A Role for the Cyclin Box in the Ubiquitin-Mediated Degradation of Cyclin G1

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Abstract

Cyclin G1 was identified as a transcriptional target of p53 that encodes a protein with strong homology to the cyclin family of cell cycle regulators. We show that either ectopically expressed or endogenous cyclin G1 protein is very unstable, undergoes modification with ubiquitin, and is likely degraded by the proteasome. Ectopic cyclin G1 protein stability is increased by cyclin box mutation or by association with inactive cyclin-dependent kinase (CDK) subunits, suggesting that a function of cyclin G1 as a CDK regulator may be required for its rapid turnover. Furthermore, cyclin G1 and the cyclin box mutant interact with and are ubiquitinated by MDM2, another transcriptional target of p53 that acts as a negative regulator of p53 stability. These data suggest that the cyclin box has a role in the proteasome-mediated degradation of cyclin G1 and thus suggest a putative role for a CDK in cyclin G1 metabolism and function. [Cancer Res 2008;68(14):5581–90]

Introduction

The importance of the p53 pathway in suppression of tumorigenesis is exemplified by the fact that it is impaired in virtually all human cancers (1). Neoplasias in which p53 itself is not mutated often sustain mutations in genes that regulate p53 accumulation, thus indirectly reducing p53 function (2), p53 is normally expressed at low levels in proliferating cells, but in response to both intrinsic and extrinsic cellular stresses, the half-life of the p53 protein is increased from 6 to 20 minutes to hours (3). Three ubiquitin ligases are known to regulate the ubiquitin-mediated degradation of the p53 protein, MDM2 (HDM2), Pirh-2, and Cip-1 (4). Most of the tumor suppressor functions of p53 lie in its ability to bind DNA and regulate the expression of a set of target genes, some of which are involved in cell cycle arrest, DNA repair, cellular senescence, and apoptosis (3). Of these, p21CIP1 is the best characterized regulator of proliferation. The ability of p21CIP1 to inactivate cyclin-dependent kinase (CDK) function is clearly important in mediating growth suppressive properties of p53 (5). Nevertheless, p21CIP1 cannot totally account for p53 function as a cell cycle regulator because cells from p21CIP1-null mice retain some ability to arrest in G1 after DNA damage, a trait lost after deletion of p53 (6).

The strict regulation of p53 activity is achieved through a number of positive and negative feedback loops. Another well-characterized p53 target, MDM2, functions as an oncogene when overexpressed, consistent with its role as a negative regulator of p53 (7). MDM2 can bind to p53 and block its ability to activate transcription and can also promote its ubiquitination and subsequent proteasome-mediated degradation (8). MDM2 has a COOH terminal RING-finger protein domain that is required for its ability to function directly as an E3 ubiquitin-ligase (9). MDM2 may act to either monoubiquitinate or polyubiquitinate p53 (10). The polyubiquitination of p53 by MDM2 is a signal for degradation of p53 by proteasomes in the nucleus, whereas monoubiquitination results in nuclear export and subsequent destruction in the cytoplasm (11). These processes result in a feedback mechanism to limit p53 activity. A further level of regulation is provided by the nucleolar protein p19ARF (ARF: p14 in human), a key mediator of oncogene-activated cell cycle arrest and apoptosis (12). ARF physically interacts with MDM2 and antagonizes its ability to negatively regulate p53.

Cyclin G1 is a major transcriptional target of p53 whose role is still not completely understood (13, 14). Cyclin G1 has been categorized as a cyclin because it contains a region of protein sequence homology, the cyclin box, which is common to all members of the cyclin family of cell cycle regulators (15). Cyclins are dependent on the cyclin box to associate with specific kinase partners CDKs and regulate their kinase activity during the cell cycle (16). The p53-responsiveness of cyclin G1 is well documented. At the transcriptional level, cyclin G1 is one of the most highly induced genes in response to DNA damage (17, 18). However, no functional CDK partner has been identified for cyclin G1 to date.

Cyclin G1 has been found in complex with a number of proteins that are involved in cell cycle regulation. Cyclin G1 has been shown to interact with cyclin G-associated kinase and CDK5, although the physiologic significance of these interactions remains unclear (19). Interaction of cyclin G1 with tumor suppressor proteins, such as p53, p73, and ARF, has been reported, although whether the binding of these proteins is direct is contentious (20, 21). Okamoto and colleagues showed an association of cyclin G1 with two regulatory subunits of protein phosphatase 2A (22). Indeed, cyclin G1, as well as cyclin G2, associates with MDM2 and the active PP2A trimeric complex (21, 23–25). Although cyclin G2 is not p53-responsive, the fact that cyclin G2 is able to form similar complexes suggests that it may be able to compensate for cyclin G1 loss, perhaps contributing to the minimal phenotype of cyclin G1 knockout animals.

