p53-Dependent Association between Cyclin G and the B' Subunit of Protein Phosphatase 2A

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We and others previously showed that cyclin G is a transcriptional target of the p53 tumor suppressor protein. However, cellular proteins which might form a complex with cyclin G have not yet been identified. To gain insight into the biological role of cyclin G, we used the yeast two-hybrid screen and isolated two mouse cDNAs encoding cyclin G-interacting proteins. Interestingly, both positive cDNAs encoded B' regulatory subunits of protein phosphatase 2A (PP2A). One clone encodes B'α, while the other clone codes for a new member of the B' family, B'β. B'β is 70% identical to other members of the B' family. B'α associated both in vitro and in vivo with cyclin G but not with the other mammalian cyclins. Furthermore, cyclin G formed a complex with B'α only after induction of p53 in p53 temperature-sensitive cell lines. These results indicate that cyclin G forms a specific complex with the B' subunit of PP2A and that complex formation is regulated by p53. Potential roles for the cyclin G-B' complex in p53-mediated pathways are discussed.

Alteration of the p53 tumor suppressor gene is the most common genetic change in malignancy (reviewed in reference 15). p53 is involved in a variety of biological activities, including cell cycle arrest, apoptosis, genomic instability, immortalization, differentiation, and stress response (reviewed in reference 22). p53 can function as a transcription factor, and it is likely that the ability of p53 to suppress tumorigenesis is at least partly attributable to its transcriptional activity, since the mutational inactivation of the DNA binding activity of p53 tends to impair its function as a tumor suppressor protein (reviewed in reference 37). To date, several genes have been shown to be transcriptional targets of p53. These include *p21* (7, 12, 27, 43), *mdm2* (17, 42), *bax* (26), *gadd45* (19), *IGF-BP3* (2), and the cyclin G gene (28, 45). Mdm2, by physically interacting with the p53 protein, inhibits the transcriptional activity of p53, thereby forming a negative-feedback loop (17, 42). On the other hand, p21, an inhibitor of cyclin-dependent kinase (Cdk) is partly responsible for p53-dependent cell cycle arrest (1, 4, 6), and Bax is likely to be involved in the induction of p53-dependent apoptosis (41). However, in contrast to p53-deficient mice, knockout mice deficient in either *p21* or *bax* do not show a marked increase in susceptibility to tumorigenesis (1, 4, 21), suggesting that these genes alone are not sufficient to explain the tumor suppressor function of p53. It is likely, therefore, that each target gene of p53 plays a role in suppressing tumorigenesis either in certain cell types or in combination with other target genes or other functions of p53.

Transactivation of the cyclin G gene by p53 is intriguing, since other cyclins have been shown to play crucial roles in the cell cycle (13). In general, cyclins form heterodimeric complexes with Cdks, thereby regulating Cdk enzymatic activity (5). Although cyclin G was so named because of its cyclin box

and although cyclin G mRNA is induced by serum (35), its mRNA does not change significantly during the cell cycle (16, 35). So far, no candidate for a Cdk partner has been identified, and a physiological role of cyclin G has not yet been defined.

In order to probe the biological roles of cyclin G, we have used a yeast two-hybrid screen to isolate cDNAs encoding mouse proteins that interact with this cyclin. Through this approach, we found that cyclin G binds to two members of the family of B' regulatory subunits of protein phosphatase 2A (PP2A). One of these is a B'α subunit, and the other is a new member of the B' family. Our data further suggest that this novel interaction is mediated by p53.

MATERIALS AND METHODS

Cell culture. LTR-6 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 39 or 32°C in 5% CO₂. COS-1 cells and clone 6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO₂. Insect *Spodoptera frugiperda* (Sf9) cells were cultured in Grace's medium supplemented with 10% fetal bovine serum at 27°C.

Antibodies. For the production of the anti-cyclin G antibodies, a DNA fragment corresponding to either the N-terminal domain (amino acids 1 to 45) or the cyclin box–C-terminal domain (amino acids 46 to 294) of cyclin G was cloned into pGEX-KG (10). Each glutathione *S*-transferase–cyclin G fusion protein was expressed in *Escherichia coli* BL21, purified on glutathione–Sepharose (33), and used to immunize rabbits. To obtain the purified antibody against the N-terminal domain, the antiserum was depleted extensively with glutathione *S*-transferase protein coupled to cyanogen bromide (CNBr)-activated Sepharose (Pharmacia) and the specific antibody was subsequently purified on GST–cyclin G coupled to CNBr–Sepharose as previously described (46). To obtain purified antibody against the cyclin box–C-terminal domain, the corresponding cDNA fragment was cloned into pMAL-c2 (New England Biolabs) to express the maltose-binding protein (MBP)–cyclin G fusion protein. The MBP–cyclin G fusion protein was then purified on an amylose column (New England Biolabs) and coupled to CNBr–Sepharose. MBP–cyclin G coupled to CNBr–Sepharose was subsequently used to purify the antibody. The affinity-purified antibody against either the N-terminal domain (2 μg per reaction) or the cyclin box–C-terminal domain (8 μg per reaction) was used for immunoprecipitations. The antiserum against the N-terminal domain was used for Western blot (immunoblot) analyses at a 1:2,000 dilution.

M880 anti-B'α antiserum was produced in a rabbit against a synthetic peptide corresponding to amino acids 471 to 480 of B'α1 (36). This antiserum also recognizes B'α2 but with a weaker affinity. A B'α1-specific rabbit polyclonal antiserum (P375) was raised against a synthetic peptide corresponding to residues 455 to 468 (CFEDVQMLRKTVKDE) of human B'α1. The amino-terminal

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cysteine was added to facilitate coupling to keyhole limpet hemocyanin (36). M880 was used for Western blot analyses at a 1:10,000 dilution for the experiments whose results are shown in Fig. 4 and 5. A mixture of M880 and P375 was used for Western blot analysis (1:10,000 dilution for each antibody) and for immunoprecipitations (1 μ l per reaction volume for each antibody) for the experiments whose results are shown in Fig. 6 and 7.

