRNA were used, and RNA interference studies in HCT116 cells were performed. This study selected four different target regions of human CRIF1 for the synthesis of the RNA duplexes as shown in Fig. 6A. The activities and specificity of the CRIF1-siRNAs was evaluated by the cotransfection of four different forms of siRNA duplexes with the expression plasmids, pFLAG-CRIF1 and pEGFP-CRIF1 (Fig. 6, B and D). As shown in Fig. 6B, two kinds of CRIF1-siRNAs, siRNA-263 and -379, effectively inhibited the exogenous FLAG-CRIF1 expression. However, siRNA-578 was not effective in inhibiting exogenous FLAG-CRIF1 expression. In similar experiments, siRNA-263 and siRNA-379 were effective in the reducing the green fluorescence protein fluorescence by inhibiting green fluorescence protein-tagged CRIF1 expression, as shown in Fig. 6D. However, siRNA-578 transfection did not alter the green fluorescence protein-CRIF1 fluorescence intensity compared with the control. These observations suggest that siRNA-263 and -379 are effective in inhibiting the exogenous CRIF1 expression. Therefore, the effects of siRNA-263 and -379 on the endogenous CRIF1 levels were investigated. The HCT116 cells transfected with CRIF1-siRNA-263 and -379 showed a very low level of endogenous CRIF1 compared with the nontransfected and siRNA-578-transfected cells (Fig. 6C). Collectively, these observations suggest that siRNA-263 and -379 are effective siRNA duplexes for the suppression of exogenous and endogenous CRIF1 expression.

During the G1 phase, the growth factor-initiated signals promoted the accumulation and assembly of the α-type cyclins (D1, D2, D3) with their cognate cyclin-dependent kinases (CDK4 and -6). The active holoenzyme promoted the G1 phase progression and S phase entry by virtue of its ability to phosphorylate the retinoblastoma protein Rb and titrate the CDK inhibitors p27Kip1 and p21Cip1. The initial phosphorylation of Rb and titration of the CDK inhibitors expedited the cyclin
E-CDK2 kinase activation, thereby completing the Rb phosphorylation and inactivation prior to the initiation of DNA replication. As described above, the exogenous CRIF1 overexpression decreased the Cdk2-cyclin E kinase activity. This study observed the role of CRIF1 in the phosphorylation of Rb by the overexpression and down-regulation of CRIF1. As shown in Fig. 7A, CRIF1 expression in the HCT116 cells resulted a decrease in the phosphorylated forms of Rb with an increase in the CRIF1 expression level. The decrease in phosphorylated Rb level by CRIF1 overexpression was completely inhibited by CRIF1-siRNA-263 and -379 cotransfection but not by CRIF1-siRNA-578, which was unable to suppress CRIF1 expression (Fig. 7B). The level of Rb phosphorylation after transfection of the siRNA duplexes, siRNA-263, -379, and -578 was analyzed in order to observe the effect of the down-regulation of the endogenous CRIF1 on Rb phosphorylation. The HCT116 cells transfected with siRNA-263 and -379 duplexes showed an increase in Rb phosphorylation, but siRNA-578 did not alter the endogenous CRIF1 and phosphorylated Rb levels (Fig. 7C). These siRNA experiments suggest that modulating the intracellular level of CRIF1 results in the alteration of the Rb phosphorylation status, which is involved in regulating the G1/S phase of the cell cycle.

CRIF1 overexpression resulted in the accumulation of the G1 phase populations as shown in Fig. 5A. This study analyzed the cell cycle in the cells transfected with the CRIF1-siRNA duplexes to observe the roles of the endogenous CRIF1 in regulating the cell cycle. As shown in Fig. 7D, CRIF1 overexpression resulted in increased G1 phase populations, but the transfection of siRNA-263 and -379, which was highly effective in decreasing the endogenous CRIF1, resulted in a reduction of the G1 phase cell populations. However, transfecting the inefficient siRNA duplex, siRNA-578, did not change the G1 phase cell populations compared with the control. These observations suggest that the down-regulation of the endogenous CRIF1 promoted the cell cycle progression.

CRIF1 Expression in Endocrine Tumors—As shown in Fig. 2C, adrenal and thyroid gland tissue express CRIF1 at a high level. Therefore, the cellular distribution of CRIF1 in adrenal and thyroid glands was examined by immunohistochemistry in
CRIF1 Interacts with Gadd45