Although mice lacking cyclin G1 protein expression develop normally, cells derived from these mice have proved useful in uncovering the function of cyclin G1 (26, 27). Cyclin G1-null cells exhibit growth retardation after DNA damage, an abnormal G2-M arrest, decreased survival, and altered kinetics of p53 accumulation (24, 26, 27). Despite some contradictory findings, the preponderance of evidence seems to support a role for cyclin G1 in growth promotion rather than arrest. Indeed, cyclin G1-decorient mice have decreased tumor incidence, size and, malignancy (27). This result is in accordance with reports of cyclin G1 overexpression in
cancer (28–30). Ectopic overexpression of cyclin G1 has also been reported to accelerate cell proliferation (31–33).

The observation that cyclin G1 stimulates the dephosphorylation of MDM2 at T216 through its association with enzymatically active PP2A (23) supports a proproliferative role for cyclin G1. Dephosphorylation of MDM2 at T216 results in increased complex formation between MDM2 and p53 and subsequent degradation of p53. Furthermore, it has been shown that cyclin G1-null MEFs have increased p53 levels and increased MDM2 phosphorylation at T216 (23, 27). These data suggest that cyclin G1 is yet another component of the feedback regulation of p53 abundance.

Despite the progress in understanding the role of cyclin G1 as a p53 mediator, little is known about the posttranscriptional regulation of this protein or about the role of the conserved cyclin box domain of cyclin G1. Here, we report that cyclin G1 is subject to MDM2-mediated ubiquitination and degradation that can be inhibited by ARF. The cyclin box domain of ectopically expressed cyclin G1 plays an important role in localization and degradation, but not ubiquitination, of cyclin G1, suggesting that functional CDK interaction may regulate this target of p53 in normal and tumor cells.

Materials and Methods

Cell culture and plasmids. U2OS, MRCS, W38R, C2C12, and A1-5 cells were maintained in DMEM (JRH Biosciences), 10% fetal bovine serum (FBS) and 100 units/mL pen/strep (Life Technologies-Bethesda Research Laboratories) at 37°C, 5% CO₂, NIH-3T3 cells were grown in DMEM containing 10% calf serum with antibiotics, Saos2 cells in DMEM with 15% FBS. U2OS cells were transfected using BBS and calcium chloride. For induction of p53 in A1-5 cells, 500K cells were plated per 10-cm dish and incubated at 37°C for 12 h. The cells were shifted to 32°C for 36 h before harvesting.

Human cyclin G1 was a gift from Alan Walsh and was subcloned into the CMVNeoBam vector. Murine cyclin G1 was expressed using the pCDNA3 vector (Invitrogen). The K106R and K106D mutants and Flag-tagged versions were generated by PCR and sequenced. The MDM2 expression plasmid was a generous gift of Arnold Levine and the HA-tagged ubiquitin construct and MDM2-D Ring were kindly provided by Carl Maki. p19ARF was a gift from Karl Munger, and p14ARF was kindly provided by Martine Roussel. CDK2 and dnCDK2 were provided by Sander van den Heuvel. CDK5 and dnCDK5 were both generous gifts of Li-Huei Tsai. Roussel. CDK2 and dnCDK2 were provided by Sander van den Heuvel.

Immunoprecipitation and immunoblotting. Lysis, immunoprecipitation, and immunoblotting were performed essentially as described (34). The following primary antibodies were used: Flag (M2, Sigma), cyclin G1 (H-46, Santa Cruz), CDK2 (M2, Santa Cruz), CDK5 (C8, Santa Cruz), tubulin (Ab-1, Oncogene), HA (supernatant from clone12CA5), and MDM2 (smp-14, Santa Cruz). Horseradish peroxidase–conjugated donkey anti-rabbit or anti-mouse secondary antibodies (Jackson Immunoresearch) were diluted 1:10,000 or 1:5,000 in NET and detected by enhanced chemiluminescence (NEN).

Half-life experiments. Cycloheximide (Sigma) was prepared in 100% ethanol and used at a final concentration of 50 μg/mL. For half-life experiments in transfected U2OS cells, a single transfected 10-cm dish was trypanzied and plated in five wells of a six-well dish. Medium containing cycloheximide was added and incubated for the indicated time points before harvest. For 3T3 experiments, cells were grown for 24 h at 37°C and treated with a final concentration of 0.2 ng/mL doxurubicin (Calbiochem) for 12 to 24 h before addition of cycloheximide. MG-132 (Peptides International) and lactacystin (Calbiochem) were reconstituted in DMSO and added to cell cultures at a final concentration of 25 and 10 μmol/L, respectively, 1 h before addition of cycloheximide.