Anti-FLAG antibody (M2) is a mouse immunoglobulin G monoclonal antibody directed against the FLAG epitope (Kodak). A 3- μ g amount of the antibody was used for immunoprecipitations. Anti-cyclin A, B1, D1, and E antibodies are rabbit polyclonal antisera and were used for Western blot analyses at a 1:3,000 dilution. The peptide corresponding to the C-terminal region of PCTAIRE-1 was purchased from Santa Cruz.

Construction of cDNA library and hybridization screening. Total RNA was isolated from LTR-6 cells 12 h after a temperature shift to 32°C as described previously (28). Subsequently, poly(A)⁺ RNA was isolated and 4 μ g was used as a substrate for cDNA synthesis with a Zap cDNA synthesis kit (Stratagene). Size-fractionated cDNA (>2 kb) was ligated into the Uni-Zap XR vector (Stratagene) and packaged with Gigapack II Gold packaging extract (Stratagene). A phage library was obtained by infection of *E. coli* XL1blue.

Mouse cyclin G cDNA clones were isolated by hybridization screening of 5 $\times$ 10⁵ recombinants from the LTR-6 cell cDNA library as described previously (28). A 5' fragment of cyclin G derived from mouse embryonic stem cells was used as a probe.

Two-hybrid screening. The complete open reading frame of mouse cyclin G was fused to the GAL4 DNA-binding domain of pGBT9 (11). For the construction of a cDNA library, cDNA derived from embryonic fibroblasts from a p53-deficient mouse was fused to the GAL4 DNA activation domain of pGAD-GH (11). *S. cerevisiae* HF7c was cotransformed with the GAL4 DNA-binding domain–cyclin G fusion plasmid and cDNA library plasmids. Screening was performed as previously described (11). Primary positives were examined for target specificity by retransformation into HF7c together with various GAL4 DNA-binding domain–target fusion plasmids. These included fusions with Snf4, cyclin E, cyclin D1, and cyclin G. Leu⁺ Trp⁺ transformants, which contain both GAL4 DNA-binding and GAL4 DNA activation domain plasmids, were streaked onto plates with or without histidine.

Baculovirus infection. The complete open reading frame of cyclin G was cloned into the baculovirus expression vector pVL1392 (Pharmingen). A baculovirus expressing cyclin G was produced in insect Sf9 cells with a Baculo-Gold kit (Pharmingen). The B' α 1-expressing baculovirus was previously described (36). Sf9 cells grown in a monolayer were infected at a multiplicity of infection of 5 with wild-type baculovirus alone or in combination with baculoviruses directing the expression of cyclin G and B' α 1. Infected cells were harvested 40 h postinfection.

COS-1 cell transfection. The complete open reading frame of cyclin G cDNA was inserted in expression vector pRCMV (Invitrogen) to create plasmid pCMV-cyclin G. pCMV-FLAG-B' α 1 was described previously (36). pCMV-FLAG-B' α 1 was digested with *Sall* and *Clal*, treated with Klenow enzyme, and self-ligated to create pCMV-FLAG. pCMV-cyclin A, B1, D1, and E were gifts from Robert Weinberg and Clayton Small. COS-1 cells were grown in 100-mm-diameter plates to ~20% confluence and transfected by the calcium phosphate method with a total of 20 μ g of DNA. Glycerol shock was performed 8 h after transfection, and the cells were harvested 48 h posttransfection.

Immunoprecipitation, Western blot analyses, and V8 protease mapping. For immunoprecipitation and Western blot analyses of infected insect Sf9 cells, transfected COS-1 cells, and clone 6 cells, the harvested cells were washed three times with phosphate-buffered saline (PBS) and lysed in PL buffer [20 mM Tris-HCl (pH 7.5), 0.2% Nonidet P-40 (NP-40), 200 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml, 1 μ g of pepstatin per ml, 10 μ g of aprotinin per ml, 1 mM benzamidine, 10 μ g of trypsin inhibitor per ml]. Lysates were centrifuged at 14,000 $\times$ g for 10 min, and the supernatants were used for either Western blotting or immunoprecipitations. For immunoprecipitations, cleared lysates were incubated with the appropriate antibody for 12 h at 4°C. Immune complexes were then precipitated by the addition of 15 μ l of protein A-Sepharose 4B (Pharmacia), washed four times with PL buffer, and boiled in sodium dodecyl sulfate (SDS) sample buffer. The eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot analyses, the immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 2% nonfat dry milk in TBST (10 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Tween 20) and probed with antibodies. Protein A-horseradish peroxidase (Amersham) and an ECL detection kit (Amersham) were used to detect bound antibody.

For metabolic labeling with [³⁵S]methionine, ~5 $\times$ 10⁷ LTR-6 cells were incubated at either 39 or 32°C for 12 h. The cells were then washed once with PBS and incubated with 5 ml of labeling medium (methionine-free, cysteine-free Dulbecco's modified Eagle's medium [ICN] supplemented with 10% dialyzed fetal bovine serum [JRH Biosciences]). After a 30-min incubation with the labeling medium, [³⁵S]methionine (200 μ Ci/ml) was added, and the LTR-6 cells were further incubated for 5 h. The harvested cells were washed three times with PBS and lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 7.5], 0.5% NP-40, 250 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml,

1 μ g of pepstatin per ml, 10 μ g of aprotinin per ml, 1 mM benzamidine, 10 μ g of trypsin inhibitor per ml). The lysates were centrifuged at 14,000 $\times$ g for 10 min, precleared by incubation with 200 μ l of protein A-Sepharose (1 h), and centrifuged at 14,000 $\times$ g for 10 min. The cleared lysates were then incubated with the appropriate antibodies for 12 h at 4°C. The immune complexes were collected as described above except that the washes were done with NP-40 lysis buffer. The immunoprecipitates were resolved by SDS-10% PAGE as described above. V8 protease mapping of in vivo- and in vitro-labeled cyclin G was performed as described previously (47). Western blot analysis of LTR-6 cell lysate was performed as described above.