normal and cancerous tissue from patients who underwent surgery for nonfunctioning adrenal tumor or papillary thyroid cancer. Deparaffinized adrenal adenoma (n = 2) and thyroid (n = 7) papillary cancer tissue was sequentially incubated with ant-CRIF1 antiseraum (1:800), biotinylated secondary antibody, streptavidin peroxidase, and 3,3′-diaminobenzidine substrate. High level expression of CRIF1 was expected in adrenal and thyroid tissue based on previous Northern blot results (Fig. 2C). CRIF1 immunoreactivity was mainly confined to the cortex of the adrenal gland (C) (Fig. 8). Expression was highest in the zona glomerulosa region of the cortex, and CRIF1 expression was very low in adrenal medulla (M). CRIF1 immunoreactivity was noted at cytoplasm and nucleus. However, the adenoma (A) in the adrenal cortex (C) expressed CRIF1 at a significantly lower level than the surrounding tissue (Fig. 8). In the thyroid gland, CRIF1 immunoreactivity was high within the nucleus in follicular epithelial cells (Fig. 8); however, papillary cancer cells within the follicular epithelium had much lower CRIF1 immunoreactivity than the surrounding cells as shown in Fig. 8. Immunohistochemical findings from three patients are shown in Fig. 8; in addition, CRIF1 immunoreactivity was lower in seven papillary thyroid cancers than in normal thyroid follicular epithelial cells. The DNA sequences of exon 1 and exon 2 in CRIF1 in thyroid papillary cancer tissues from seven patients did not carry detectable mutations (data not shown).

DISCUSSION

This study reports the identification of CRIF1, a novel nuclear protein that interacts with Gadd45 family proteins. Gadd45 plays several important biological functions including maintaining genomic stability and regulating cell cycle progression and apoptosis. Gadd45 interacts with several cell cycle regulatory proteins such as p21WAF1/CIP1 (17, 20), Cdc2 (21, 22), and proliferating cell nuclear antigen (10). MTK1/MEKK4 is a member of the mitogen-activated protein kinase kinase kinase family that also interacts with Gadd45 and activates Jun N-terminal kinase and p38 (5). These findings suggest that proteins that interact with Gadd45, including CRIF1, could play critical roles in regulating cell cycle progression.

A partial human CRIF1 cDNA was isolated using a yeast two-hybrid screen with Gadd45γ as bait. CRIF1 genomic sequence, chromosomal location, and a mouse CRIF1 homologue were identified by searching DNA databases. Evidence presented here indicates that CRIF1 interacts with Gadd45γ, -β, and -γ proteins in vitro and in vivo. In addition, CRIF1 colocalizes with Gadd45γ in the nucleus. This result is consistent with the possibility that CRIF1 and Gadd45 proteins co-regulate nuclear functions such as cell cycle progression, replication, and transcription (29).

The function of Gadd45 proteins has been implicated in activating a G2/M checkpoint in response to genotoxic stress agents such as UV radiation or methylmethane sulfonate. However, the function of Gadd45 proteins in negative growth control is not fully understood. Recently, it was shown that Gadd45 proteins α, β, and γ interact with and inhibit Cdc2-cyclin B1 kinase. This study shows that CRIF1 suppresses the histone H1 kinase activity of Cdc2-cyclin B1 and Cdk2-cyclin E complexes in the presence or absence of Gadd45 proteins. CRIF1 and Gadd45 inhibition was additive for Cdc2-cyclin B1 kinase but not for Cdk2-cyclin E kinase. These findings suggest that CRIF1 may modulate the G2/M transition in dividing cells. Overexpression of CRIF1 alters cell cycle progression in NIH3T3 cells and increases the proportion of cells in G2; thus, CRIF1 may participate in regulating the G2/M transition, which is when cells commit to entering S phase and completing DNA replication and cell division. Small interfering RNA experiments revealed that the down-regulation of endogenous CRIF1 is related to the increase in Rb phosphorylation. This observation suggests that CRIF1 has a constitutive inhibitory role in G1 cell cycle regulation.

The tumor suppressor, p53, is a key protein that regulates G1 arrest in cells with DNA damage (30, 31). Genetic instability is one of the hallmarks of cancer, and cancer is often associated with aberrations in DNA repair or cell cycle checkpoint pathways (32). Proteins that regulate G2/S, such as ATM (ataxia telangiectasia-mutated) (33), Chk2 (checkpoint kinase 2) (34), BRCA1 (35), p53 (31), p16 (36), and Rb (36), are frequently altered in cancer cells. These major G2/S checkpoint transducers and effectors are tumor suppressors or protooncogenes, and loss of functional mutations in the genes encoding these proteins has been identified in many human malignancies.

CRIF1 expression is different in normal and cancer tissues. In normal tissue, thyroid and adrenal gland expressed the highest level of CRIF1 mRNA, and CRIF1 immunoreactivity is mainly localized in the nucleus by immunohistochemistry and in vitro immunofluorescence studies. However, histiocyte, osteoclast, terminally differentiated keratinocyte, and adrenal medulla expressed a very low or undetectable level of CRIF1 (data not shown), in contrast to high level CRIF1 expression in epithelial cells from thyroid follicle, colon (data not shown), and breast mammary duct (data not shown). It is interesting to note that some epithelial cell cancers in thyroid or breast expressed CRIF1 at a lower level than adjacent normal epithelial cells. Seven of seven papillary thyroid cancers expressed CRIF1 at a virtually undetectable level relative to normal epithelial cells and infiltrating T lymphocytes in the tumor, which expressed a high level of CRIF1. Reduced expression of CRIF1 was also observed in a benign adenoma from adrenal cortex. The mechanism by which CRIF1 expression is lowered in certain endocrine tumors was not evaluated in this study. No mutations that might account for altered expression of CRIF1 were found in exons 1 or 2 of CRIF1 in papillary thyroid cancer tissue.
In summary, the results presented here suggest that CRIF1 may negatively regulate cell growth by inhibiting the G1/S transition. Reduced expression of CRIF1 may occur in some cancers, and altered expression of CRIF1 may play a significant role in carcinogenesis.