For pulse-chase experiments, cell cultures were washed with PBS and incubated in methionine-free medium supplemented with 10% dialyzed FBS for 1 h. The medium was removed and 2 mL of fresh methionine-free medium was added containing 250 μCi of [35S]methionine (NEN)/mL. Cells were labeled for 1 h. After washing with PBS, chase medium was added (DMEM plus 10% FBS supplemented with 15 μg/mL cold methionine) and samples were harvested. Lysates were precleared using rabbit immunoglobulin prebound to Staphylococcus aureus cells for 1 h at 4°C. Immunoprecipitations were performed as described using an agarose-conjugated Flag antibody (M2, Sigma) and separated by SDS-PAGE. Quantitation of bands was performed using ImageJ.

Indirect immunofluorescence. Cells were plated onto glass coverslips 24 h after transfection and fixed – 24 h later in 4% PEA/PBS and permeabilized with 0.1% TritonX-100 in PBS. Coverslips were blocked with 5% normal goat serum in PBS and incubated with primary antibody for 1 h at room temperature. Antibodies used were CDK5 (J3), cyclin G1 (C-18), and fibrillarin (AFBO1, Cytoskeleton). Coverslips were washed thrice, immunolabeled with fluorochrome-conjugated secondary antibodies (Alexa Fluor 555 goat anti-rabbit, 488 goat anti-mouse; Molecular Probes), and stained with bisbenzimide (Hoechst). Images were collected on a Leica SP2 laser scanning confocal microscope.

Cell synchronization and irradiation. U2OS cells were synchronized by double thymidine block. Briefly, cells were plated at 500 K per 10-cm dish and allowed to grow overnight. The cells were treated with excess thymidine (2 mmol/L; Sigma) for 17 h, washed, and allowed to recover for 9 h before readdition of thymidine (2 mmol/L). The cells were infected with adenovirus for 10 h after the second thymidine treatment and released from the thymidine block 4 h later. The cells were exposed to 6 Gy γ- irradiation from a 320Co γ-ray source (U.S. Nuclear) 2 h after release from thymidine block and harvested for flow cytometry 24 h later.

Generation of adenovirus. Wild-type cyclin G1 and the KD mutant were cloned into the pAdTrack-CMV shuttle vector, and this was cotransformed with an adenoviral backbone plasmid (pAdEasy) by electroporation into electrocompetent BJ5183 Escherichia coli cells. Recombinants were selected using kanamycin and confirmed by restriction digest. Recombinant plasmids were linearized and transfected into the packaging cell line (293) using Fugene (Roche) per manufacturer’s instructions.

Flow cytometry analysis. Medium was collected, and cells were washed with PBS followed by PBS + 0.1% EDTA. Cells were dislodged from the plate by incubation with PBS + 0.1% EDTA. All cells were pooled and centrifuged for 5 min at 1,600 rpm. The pellet was resuspended and washed twice in PBS supplemented with 1% calf serum and 0.1% sodium azide. Fixation was achieved by mixing the cell suspension dropwise into 90% ethanol (−20°C) while vortexing. For DNA staining, the cells were pelleted and resuspended in a solution of propidium iodide (20 μg/mL) and RNase A (200 μg/mL). DNA content was measured using a Becton Dickinson FACScan with CellQuest software and analyzed using ModFit.

Results

Cyclin G1 is an unstable protein. The homology between cyclin G1 and known cyclins suggests that cyclin G1 may activate a CDK. In effort to uncover a CDK-activating role for cyclin G1, we mutated a conserved lysine residue in murine cyclin G1 (K106) analogous to a residue in cyclin D1 that is necessary for activating, but not binding, a CDK partner (34). 1 This residue is predicted from the cyclin A/CDK2 structure to form contacts that allow the proper orientation of ATP in the active site of the enzyme complex (35). We created both a nonconservative mutation of lysine to aspartic acid and a conservative mutation to arginine, an amino acid that naturally exists at this position in cyclin F and cyclin G2. Upon transfection of these constructs into U2OS cells, we observed an alteration in the mobility of K106D (KD) compared with the wild-type or K106R (KR). Additionally, we noted a substantial increase in the steady-state level of the KD mutant, suggesting an increase in protein stability. The instability of cyclin G1 was not predicted by

1 Our unpublished observation.
its sequence, as it encodes neither a PEST sequence nor a destruction box motif found in mitotic cyclins (36).

The half-lives of the wild-type and mutant cyclin G1 proteins were determined by treating transfected U2OS cells with cycloheximide (Fig. 1A). Wild-type cyclin G1 and the KR mutant both had half-lives between 15 and 20 minutes, whereas the KD mutant had a half-life 3-fold to 4-fold longer. Similar results were obtained by pulse-chase analysis (data not shown). Thus, the instability of cyclin G1 depends at least in part on the integrity of the cyclin box, suggesting that cyclin G1 degradation depends on CDK activation or association, as has been seen with other cyclins (37–39). We observed that the half-life of cyclin G1 was also dependent on the functional integrity of cotransfected CDKs (Fig. 1B). Coexpression of dominant-negative CDK2 or CDK5 with cyclin G1 in U2OS cells resulted in a substantial increase in the half-life of cyclin G1, whereas the half-life of cyclin G1 coexpressed with wild-type CDK2 or CDK5 remained short. Neither wild-type nor inactive versions of CDK4 or CDK6 seemed to have a profound effect on cyclin G1 stability. We found that, in U2OS cells, the CDK inhibitor p27KIP1 could stabilize cyclin G1 when coexpressed with CDK2 or CDK5 and that p21CIP1 could stabilize cyclin G1 in combination with CDK2 (Fig. 1B and C). The steady-state level of cyclin G1 was also increased by dnCDKs in the p53-deficient Saos2 cell line (Fig. 1C).