In vitro binding assay. For the production of in vitro-translated cyclin G, the complete open reading frame of cyclin G was inserted into pBluescript SK⁺. pBluescript-cyclin B1 was a gift from Konstantin Galaktionov. pBluescript-cyclin D1 was a gift from Hui Zhang, and pCITE-cyclin E was kindly provided by Jim Roberts. In vitro-translated cyclin proteins were synthesized by using the TNT coupled reticulocyte lysate systems (Promega) in the presence of ³⁵S-labeled methionine.

For the experiments whose results are shown in Fig. 4B, Sf9 cell lysates were prepared after infection with either the wild-type baculovirus or the virus expressing B' α 1 as described above. Each lysate was then subjected to immunoprecipitation in PL buffer with anti-B' α 1 antibody. The immunoprecipitates were then incubated with in vitro-translated cyclins in 200 μ l of PL buffer for 12 h at 4°C. Complexes were then washed four times in PL buffer, and bound cyclins were eluted by being boiled in SDS sample buffer and separated by SDS-10% PAGE.

Nucleotide sequence accession number. The GenBank accession numbers of the newly isolated mouse cyclin G cDNA and B' β are Z37110 and U49728, respectively.

RESULTS

The induction of cyclin G protein by p53. The mouse cyclin G cDNA was previously isolated as a p53-inducible transcript (28). The cDNA analyzed in that study was derived from mouse embryonic stem cells and encoded a protein of 249 amino acids (28). We subsequently analyzed the sequence of two additional cyclin G cDNAs derived from mouse embryonic stem cells and three cyclin G cDNAs derived from LTR-6 mouse leukemic cells incubated at 32°C (see Materials and Methods). All of the newly analyzed cDNAs contained a 1-nucleotide insertion at position 246 of the previously identified cyclin G cDNA sequence (28). The nucleotide insertion, which is located in the 5' untranslated region of the previously identified cDNA, results in a 45-amino-acid extension of the open reading frame at the N terminus (see Materials and Methods). As a result, the newly analyzed cyclin G cDNAs encode a protein of 294 amino acids. The corresponding open reading frame was also found in rat cyclin G cDNA (45).

Since five of the six analyzed cDNAs contained the same nucleotide insertion, it is likely that these cDNAs represent the major form of the mouse cyclin G cDNA. In fact, sequence analysis of genomic DNA from mouse embryonic stem cells demonstrated that the genomic sequence contains the same nucleotide insertion (data not shown). It is therefore likely that the nucleotide deletion in the original cDNA was caused by a cloning artifact, although other regulatory mechanisms such as RNA editing cannot be ruled out. We have used exclusively the cyclin G cDNA encoding the 294-amino-acid protein in the work presented here.

We previously demonstrated that cyclin G mRNA is transcriptionally induced by p53 (28). In that study, we did not determine whether cyclin G protein was synthesized upon p53 induction. Here, to demonstrate expression of cyclin G protein, we used LTR-6 mouse leukemic cells, which harbor a temperature-sensitive p53 allele (44), and in which cyclin G RNA increases markedly upon a shift from the restrictive (39°C) to the permissive (32°C) temperature (28).

Several lines of evidence showed that cyclin G protein is induced by wild-type p53 in these cells (Fig. 1). First, when lysates were prepared from ³⁵S-labeled LTR-6 cells incubated at either temperature, immunoprecipitation with affinity-puri-

FIG. 1. Induction of cyclin G protein in LTR-6 cells. (A) Lysates from ^{35}S -labeled LTR-6 cells incubated at 39°C (lane 1) or 32°C (lane 2) were immunoprecipitated by the antibody against the N-terminal domain of cyclin G. Immunoprecipitates were boiled in SDS buffer and analyzed by SDS-10% PAGE. The 33-kDa polypeptide induced after the temperature shift to 32°C is indicated (arrowhead). (B) In vitro-translated cyclin G (lane 1) comigrates with the 33-kDa polypeptide shown in panel A (lane 2) on SDS-10% PAGE. (C) V8 protease mapping. In vitro-translated cyclin G (lanes 1 to 3) and the 33-kDa polypeptide shown in panel A (lanes 4 to 6) were digested with the amounts of V8 protease indicated above the lanes. Digested proteins were separated by SDS-17.5% PAGE. (D) Western blot analysis of total lysates from LTR-6 cells after the temperature shift from 39 to 32°C . LTR-6 cells were harvested at the indicated times after the temperature shift, and lysates were separated by SDS-10% PAGE. The blot was probed with the anti-cyclin G serum. IVT, in vitro translation; IP, immunoprecipitation.

fied anti-cyclin G antibody yielded a polypeptide of 33 kDa that appeared only after a shift to the permissive temperature (Fig. 1A). As a control, a similar immunoprecipitation experiment was performed using ^{35}S -labeled lysates of M1 (S6 subclone) cells (44). M1 is the parental cell line of LTR-6 and does not have any detectable p53 protein. In contrast to the case for LTR-6 cells, induction of the 33-kDa polypeptide after the temperature shift to 32°C was not observed in S6 cells (data not shown). Second, the 33-kDa polypeptide induced in LTR-6 cells comigrated with in vitro-translated cyclin G (Fig. 1B). Third, the identity of the 33-kDa polypeptide in the immunoprecipitate was confirmed by partial proteolytic mapping (Fig. 1C). Finally, to determine whether the steady-state level of cyclin G protein was increased upon induction of p53, we monitored cyclin G expression in LTR-6 cells by Western blot analysis (Fig. 1D). The abundance of cyclin G protein increased by 2 h and reached its peak at 6 h after the shift to the permissive temperature. The decrease of cyclin G protein expression at 24 h probably resulted from apoptosis caused by p53 induction. Taken together, these results demonstrate that the expression of cyclin G protein is increased upon induction of p53 in LTR-6 cells.