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REFERENCES

1. Fornace, A. J., Jr., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasious, M., Fargnoli, J., and Holbrook, N. J. (1989) Mol. Cell. Biol. 9, 4196–4203

2. Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) Oncogene 6, 165–167

3. Beadling, C., Johnson, K. W., and Smith, K. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2719–2723

4. Nakayama, K., Hara, T., Hibi, M., Hirano, T., and Miyajima, A. (1999) J. Biol. Chem. 274, 24766–24772

5. Takeda, M., and Saijo, H. (1998) Cell 95, 521–530

6. Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8800–8804

7. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Almo, I., Rajasekaran, A., and Zhan, Q. (2001) Oncogene 19, 3083–3090

8. Jin, S., Antinore, M. J., Lung, F. D., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000) J. Biol. Chem. 275, 16602–16608

9. Hollander, M. C., Sheikh, M. S., Bulavin, D. V., Lundgren, K., Auger-Hemmelmueller, L., Shehee, R., Molinaro, A. T., Kim, E. K., Tolosa, E., Ashwell, J. D., Rosenberg, M. P., Zhan, Q., Fernandez-Salguero, P. M., Morgan, W. F., Deng, C. X., and Fornace, A. J., Jr. (1999) Nat. Genet. 23, 176–184

10. Jin, S., Fan, P., Fan, W., Zhao, H., Tong, T., Blanck, P., Almo, I., Rajasekaran, A., and Zhan, Q. (2001) Oncogene 20, 2683–2690

11. Fan, W., Richter, G., Cereseto, A., Beadling, C., and Smith, K. A. (1999) Oncogene 18, 6573–6582

12. 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

13. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) Nature 414, 308–313

14. Hall, P. A., Kearsey, J. M., Coates, P. J., Norman, D. G., Warbrick, E., and Cox, L. S. (1995) Oncogene 10, 2427–2433

15. Azam, N., Vairapandi, M., Zhang, W., Hoffman, B., and Liebermann, D. A. (2001) J. Biol. Chem. 276, 2766–2774

16. Vairapandi, M., Azam, N., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2000) J. Biol. Chem. 275, 16810–16819

17. Vairapandi, M., Balliet, A. G., Fornace, A. J., Jr., Hoffman, B., and Liebermann, D. A. (1996) Oncogene 12, 2579–2594

18. Yang, Q., Manicone, A., Coursen, J. D., Linke, S. P., Nagashima, M., Forgues, M., and Wang, X. W. (2000) J. Biol. Chem. 275, 36882–36888

19. Vairapandi, M., Balliet, A. G., Fornace, A. J., Jr., and Liebermann, D. A. (1995) Oncogene 11, 1931–1937

20. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000) J. Biol. Chem. 275, 16602–16608

21. Jin, S., Antinore, M. J., Lung, F. D., Dong, X., Zhao, H., Fan, F., Colchagie, A. B., Blanck, P. P., Fornace, A. J., Jr., and Zhan, Q. (2000) J. Biol. Chem. 275, 16602–16608

22. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999) Oncogene 18, 2892–2900

23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. 18.6–18.38, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

24. Vairapandi, M., Balliet, A. G., Hoffman, B., and Liebermann, D. A. (2002) J. Cell. Physiol. 192, 327–338

25. Fields, S., and Song, O. (1989) Nature 340, 245–246

26. Altshuler, S. F., Madden, T. L., Schiffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402

27. Kearsey, J. M., Coates, P. J., Prescott, A. R., Warbrick, E., and Hall, P. A. (1995) Oncogene 11, 1675–1683

28. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Cell 64, 615–623

29. Y.T.W. Kim, D., Jung, N., Hong, S. S., Lee, H. S., and Bae, I. (2000) Biochem. Biophys. Res. Commun. 272, 193–198

30. Hartwell, L. H., and Kastan, M. B. (1984) Science 226, 1821–1828

31. Vogelstein, B., Lane, D., and Levine, A. J. (1990) Science 253, 327–332

32. Zhou, B. B., and Elledge, S. J. (2000) Science 289, 630–637

33. Birck, J. M., Li, F. P., Garber, J. E., and Haber, D. A. (1999) Science 282, 2328–2331

34. Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verseli, S., Isselbacher, K. J., and Frauenin, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. (1999) Science 282, 2328–2331

35. Scully, R., and Livingston, D. M. (2000) Nature 408, 429–432

36. Sherr, C. J. (2000) Cancer Res. 60, 3689–3695