The inhibitors p21CIP1, p27KIP1, or p16INK4a were not able to stabilize cyclin G1 when expressed alone. This suggests that cyclin G1 is stabilized under conditions in which it may be confined in an inactive complex and not as a result of altered cell cycle profile of transfected cells or by cell cycle arrest. Intriguingly, wild-type cyclin G1, but not the KD mutant, was found to be phosphorylated in vivo, consistent with a putative autoregulatory function of cyclin G1 (Supplementary Fig. S1A). Furthermore, using a baculovirus expression system, we detected a CKI-inhibitable kinase activity that coprecipitated with wild-type cyclin G1, but not the KD mutant (Supplementary Fig. S1B). The nature of this activity remains to be identified.

We next assayed the ability of cyclin G1 to bind CDK1, CDK2, CDK4, CDK5, and CDK6. Coimmunoprecipitation experiments showed that cyclin G1 was able to interact with all of the kinases tested, including dominant-negative versions. An example experiment with CDK2 is shown in Fig. 1D (others are shown in Supplementary Fig. S2A–C). The levels of cyclin G1 were increased upon coexpression of dnCDK2 or the CDK inhibitors p21CIP1 or p27KIP1, resulting in increased communoprecipitation of CDK2. However, no activity was detected in in vitro kinase assays using histone H1 and GST-Rb (COOH terminus) as substrates (data not shown). We cannot rule out the possibility

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**Figure 1.** Half-life of exogenous cyclin G1 and mutants. A, U2OS cells were transfected with wild-type cyclin G1 or the mutants KD or KR. Cells were treated with cycloheximide (50 μg/mL) for the indicated times to determine protein half-life. The images shown are representative examples. The experiments were performed at least thrice and quantitated using NIH image software. B, wild-type cyclin G1 was cotransfected with either functional or dominant negative (dn) isoforms of CDK2, CDK4, CDK5, or CDK6 and in combination with the CDK inhibitors p21CIP1 and p27KIP1. The transfected cells were treated with cycloheximide and blotted for cyclin G1 to assess half-life. C, top, U2OS cells transfected with cyclin G1 and combinations of CDKs and CKIs and blotted for steady-state levels. Bottom, steady-state levels of cyclin G1 protein in Saos2 cells cotransfected with wt and dn forms of CDK2 or CDK5. D, U2OS cells were transfected with the indicated expression vectors, lysates were immunoprecipitated with Flag antibodies, and blots were probed for CDK2 or Flag–cyclin G1.
that cyclin G1 can activate these kinases but acts on an undetermined substrate.

We next considered the possibility that cyclin G1 may act as a negative regulator of other cyclins. However, overexpression of cyclin G1 does not interfere with the associated kinase activities of either cyclin E or p35 (Supplementary Fig. S2D and E). Furthermore, we found no cell cycle effect of cyclin G1 overexpression by either transient transfection or adenoviral expression in any normal or tumor cell line as analyzed by flow cytometry (Supplementary Fig. S3A). These data are consistent with reports by others (13, 40). However, we did detect abnormal nuclei in some cyclin G1 overexpressing cells similar to those described for cyclin G2 overexpressing cells (data not shown; ref. 25). Overall, it seems unlikely that cyclin G1 negatively regulates cyclin-CDK complexes required for cell cycle progression under routine culture conditions. Thus, although a specific CDK partner and activity of cyclin G1 remains to be identified, functional association with such a kinase may contribute to cyclin G1's markedly short half-life.

Endogenous cyclin G1 induced by p53 is also unstable. Because exogenous cyclin G1 was unstable in U2OS cells, we asked if the endogenous protein was equally labile. We treated NIH-3T3 cells with doxorubicin to induce p53 and cyclin G1 and the resulting increase in cyclin G1 protein level is apparent (Fig. 2A, left). We found that the stability of the endogenous protein was similar to that of the exogenous protein in U2OS cells (Fig. 2A, right).