Isolation of cyclin G-binding proteins. In order to gain insight into the biological role of cyclin G, we looked for proteins that associate with cyclin G. For this purpose, we used a yeast two-hybrid screen. Cyclin G was fused in frame to the GAL4 DNA-binding domain of pGBT9 (11) and cotransformed together with cDNAs fused to the GAL4 transactivation domain of pGAD-GH into the yeast host strain HF7c (11). Three cDNAs were isolated as clones encoding proteins that inter-

acted with cyclin G strongly enough to allow the growth of transformants on medium lacking histidine and expression of β -galactosidase activity (11).

Sequence analysis of the cDNAs revealed that two of the three positive clones encode a polypeptide, B' α , which belongs to the B' regulatory subunit family of PP2A (25, 36). B' α comprises at least three different forms generated by alternative splicing (B' α 1, B' α 2, and B' α 3) (36). Figure 2 shows the sequences of several B' family members, including our two positive clones which encode the B' α 3 form. The third clone that we obtained from our screen encodes a novel protein with a partial open reading frame of 388 amino acids. This clone shows a high ($\sim 70\%$) degree of identity with other members of the B' family, and we have designated this clone B' β (Fig. 2).

Both B' α 3 and B' β interacted strongly with cyclin G and weakly with cyclin D1 in yeast cells (Fig. 3). In contrast, no interaction was detected with cyclin E, Snf4, or the GAL4 transactivation domain (Fig. 3). These results indicate that the interactions between cyclin G and the B' proteins in yeast cells are specific.

Cyclin G associates with B' α in insect cells. We took several approaches to confirm the existence of the interaction between cyclin G and B' subunits of PP2A. For this purpose, we used human B' α 1, since a specific antibody against B' α 1 was available. B' α 3 is identical to B' α 1 except that B' α 3 lacks 68 amino acid residues of the C terminus (Fig. 2) (36).

Initially, we examined the interaction between cyclin G and B' α 1 in insect cells, using baculoviruses that direct expression of the recombinant proteins. Wild-type virus or the cyclin G-expressing virus was used to infect cells in either the presence

B56 α	MSSSSPPAGAASAAISASEKVDGFT	25
B56 β	METKLPPASTPTSPSSPGLSPVPPDKVDGFS	32
SCS1	ENGNSNNNNMINTSNTQDANHASSQSIDI PRSSHSFERLPTPTKLNPD	250
B' β	..KDVPTSEQPELFLK	15
B' α	MVDAASSNGPFQVALLHTRDVPADQEKLEFIQ	34
B56 α	RKSVRKAQRKRSQSSQFRSQGSQAELHPLQL..KDATSNEQPELFLCQ	73
B56 β	RRSLRRA.RPRRSHSSSQFRYQSNQQLTLPPLL..KDVPASELHPLLSR	79
SCS1	DLELIKTPQRHSSSRFEPSRY...TPLTKLPNF..NEVSPERIPFLIA	294
B' β	KLQQCCVICDQMDLTLSDLKMKEMKRSTLNELVDYITISRGCLTEQTYPE	64
B' α	KLRQFCVDFDFVSDPLSDLKMKEMKRAALSEMVEYITHNRNVITEPIYPE	84
B56 α	KLQQCCILFDHM.DSVSDLKSKETIKRATLNELVEYVSTNRGVIVESAYS	122
B56 β	KLAQCGVMDFDL.DCVADLKGEVKRAALNELVECVGSTRGVLTPEVYPD	128
SCS1	KVDCQNTMDEH.NDPSFDIQCKEKKRSTLDELTEFLVTNRFTYTNEMYAH	343
B' β	VVRMVSNIFRTLPSPDSN...EFDPEEDEPTLEASMPHLQVLYEFFIRF	111
B' α	AVHMFVNMFRTPPPSSNPTGAEPDPEDEPTLEAAMPHLQVLYEFFLRF	134
B56 α	IVKMISANIFRTLPSPDNP...DFDPEEDEPTLEASMPHLQVLYEFFLRF	169
B56 β	IIRMISVNIIFRTLPSPENP...EFDPEEDEPTLEASMPHLQVLYEFFLRF	175
SCS1	VVMFKINLFRPIEPVNVGDIYDFEPEDEPTLEASMPHLQVLYEFFLRF	393
B' β	VGKPRIPTQHCQILHRSANLYSYFWELFDSQEDPRERDYLKTVLHRIYKGF	161
B' α	LESDFQPN.IAKRYLDQRFVLQLLELFDSEDPREERDLKTLHRIYKGF	183
B56 α	LESDFQPS.IAKRYLDQRFVQQLLELFDSEDPREERDLKTVLHRIYKGF	218
B56 β	LESDFQPS.VAKRYLDQRFVLMLELFDSEDPREERYLKLHRIYKGF	224
SCS1	VESDFNHQ.IAKRYLDQFLLKLELFDSEDIRERDLKTLHRIYKGF	442
B' β	LGLRAFIKQNNIFLRFIYETEHFNGVAELLEILGSIINGFALPLKAEH	211
B' α	LGLRAYIRKQNNIFLRFIYETEHFNGVAELLEILGSIINGFALPLKAEH	233
B56 α	LGLRAFIKQNNIFLRFIYETEHFNGVAELLEILGSIINGFALPLKAEH	268
B56 β	LGLRAYIRKQNNIFLRFIYETEHFNGVAELLEILGSIINGFALPLKAEH	274
SCS1	LSLSRFIRSMNNIFLRFIYETEHFNGVAELLEILGSIINGFALPLKAEH	492
B' β	KQFLVKVLIPLHVRSLSLFHAQLAYCIVQFLEKDPSTLTFVIRGLLKYM	261
B' α	KHFLKVLPLHVRKSLSVYHPOLAYCVVQFLEKDPSTLTFVIRGLLKYM	283
B56 α	KQFLMKVLIPLHVRKSLSVYHPOLAYCVVQFLEKDPSTLTFVIRGLLKYM	318
B56 β	KQFLVRVLIPLHVRKSLSVYHPOLAYCVVQFLEKDPSTLTFVIRGLLKYM	324
SCS1	KVFLVRVLIPLHVRKSLSVYHPOLAYCIVQFLEKDPSTLTFVIRGLLKYM	542
B' β	PKTCSQKEVMFLGEIEILDVIEESQGVKIQEPLKQIAKCVSSPHFQVA	311
B' α	PKTHSPKEVMFLGEIEILDVIEESQGVKIMEPLBROLAKCVSSPHFQVA	333
B56 α	PKTCSQKEVMFLGEIEILDVIEESQGVKIQEPLKQIAKCVSSPHFQVA	368
B56 β	PKTCSQKEVMFLGEIEILDVIEESQGVKIQEPLKQIAKCVSSPHFQVA	374
SCS1	PRINSTKBIIMFLNEIDDFEIVIEBPIKVEVPELVQLKQISSPHFQVA	592
B' β	ERALLYWNNYEIMSLIEENSNVILPIMSSSLYR.....	344
B' α	ERALLYWNNYEIMSLIEENSNVILPIMSSSLYR.....	366
B56 α	ERALLYWNNYEIMSLIEENSNVILPIMSSSLYR.....	401
B56 β	ERALLYWNNYEIMSLIEENSNVILPIMSSSLYR.....	407
SCS1	ERALLYWNNYEIMSLIEENSNVILPIMSSSLYR.....	642
B' β	TSKBHWNPTVALVTVLKAFFMEMNMTFDELTAITYKSDRQ	385
B' α	TSKBHWNKTTHGLINAKLFFMEMNOKLFDCTQQAERKL	407
B56 α	TSKBHWNPTVALVTVLKTLMEMNGKLFDDLTSYKAEERQ	442
B56 β	TSKBHWNQTVSLIKNVLKTFFMEMNGKLFDELTAITYKLEKQ	448
SCS1	DPYMLVEQAINSQSMNRAHAMAFKALKTELETNFVLYENCNALLYSSVK	692
B' β	RKK	388
B' α	KKKLMMKERBAAVVKIENLAKANQYTVYSQASTMSIPVAMETDGLPFED	457
B' α	KKKLMMKERBAAVVKIENLAKANQVQLKRVTRC	442
B56 α	RKKKKELERDELAKKLEELKLLKALEKQNSAYNMHSILSNTSAE	486
B56 β	QEQQAQERDELQGLLELRRLRLQGQGAKEAPLQRLTPQVAASGGQS	497
SCS1	ETQQRKVQREEMSKLEEYVKNLRINNDKQYTIKNPELRNSFNTASENN	742
B' α	VQMLRKTVKDEAHQAQKDPKDRPLALRKSELQDPHTKALEAHGRADE	507
SCS1	TLNEENENDCSEIQ	757
B' α	LASQDGR	514

FIG. 2. Alignment of amino acid sequences of the B' family proteins. The B' family contains four mammalian proteins (B' α , B' β , B56 α , and B56 β) and one yeast protein (SCS1). Amino acid sequences were aligned by using the PILE-UP program (Wisconsin Genetics Computer Group). Gaps introduced to optimize the alignments (dashes) and amino acids found in four or five of the five sequences (highlighted) are indicated. B' α 1 and B' α 3 are splice variants of B' α , and their sequences diverge at the position of amino acid 433.

or the absence of the B' α 1-expressing virus. The interaction between cyclin G and B' α 1 in infected cells was assessed by immunoprecipitation-Western blot assays (Fig. 4A). B' α 1 was immunoprecipitated by anti-cyclin G antibody in the presence of cyclin G but not in the absence of cyclin G (Fig. 4A, lanes 9 to 12). Approximately the same amount of cyclin G protein was immunoprecipitated by anti-cyclin G antibody in either the presence or the absence of B' α 1 (Fig. 4A, lanes 2 and 4), and the same amount of B' α 1 was detected in the lysates regardless of whether cyclin G was present (Fig. 4A, lanes 7 and 8).

Similar coinfection experiments were performed using the cyclin G-expressing baculovirus in combination with recombinant baculoviruses expressing either the A or the C subunit of PP2A. We did not observe coimmunoprecipitation of these PP2A subunits with cyclin G (data not shown), suggesting that cyclin G binds to B' α 1 directly, not indirectly via the A or C subunit of PP2A.

The association between cyclin G and B' α is unique among mammalian cyclins in vitro and in vivo. Next, we tested the interaction between cyclin G and B' α 1 in a cell-free system. Either the B' α 1-expressing virus or the wild-type virus was used to infect Sf9 cells, and the lysates from infected cells were immunoprecipitated by anti-B' α 1 antibody. Each immunoprecipitate was further incubated with in vitro-translated cyclin G, B1, D1, or E protein, and 35 S-labeled cyclins that bound to the B' α 1 immunoprecipitate were analyzed (Fig. 4B). In vitro-translated cyclin G bound to the immunoprecipitate from cells infected with the virus expressing B' α 1 but not to the immunoprecipitate from cells infected with the wild-type virus. None of the other cyclin proteins bound to either immunoprecipitate. These results indicate that the interaction between cyclin G and B' α 1 is unique among mammalian cyclins in vitro.