We also examined endogenous cyclin G1 protein in the A1-5 rat fibroblast cell line. A1-5 cells express a temperature-sensitive allele of murine p53, val135 (41). In these cells, p53 is inactive at 37°C, but assumes a wild-type conformation at 32°C, activates transcription of target genes, and arrests cells in G1 phase. Cyclin G1 protein is barely detectable at the nonpermissive temperature but is abundant at 32°C (Fig. 2B, top). When A1-5 cells maintained at 32°C for 36 hours were treated with cycloheximide, the cyclin G1 protein was short-lived (Fig. 2B, left middle, long-time course; bottom, short-time course). Although p53 increases the level of cyclin G1 transcript, it does not seem to create a stabilizing environment for the cyclin G1 protein in either A1-5 or 3T3 cells. Furthermore, cyclin G1 stability does not correlate with proliferative state because there is no difference in the half-life of cyclin G1 in A1-5 and 3T3 cells.
in proliferating transfected U2OS cells compared with that in p53-arrested NIH-3T3 or A1-5 cells.

Inhibitors of the proteasome stabilize exogenous and endogenous cyclin G1 protein. As degradation of numerous proteins is dependent on the activity of proteasomes, we tested whether the proteasome inhibitor MG-132 could prevent the rapid destruction of cyclin G1. Treatment of A1-5 cells at 32°C with MG-132 resulted in a substantial increase in the stability of cyclin G1 compared with the DMSO treated control (from ~20 minutes to ~2 hours; Fig 2B). A similar response of cyclin G1 to MG-132 was observed in transfected U2OS cells (Fig 2C), suggesting cyclin G1’s short half-life is proteasome-dependent. However, because MG-132 inhibits thiol proteases in addition to the proteasome complex, we tested whether a specific inhibitor of the proteasome, lactacystin, was also capable of extending the half-life of transfected cyclin G1 in U2OS cells. Comparable with the effect of MG-132, lactacystin also increased the half-life of cyclin G1 from 15 to 20 minutes to nearly 2 hours (Fig 2C).

To test whether proteasome inhibitors could increase the levels of endogenous cyclin G1 in undamaged cells, we treated a variety of cell lines with MG-132 and observed a consequent increase in cyclin G1 protein (Fig 2D). The cells used in this study were C2C12 murine myoblasts, U2OS human osteosarcoma cells, A1-5 (ts-p53) rat fibroblasts cultured at the nonpermissive (37°C) temperature, and the human diploid fibroblast cells, MRC5 and WI38. All of these cell lines express wild-type p53 protein. As p53 is also stabilized by inhibition of the proteasome, we cannot discount the possibility that stabilization of p53 results in increased transcription of cyclin G1 and hence an increase in protein. However, A1-5 cells produce high levels of dominant-negative mutant p53 at 37°C, likely inactivating wild-type p53 through oligomerization, rendering the cells effectively p53-deficient. To test this p53-independent increase more directly, we examined the endogenous cyclin G1 protein in Saos2 cells that lack p53. MG-132 treatment increases cyclin G1 protein in Saos2 cells, supporting a role for proteasome-mediated degradation in controlling steady-state levels of cyclin G1 (Fig 2D, right). We conclude that the short half-life of basal and induced endogenous cyclin G1, as well as that produced ectopically, is dependent on proteasome activity.

Ubiquitination of cyclin G1 in U2OS cells. The proteasome-dependent degradation of cyclin G1 suggested that it was likely to be a direct target of the ubiquitination machinery. To confirm this,
Flag-tagged wild-type cyclin G1, KR, and KD were transfected into U2OS cells, both in the presence and absence of HA-tagged ubiquitin. When cyclin G1 was immunoprecipitated, anti-HA reactive bands and smears of high molecular weight material likely to be polyubiquitinated cyclin G1 were detected in the presence, but not the absence, of HA-ubiquitin (Fig. 3A, left). Consistent with the degradation of ubiquitinated cyclin G1 by the proteasome, the intensity of these bands was enhanced by treating the cells with MG-132. In a similar experiment, HA antibodies were used to immunoprecipitate the tagged ubiquitin and blots were probed for Flag–cyclin G1 (Fig. 3A, right). Flag reactive bands are present at the top of the gel in the presence of HA-tagged ubiquitin, indicating polyubiquitinated cyclin G1 and mutants. Unmodified cyclin G1 is also detected, indicating that it is being immunoprecipitated through interaction with another ubiquitinated protein. We hypothesize that the reason monoubiquitinated and intermediate forms of cyclin G1 are not detected in this blot is likely due to the large number of cellular proteins that are ubiquitinated, such that the HA antibody is limiting.