Since the interaction between cyclin G and B' α 1 appeared to be specific, we tested whether cyclin G specifically associates with B' α 1 in mammalian cells. We chose COS-1 cells because they are easily transfectable. Mammalian cyclins (cyclin G, A, B1, D1, or E) were expressed in COS-1 cells by transient transfections in combination with a B' α 1 construct bearing a FLAG epitope tag at its N terminus (FLAG-B' α 1). Lysates of the transfected cells were then immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were blotted with anti-B' α 1 antibody or the corresponding anticyclin antibodies (Fig. 5, lanes 1 to 3 and 5 to 7). To verify that similar amounts of each cyclin were expressed in each experiment, the total lysates were also used for Western blot analysis with anticyclin antibodies (Fig. 5, lanes 9 to 12). In parallel, to estimate the total amount of cyclin proteins in the lysates from transfected COS-1 cells, the lysates were immunoprecipitated with the corresponding anticyclin antibodies and then the immunoprecipitates were blotted with the corresponding anticyclin antibodies (Fig. 5, lane 8). Transfected FLAG-B' α 1 was detected in anti-B' α 1 immunoprecipitates, and the immunoprecipitation of FLAG-B' α 1 was abrogated if we preabsorbed the anti-FLAG antibody with competing peptide (Fig. 5, lanes 1 to 3). Cyclin G was coimmunoprecipitated by anti-FLAG antibody in the presence of FLAG-B' α 1 (Fig. 5, lanes 5 to 7), suggesting that cyclin G bound to B' α 1 in COS-1 cells. In contrast, none of the other cyclins (cyclin A, B1, D1, or E) were coimmunoprecipitated by anti-FLAG antibody in the presence of FLAG-B' α 1 (Fig. 5, lanes 5 to 7). These results indicate that the interaction between cyclin G and B' α 1 is unique among mammalian cyclins in vivo.

Endogenous cyclin G and B' α form a complex in mammalian cells. In order to determine whether cyclin G associates with B' α under physiological conditions, we attempted to detect whether an endogenous complex forms between cyclin G

FIG. 3. Interaction of cyclin G with the B' proteins in yeast cells. Yeast cells were cotransformed with the plasmid expressing the GAL4 activation domain-B' fusion proteins, and the plasmids expressing the GAL4-binding domain fused to cyclin G, cyclin D1, cyclin E, or yeast protein Snf4 or the nonfused GAL4-binding domain. Host cells containing both plasmids were streaked onto the plate with or without histidine. The host strain contains a GAL4-dependent *HIS3* gene and grows on medium lacking histidine in the presence of the functional GAL4 transcriptional activator. Cotransformations with B' α 3 (top) and with B' β (bottom) are shown.

and B' α 1 in mammalian cells. We expressed FLAG-B' α 1 in COS-1 cells and assessed the association of endogenous cyclin G with exogenously expressed B' α 1 by immunoprecipitation-Western blot assays (Fig. 6A). The lysates from transfected cells were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were blotted with either anti-cyclin G antibody or anti-B' α 1 antibody. FLAG-B' α 1 was immunoprecipitated with the anti-FLAG antibody from the FLAG-B' α 1-transfected cells. The immunoprecipitation of FLAG-B' α 1 was abrogated if the anti-FLAG antibody was preabsorbed with the competing peptide (Fig. 6A, lanes 4 to 6). Cyclin G was coimmunoprecipitated by the anti-FLAG antibody if FLAG-B' α 1 was present in the immunoprecipitates (Fig. 6A, lanes 1 to 3), indicating that endogenous cyclin G forms a complex with introduced B' α 1 in COS-1 cells.

Stable complex formation between cyclin G and B' α is regulated by p53. We used p53 temperature-sensitive cell lines to determine whether a complex between cyclin G and B' α is formed in cells without overexpression of transfected B' α or cyclin G. A rat fibroblast cell line, clone 6 (45), which harbors

the p53 temperature-sensitive allele (Val-135), was incubated at the permissive temperature (32°C), at which p53 is in its wild-type conformation. Lysates from clone 6 cells incubated at 32°C for 12 h were immunoprecipitated with anti-B' α 1 antibody. The immunoprecipitates were then blotted with either anti-B' α 1 antibody or anti-cyclin G antibody. B' α 1 was immunoprecipitated with anti-B' α 1 antibodies but not with the pre-immune serum (Fig. 6B, upper panel, lanes 3 and 4). As expected, the immunoprecipitated B' α from clone 6 cells comigrated with the B' α 1 produced in insect cells (Fig. 6B, upper panel, lanes 4 to 6). The immunoprecipitation of B' α 1 was abrogated if the anti-B' α 1 antibody was preabsorbed with the competing peptide but not with the unrelated peptide (Fig. 6B, upper panel, lanes 1 and 2). These results indicate that the anti-B' α 1 antibody specifically immunoprecipitated B' α 1. Cyclin G was immunoprecipitated by anti-B' α 1 antibody if B' α 1 was present in the immunoprecipitates (Fig. 6B, lower panel, lanes 1 to 4), suggesting that endogenous cyclin G formed a complex with endogenous B' α 1 in clone 6 cells at the permissive temperature. Quantitative analysis of the complex by

FIG. 4. (A) Association of cyclin G with B'α1 in insect cells. Either the wild-type virus or the cyclin G-expressing virus was coinfecting with or without the B'α1-expressing virus in Sf9 insect cells. The lysates from infected cells were subjected to immunoprecipitation with the antibody against the N-terminal domain of cyclin G (lanes 1 to 4 and 9 to 12). The immunoprecipitates were boiled in SDS sample buffer, separated by SDS-10% PAGE, and transferred to a nitrocellulose membrane. The membrane was probed with anti-B'α1 antibody (lanes 9 to 12) or with anti-cyclin G antibody (lanes 1 to 4). As a control, the total lysate was subjected to SDS-10% PAGE and probed with anti-B'α1 antibody (lanes 5 to 8). The endogenous protein in Sf9 cells that cross-reacts with anti-B'α1 antibody is indicated (asterisk, lanes 5 to 8). (B) Association of cyclin G with B'α1 in vitro. Lysates from Sf9 cells expressing B'α1 or the wild-type baculovirus were immunoprecipitated with anti-B'α1 antibody, and each immunoprecipitate was further incubated with ³⁵S-labeled in vitro-translated cyclin G, B1, D1, or E. Bound cyclins were released by being boiled in SDS sample buffer and were analyzed by SDS-10% PAGE (lanes 1 to 8). Aliquots of in vitro translation products were also analyzed by SDS-10% PAGE (lanes 9 to 12). An unrelated protein that is produced during the in vitro translation reaction if the pBluescript-based plasmid and T7 RNA polymerase are used in the reaction is indicated (asterisk, lanes 9 and 11). Molecular masses are indicated in kilodaltons. IP, immunoprecipitation; wt, wild type.

Western blot analysis, derived by comparing the amounts of cyclin G in the lysate and the immunoprecipitate using serial dilution, indicated that approximately 10% of the cyclin G in clone 6 cells at the permissive temperature participated in the cyclin G-B'α1 complex (data not shown).

In order to examine the stability of the interaction, the complex formed in the standard buffer (PL buffer; see Materials and Methods) was challenged by a washing with buffer containing increasing amounts of NaCl or NP-40. The complex was stable up to 350 mM NaCl (data not shown). Notably, the complex was as stable in the presence of 2.0% NP-40 as it was in the standard buffer (data not shown). Taken together, our results indicate that a stable cyclin G-B'α1 complex exists in mammalian cells under physiological conditions.

Finally, we attempted to determine whether cyclin G-B'α1 complex formation depends upon induction of wild-type p53 in cells. Clone 6 cells bearing the temperature-sensitive p53 were incubated at the restrictive (38°C) and the permissive (32°C) temperatures, and the lysates prepared from cells at either temperature were immunoprecipitated with either anti-cyclin G antibody or anti-B'α1 antibody. The immunoprecipitates were subjected to Western blotting with either antibody. As

expected, p53 induced cyclin G in clone 6 cells (Fig. 7, lower panel, lanes 1 and 2), and the association of cyclin G with B'α1 was detected upon induction of p53 (Fig. 7, lower panel, lanes 3 and 4). In reciprocal immunoprecipitation-Western blot assays, the association of B'α1 with cyclin G was detected only after the shift from 38 to 32°C (Fig. 7, upper panel, lanes 1 and 2), while there was no change in the level of B'α1 upon the temperature shift (Fig. 7, upper panel, lanes 3 and 4). These results indicate that induction of p53 leads to complex formation between cyclin G and B'α1 via the induction of cyclin G in clone 6 cells.

Similarly, induction of p53 in LTR-6 cells after the temperature shift led to complex formation between cyclin G and B'α1 (data not shown). Thus, upon induction of p53, cyclin G protein is expressed and forms a complex with B'α1 in two different cell lines.

DISCUSSION

Cyclin G is transcriptionally regulated by p53 (28, 45). In this paper, we have shown that the expression of cyclin G protein was also increased upon induction of p53 in mouse leukemic

FIG. 5. Specificity of association between cyclin G and B'α1 in vivo. A 10-μg sample of the FLAG-B'α1 plasmid or the FLAG control plasmid was cotransfected into COS-1 cells with 10 μg of plasmids expressing cyclin G, A, B1, D1, or E. The lysates from transfected cells were immunoprecipitated with 3 μg of anti-FLAG antibody (lanes 1 to 3 and 5 to 7). In lanes 2 and 6, anti-FLAG antibody was preabsorbed with the FLAG peptide before immunoprecipitation. As a control, the lysates from cells cotransfected with each cyclin and B'α1 were immunoprecipitated with the corresponding anticyclin antibodies (lanes 4 and 8). Antibody against the N-terminal domain of cyclin G was used for the immunoprecipitation of cyclin G. Anti-FLAG and anticyclin immunoprecipitates were then probed with the corresponding anticyclin antibodies (lanes 5 to 8) or anti-FLAG antibody. To ensure that the same amounts of cyclins were expressed in each transfection, the total lysates were probed with the corresponding anticyclin antibodies (lanes 9 to 12). The heavy chain of immunoglobulin G is indicated (asterisks). IP, immunoprecipitation.

cells. To investigate the biological function of cyclin G, we isolated cyclin G-binding proteins, using the two-hybrid protein interaction assay. Two interacting proteins code for B' subunits of PP2A, B'α3 and B'β. Both proteins have a high level of sequence identity (~70%) with the other members of the B' family (25, 36). Cyclin G bound to B'α1 in in vitro binding assays, and this interaction was detected in both insect cells and mammalian cells. Other mammalian cyclins (cyclins A, B1, D1, and E) were not capable of forming a stable complex with B'α1 either in vivo or in vitro. Following induction of cyclin G by p53, complex formation between cyclin G and B'α1 was induced in two different cell lines. Although the binding experiments were performed with B'α1, the specific interaction of cyclin G with both B'α3 and B'β in yeast cells and the high degree of homology among members of the B' family suggest that cyclin G might interact with a variety of B' subunits.

PP2A, one of the major serine/threonine protein phosphatases, regulates many cellular processes, including signal transduction, transcription, cell cycle, metabolism, and development (24, 39). PP2A consists of three subunits, a catalytic subunit

(C), a structural subunit (A), and a regulatory subunit (B) (38, 39). At least three distinct groups of B subunits (B/PR55, B', and B''/PR72) have been biochemically isolated from mammalian cells. Sequence analysis of cloned cDNAs demonstrated that there is no significant homology between the primary sequences of the three groups of B subunits (14, 23, 25, 36). B subunits are believed to determine both the substrate specificity and the enzymatic activity of PP2A (18). In addition, genetic evidence from *S. cerevisiae* and *Drosophila melanogaster* suggests that each B subunit plays a distinct role (24). It is likely, therefore, that association with individual B subunits determines the biological function of PP2A.

Three mammalian B'-subunit genes have recently been identified (25, 36). The encoded proteins, B'α/B56γ, B56α, and B56β, show a high level of identity (~70%) to each other. The yeast protein Scs1 (32) is homologous to the B' subunits and appears to control stress response pathways (32).