In direct immunoblots, a higher molecular weight cyclin G1–reactive band appears when the cells are treated with MG-132 (Fig. 3B), consistent with the addition of a single ubiquitin molecule to cyclin G1. The coexpression of tagged ubiquitin converts this single band to a doublet in cells expressing either cyclin G1 or KD. These experiments have been performed with different antibodies and tags with the same result (data not shown). Modified forms of cyclin G1 and KD are also seen in complex with CDK5 in the presence of MG-132 (Fig. 3C). Coexpression of p21CIP1 or p27KIP1 does not alter this ubiquitination. In fact, the modified forms are increased in the presence of KD or cyclin G1/CDK5/p27KIP1, just as the steady-state levels and half-lives of cyclin G1 are increased for these combinations of proteins. Collectively with proteasome-dependent degradation of cyclin G1, these data strongly suggest that transfected cyclin G1 is a direct ubiquitin-dependent substrate of the proteasome. Furthermore, long exposure of DNA damage–induced, MG-132–treated endogenous cyclin G1 shows the presence of slower migrating bands consistent with addition of ubiquitin (Fig. 3D). The stabilized bands that react with the cyclin G1 antibody are present as doublets (Fig. 3C and D), indicating that the cyclin G1 protein probably undergoes other modifications in addition to ubiquitination. The nature of these modifications is currently under investigation. Interestingly, despite profound differences in cyclin G1 half-life, there is no apparent difference in the level of ubiquitination of wild-type cyclin G1 and KD. Hence, mutation of the conserved lysine does not inhibit ubiquitination of cyclin G1.

**Cyclin G1 cellular localization may regulate its stability.** In an attempt to understand why complex formation with inactive CDKs or a nonconservative mutation of lysine 106 altered the half-life, but not the ubiquitination of cyclin G1, we examined the cellular distribution of KD and wild-type cyclin G1 in the presence and absence of excess CDK using confocal microscopy. Wild-type cyclin G1 transfected alone or with active CDK was distributed throughout the nucleus (Fig. 4). The staining seemed largely homogeneous but was occasionally punctuated with small bright foci. A similar pattern was seen by immunostaining wild-type MEFs cells treated with DNA-damaging agents to induce endogenous cyclin G1 (Supplementary Fig. S4). The KD mutant also localized to the nucleus but lacked focal staining and was notably excluded from large subnuclear regions. Immunolabeling of these cells with the fibrillarin antibody indicated that these regions are nucleoli. Interestingly, the cyclin G1 protein stabilized by inactive kinase also

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**Figure 4.** Subcellular localization of transfected wild-type cyclin G1 and KD in U2OS cells. U2OS cells were transfected with wild-type cyclin G1 and KD and immunostained for cyclin G1 and fibrillarin, a marker of the nucleolus. Hoechst was used as a counterstain. Wild-type cyclin G1 was also cotransfected with functional and dominant-negative CDK5. The panels show cells stained for cyclin G1, fibrillarin, and a merged image. The expression of CDK5 was confirmed by immunofluorescence. No effect on the localization of either CDK5 isoform was observed in the presence of cyclin G1.
seemed to be excluded from these regions of the nucleus (Fig. 4). Cyclin G1 cotransfected with CDK5 and p27KIP1 also showed this phenotype, whereas cyclin G1 cotransfected with CDK5 and p21CIP1 or either inhibitor alone did not (Supplementary Fig. S5). Furthermore, the increase in steady-state levels of cyclin G1 seen by immunoblot is also evident by immunofluorescence.

Ubiquitination of cyclin G1 is enhanced by MDM2. A direct physical association between cyclin G1 and the MDM2 ubiquitin ligase has been shown by a number of groups (21, 23, 24). Indeed, we were readily able to confirm this observation using extracts of A1-5 cells expressing endogenous cyclin G1 and MDM2 (Supplementary Fig. S6). To test for an ability of MDM2 or an ubiquitination-deficient version (ΔRing; refs. 9, 42–44) of MDM2 to modify cyclin G1, both Cos and U2OS cells were cotransfected with tagged cyclin G1 and either MDM2 or ΔRing and modification of cyclin G1 was assessed by immunoblot. Overexpression of MDM2 resulted in the generation of slower migrating species of cyclin G1, consistent with MDM2-mediated ubiquitination of cyclin G1 (Fig. 5A and B). Expression of the ΔRing mutant resulted in a reduced level of both polyubiquitinated and monoubiquitinated cyclin G1 in both cell lines when compared with wild-type MDM2. Interestingly, in our hands, the expression of MDM2 did not result in diminution of cyclin G1 levels as it does with p53 (Fig. 5C), once again indicating that polyubiquitination of cyclin G1 is not sufficient to increase degradation.