In addition to cyclin G, several viral proteins are known to associate with PP2A. Simian virus 40 small t antigen and mouse polyomavirus small t and middle T antigens are able to form stable complexes with the heterodimeric form (AC) of

FIG. 6. (A) Association of endogenous cyclin G with B'α1 in mammalian cells. COS-1 cells were transfected with 20 μg of either pCMV-FLAG or pCMV-FLAG-B'α1. The lysates from the transfected COS-1 cells were immunoprecipitated with anti-FLAG antibody. In lanes 2 and 5, anti-FLAG antibody was preincubated with the FLAG peptide before immunoprecipitation. The immunoprecipitates were boiled in SDS sample buffer, separated by SDS-10% PAGE, and probed with anti-cyclin G antibody (lanes 1 to 3) or anti-B'α1 antibody (lanes 4 to 6). (B) Association of endogenous cyclin G and endogenous B'α1 in clone 6 cells at the permissive temperature. Clone 6 cells were incubated at 32°C for 12 h after the temperature shift from 38°C (lanes 1 to 4). The lysates were subsequently immunoprecipitated with the anti-B'α1 antibody preabsorbed with 5 μg of the peptide corresponding to the C-terminal region of PCTAIRE-1 (lanes 1), the anti-B'α1 antibody preabsorbed with 5 μg of the peptide used to raise the anti-B'α1 antibody (lanes 2), preimmune serum of the anti-B'α1 antibody (lanes 3), or the anti-B'α1 antibody (lanes 4). In parallel, the lysates derived from Sf9 cells infected with the wild-type baculovirus (lane 5) or the virus expressing B'α1 (lane 6) were immunoprecipitated by the anti-B'α1 antibody. The immunoprecipitates were then separated by SDS-8% PAGE and blotted with the anti-B'α1 antibody (upper panel) or separated by SDS-10% PAGE and blotted with the anti-cyclin G antibody (lower panel). IP, immunoprecipitation; wt, wild type.

PP2A (29, 40). It has been shown that these associations are essential in cell transformation by these viral antigens (29). Another example is the protein encoded by open reading frame 4 of the adenovirus E4 gene (E4orf4), which associates with the heterotrimeric holoenzyme of PP2A (20). The interaction between PP2A and E4orf4 is responsible for the E4orf4-mediated inhibition of *junB* transcription (20).

The unique ability of cyclin G to form a complex with B'α on induction of p53 suggests the existence of specific cross-talk between PP2A and p53-mediated pathways via cyclin G. Since p53 induction results in complex formation between cyclin G

and B'α, it may be possible that p53 modulates the activity and/or the specificity of PP2A through the cyclin G-B'-subunit interaction. Alternatively, PP2A may interfere with the cyclin G-mediated p53 pathway through the cyclin G-B'-subunit interaction. In this context, it will be interesting to determine the biochemical relationship between cyclin G and B' subunits. At least three possible relationships can be envisioned. First, cyclin G might regulate the activity or the specificity of PP2A, either by forming a complex with the PP2A holoenzyme or by dissociating the B' subunit from PP2A. Second, cyclin G may be a substrate for PP2A. In this case, PP2A might dephosphorylate cyclin G or other associated proteins in the complex. Third, cyclin G or the B' subunit might regulate the localization of the complex in the cell. Interestingly, it has been shown that the B'α subunit is localized predominantly in the nuclei of transfected NIH 3T3 cells, whereas the Bα subunit is localized exclusively in the cytoplasm (36), suggesting that B subunits may regulate the function of PP2A by directing the holoenzyme to different compartments of the cell.

It has been demonstrated previously that p53 is phosphorylated at multiple serine/threonine residues in its N-terminal and C-terminal domains, and the phosphorylations at some of these sites play important roles in regulating the biochemical activity of p53 (reviewed in reference 22). On the other hand, okadaic acid, a potent inhibitor of PP2A, has been shown to change the phosphorylation status of p53 (48). It may be possible, therefore, that PP2A containing the B' subunit is responsible for dephosphorylation of these residues, thereby regulating the biochemical activity of p53. If it is the case, it will be interesting to determine whether cyclin G, through interaction with B' subunits of PP2A, affects the activity of p53, thus forming a feedback loop.

Identification of *SCS1* in *S. cerevisiae* as a gene that has a high degree of homology to the B' gene family is intriguing. *SCS1* was isolated as a high-copy-number suppressor of *ROX3*, which encodes a stress-responsive transcription regulator (31). *SCS1* was also isolated as a high-copy-number suppressor of a mutant allele of *HSP60* (32). These data indicate that *Scs1* is involved in a global stress response pathway in yeast cells. Because the B' subunits from mammalian cells show a high level of homology to *Scs1*, it is possible that the B' proteins are also involved in a similar stress response pathway in mammalian cells. Interestingly, p53 is induced by a variety of cellular

FIG. 7. Complex formation between cyclin G and B'α1 after induction of p53 in clone 6 cells. Clone 6 cells were incubated at the restrictive temperature (38°C) (lanes 1 and 3) or at the permissive temperature (32°C) for 12 h after the temperature shift (lanes 2 and 4). The lysates derived from these cells were immunoprecipitated with antibody against the cyclin box-C-terminal domain of cyclin G (lanes 1 and 2) or anti-B'α1 antibody (lanes 3 and 4). The immunoprecipitates were then separated by SDS-10% PAGE and blotted with the anti-B'α1 antibody (upper panel) or anti-cyclin G antibody (lower panel). The heavy chain of immunoglobulin G is indicated (asterisk).

stresses (9, 30, 34) and is responsible for apoptosis caused by hypoxic stress (8). Cyclin G may be involved in a stress response via interaction with the B' subunit of PP2A. p53 could modulate the stress response by inducing cyclin G protein. Future studies will hopefully reveal the connection between p53, cyclin G, and the regulatory subunit of PP2A.

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ADDENDUM

A report of novel genes encoding new members of the B' family from rabbits was published during the preparation of the manuscript (3). These genes are called B'γ and B'δ, and B'δ is identical to our B'β.

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