We next examined the effects of ARF overexpression on cyclin G1 abundance. ARF has been shown to interact with cyclin G1 (21) and, as a negative regulator of the ability of MDM2 to act as an E3 ubiquitin ligase, might alter cyclin G1 half-life. Indeed, the steady-state level of exogenous cyclin G1 protein increases in the presence of increasing amounts of transfected ARF in U2OS cells, an ARF-negative cell line (Fig. 6A). Pulse-chase experiments in transfected U2OS cells confirmed that coexpression of ARF and cyclin G1 increased the half-life of cyclin G1 to ~2 hours (Fig. 6B), further supporting a role for MDM2 in the regulation of cyclin G1 half-life. Interestingly, we found a reciprocal relationship between ARF and wild-type cyclin G1 upon cotransfection (Fig. 6C, left and Supplementary Fig. S7). Wild-type cyclin G1 levels were increased by ARF expression, but ARF protein levels were reduced by either cyclin G1 or KD. We do not yet understand how cyclin G1 might regulate ARF; however, both cyclin G1 and KD retain the ability to interact with MDM2 (Fig. 6C, right) even in the presence of overexpressed ARF. Together, these data imply that polyubiquitination of cyclin G1 stimulated by MDM2 is necessary, but not sufficient, for proteasome-mediated cyclin G1 degradation and that the integrity of the cyclin box is required for cyclin G1 rapid turnover, perhaps as a consequence of specific subnuclear localization.

In an effort to understand the potential role of cyclin G1 kinase activity in the p53-mediated response to DNA damage, we analyzed cell cycle profiles of irradiated cells overexpressing cyclin G1 or KD. U2OS cells were synchronized by a double thymidine block and infected with adenovirus coexpressing GFP marker and cyclin G1 or KD. The cells were then exposed to γ-irradiation and harvested for flow cytometry 24 hours later (Fig. 6D, top). As shown in Fig. 6D, control, cyclin G1, and KD infected cells showed similar cell cycle profiles after synchronization with thymidine before irradiation. However, 24 hours after γ-irradiation, the majority of control and cyclin G1 infected cells were blocked in G2-M, whereas KD infected cells exhibited a decreased G2-M fraction, with a concomitant increase of cells in other phases. This suggests that cyclin G1 is required for the establishment and/or maintenance of the G2-M checkpoint in response to ionizing radiation and that KD can interfere with this process. Thus, the integrity of the cyclin box of cyclin G1 seems to be important and may play a role in the ability of cyclin G1 to modulate the DNA damage response.

Figure 5. The enhanced ubiquitination of cyclin G1 by MDM2 is affected by deletion of the Ring domain. A, Cos cells were transfected with Flag-tagged cyclin G1 alone or with MDM2 or an MDM2 mutant lacking the Ring domain (ΔRing) in the presence and absence of HA-tagged ubiquitin. Lysates were blotted directly for cyclin G1 or immunoprecipitated with Flag antibodies and blotted for HA-tagged ubiquitin. High molecular weight HA-reactive bands, as well as monoubiquitinated cyclin G1, are seen with cotransfection of MDM2 only in the presence of both HA-ubiquitin and Flag–cyclin G1 and are reduced by deletion of the Ring domain. B, the reverse immunoprecipitation is shown in cell lysates from U2OS cells with a similar result. The lysates were immunoprecipitated with HA antibodies and blotted for Flag–cyclin G1. C, U2OS cells were transfected with p53 or cyclin G1 alone or in combination with MDM2 or ΔRing and immunoblotted for p53, MDM2, and cyclin G1.
Figure 6. Stabilization of cyclin G1 by ARF, interaction with MDM2, and effect on DNA damage response. A, untagged cyclin G1 was cotransfected into U2OS cells with GFP and increasing amounts of p14ARF. Lysates were immunoblotted for cyclin G1 and GFP as a control. B, U2OS cells were transfected with Flag-tagged cyclin G1 or Flag–cyclin G1 plus p14ARF and labeled with [35S]methionine. Cells were then fed excess cold methionine, lysed, and subjected to immunoprecipitation with anti-Flag antibody to determine protein half-life. C, left, U2OS cells were transfected with HA-tagged cyclin G1 and Flag-tagged p19ARF, MDM2, or both Flag-ARF and MDM2. Lysates were blotted for cyclin G1, MDM2, or Flag. Right, the lysates from A were immunoprecipitated with anti-HA antibodies and blotted for cyclin G1 and MDM2. No interaction between ARF and cyclin G1 was detected by coimmunoprecipitation. D, top, schematic representation of protocol used to synchronize, infect, and irradiate cells. Cells were stained with propidium iodide and the cell cycle profiles of GFP positive cells were determined by flow cytometry. Time points corresponding to profiles shown are indicated by arrows. Bottom, effect of cyclin G1 and KD on the cell cycle profile of synchronized U2OS cells before and after γ-irradiation. No effect is seen with either wild-type cyclin G1 or KD before irradiation, but KD alters the cell cycle profile of irradiated cells. Quantitation was performed using ModFit.
Discussion

Although transcriptional activation of cyclin G1 by p53 is well established, a complete understanding of its function remains elusive. The current study confirms and extends several novel aspects of cyclin G1 regulation. First, in addition to its transcriptional regulation by p53, the stability of the cyclin G1 protein is also regulated (21). This stability is not p53-dependent. Second, a clear relationship exists between the potential CDK-activating functions of cyclin G1 and its stability because mutation of a conserved lysine in the putative cyclin box markedly increases the half-life of exogenous cyclin G1 as does cotransfection of CDKs and p27\(^{kip1}\) or dominant-negative CDKs. Third, there is an association between the stability of exogenous cyclin G1 and its subcellular localization, both of which depend on the integrity of the cyclin box. Fourth, ARF seems to regulate both the stability and nucleolar localization of ectopically expressed cyclin G1 (ref. 21; Supplementary Fig. S8). Fifth, cyclin G1 associates with, and its ubiquitination is stimulated by, MDM2. Proteasome inhibition results in the accumulation of both exogenous and endogenous cyclin G1 and the appearance of cyclin G1–ubiquitin conjugates. Finally, mutation of the cyclin box of cyclin G1 has an effect on the establishment and/or maintenance of the G2-M arrest after DNA damage of cells overexpressing cyclin G1.

An ability of cyclin G1 to activate a CDK partner may facilitate its proteolysis. The observation that the activity of a cyclin can regulate its own degradation is not novel. For example, it is known that the stability of cyclin E is increased when it is involved in an inactive complex, either with dnCDK2 or when the cyclin E/CDK2 complex is bound and inactivated by inhibitors, such as p27\(^{kip1}\) or p21\(^{cip1}\) (37, 39, 45). Consistent with this, phosphorylation of Thr\(^{380}\) of cyclin E by CDK2 promotes its degradation by freeing cyclin E from its complex with CDK2. Cellular distribution is also a means of cyclin regulation as the subcellular relocalization of cyclin D1 to the cytoplasm is required for its ubiquitin-dependent proteolysis (46). Here too, CDK association plays a role in stability, but in contrast to cyclin E, association with either functional or nonfunctional CDK subunits stimulates cyclin D1 phosphorylation, translocation, ubiquitination, and degradation.

Our data suggest that control of cyclin G1 stability has elements of both cyclin D1 and cyclin E dependence on CDK association. Ectopically expressed, wild-type cyclin G1 bound to dnCDK2 subunits is stable and excluded from the nucleolus. Similarly, the cyclin box mutant, KD, which may be unable to form active complexes with a CDK, binds MDM2 in the nucleoplasm and is ubiquitinated, but is excluded from the nucleolus and stable. Thus, whereas kinase activation by cyclin G1 does not seem to be required for its ubiquitination, it may be necessary for its subsequent delivery to the proteasome for degradation, a process that can be blocked by ARF. It has been shown for G1 cyclins in yeast that polyubiquitination in itself is not sufficient for degradation by the proteasome, and the authors speculate that activity may be required for release of polyubiquitinated cyclin from the ubiquitin ligase complex (47). It may be that autophosphorylation of cyclin G1 is required to release it from a CDK partner before degradation. However, as we have confirmed no wild-type cyclin G1 kinase activity to date, we can currently only speculate on the possible correlation between a CDK-activating function and stability of cyclin G1.

The stabilization of cyclin G1 by ARF suggests a role for ARF in the regulation of a member of the p53 pathway other than MDM2 and p53 itself. Interesting parallels exist between p53 and cyclin G1. Both are short-lived nuclear proteins, with half-lives <30 minutes, both are degraded in an ubiquitin-mediated, proteasome-dependent manner, both bind MDM2 (48, 49) and are stabilized by ARF (12). However, the half-life of cyclin G1 is not increased by DNA damage in NIH-3T3 cells, and in contrast to p53, cyclin G1 itself is relocalized to the nucleolus upon overexpression of ARF (21). Whether stabilized cyclin G1 has a role in the nucleolus remains a question for further study.

We did not see any significant effect of cyclin G1 or KD overexpression on unirradiated U2OS cells at 24 to 48 hours after infection. However, we did detect a defect in the G2-M checkpoint after γ-irradiation in KD expressing cells. It may be the case that cyclin G1 requires other p53-induced genes and/or the environment created by p53 for it to function. The defect seen with KD was almost identical to that seen in cyclin G1 knockout MEFs (26), supporting the notion that KD is acting in a dominant-negative fashion. It is not at all surprising that overexpression of cyclin G1 had no effect after DNA damage, as cyclin G1 levels should already be high under these conditions due to activation by p53. The role of cyclin G1 at the G2-M checkpoint requires further study.

The metabolism of cyclin G1 is clearly linked to MDM2 and ARF, suggesting an additional layer of feedback regulation in the p53 pathway (21). Cyclin G1 aids in the degradation of the protein that activates it (p53) and helps to activate the protein that ubiquitinates and degrades it (MDM2; refs. 21, 23). The stringent control of cyclin G1 expression by two known tumor suppressors, both by transcriptional regulation and protein stabilization, suggests that restraining the activity of cyclin G1 is very important. The complex network of interrelationships between p53, MDM2, ARF, and cyclin G1 suggested by data presented here predict that successful assays for the function of cyclin G1 as a putative CDK activator may require the integrity of all of these proteins.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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