$G\alpha_{12/13}$ Basally Regulates p53 through Mdm4 Expression

Mi-Sung Kim,[1] Sang Min Lee,[1] Won Dong Kim,[1] Sung Hwan Ki,[1] Aree Moon,[2] Chang Ho Lee,[3] and Sang Geon Kim[1]

[1]College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University; [2]College of Pharmacy, Duksung Women's University; and [3]Department of Pharmacology and Institute of Biomedical Science, College of Medicine, Hanyang University, Seoul, Korea

Abstract

$G\alpha_{12/13}$, which belongs to the $G\alpha_{12}$ family, participates in the regulation of diverse physiologic processes. In view of the control of $G\alpha_{12/13}$ in cell proliferation, this study investigated the role of $G\alpha_{12/13}$ in the regulation of p53 and mdm4. Immunoblotting and immunocytochemistry revealed that p53 was expressed in control embryonic fibroblasts and was largely localized in the nuclei. $G\alpha_{12}$ deficiency decreased p53 levels and its DNA binding activity, accompanying p21 repression with Bcl_2 induction, whereas $G\alpha_{13}$ deficiency exerted weak effects. $G\alpha_{12}$ or $G\alpha_{13}$ deficiency did not change p53 mRNA expression. ERK1/2 or Akt was not responsible for p53 repression due to $G\alpha_{12}$ deficiency. Mdm4, a p53-stabilizing protein, was repressed by $G\alpha_{12}$ deficiency and to a lesser extent by $G\alpha_{13}$ deficiency, whereas mdm2, PTEN, β-catenin, ATM, and Chk2 were unaffected. p53 accumulation by proteasomal inhibition during $G\alpha_{12}$ deficiency suggested the role of $G\alpha_{12}$ in p53 stabilization. Constitutively active $G\alpha_{12}$ ($G\alpha_{12}$QL) or $G\alpha_{13}$ ($G\alpha_{13}$QL) promoted p53 accumulation with mdm4 induction in MCF10A cells. p53 accumulation by mdm4 overexpression, but no mdm4 induction by p53 overexpression, and small interfering RNA knockdown verified the regulatory role of mdm4 for p53 downstream of $G\alpha_{12/13}$. In control or $G\alpha_{12}$/$G\alpha_{13}$-deficient cells, genotoxic stress led to p53 accumulation. At concentrations increasing the flow cytometric pre-G_1 phase, doxorubicin or etoposide treatment caused serine phosphorylations in $G\alpha_{12}-/-$ or $G\alpha_{12/13}-/-$ cells, but did not induce mdm4. $G\alpha_{12/13}$QL transfection failed to phosphorylate p53 at serines. Our results indicate that $G\alpha_{12/13}$ regulate basal p53 levels via mdm4, which constitutes a cell signaling pathway distinct from p53 phosphorylations elicited by genotoxic stress.
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Introduction

Heterotrimeric GTP-binding proteins (G proteins) participate in cell signaling for the regulation of a variety of physiologic processes. The Gα subunits which define G proteins are divided into $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$ family members. Among the Gα families, $G\alpha_{12}$ members are activated by the stimulation of thrombin, TXA_2, lysophosphatidic acid, and thyroid-stimulating hormone receptors (1). The $G\alpha_{12}$ family members, which consist of $G\alpha_{12}$ and $G\alpha_{13}$, regulate various intracellular effectors or cellular responses such as platelet aggregation (2), actin-stress fiber formation (3), apoptosis (4), and neurite retraction (5). In spite of the mostly overlapping functions between $G\alpha_{12}$ and $G\alpha_{13}$, the proteins sometimes exhibit differential abilities to recruit signaling pathways for physiologic effectors (6). *$G\alpha_{13}$* gene knockout resulted in impaired angiogenesis and intrauterine death, whereas mice deficient in the *$G\alpha_{12}$* gene were alive (7). G proteins and their associated proteins (e.g., β-catenin), if inappropriately activated or accumulated (8, 9), may carry transforming potential and thus play a role in tumorigenesis. In fact, constitutively active mutants of $G\alpha_{12/13}$ may represent oncogenic properties (10-12). Thus, it is most likely that the G proteins play roles in signaling pathways linked to the biological processes of cell survival. However, the $G\alpha_{12/13}$ signaling pathways for cell proliferation or survival have been inadequately defined.

p53 is a well-known tumor suppressor gene that serves as a nuclear transcription factor for the regulation of target gene expression associated with cell proliferation (13). p53 is activated by various toxic stress signals, which becomes important to prevent the replication of damaged cells (e.g., oncogenic genetic lesions). p53 function is important to monitor oncogenic changes in cells or for the prevention of the growth of abnormal or damaged cells (14). Activation of p53 leads to the inhibition of cell growth through cell cycle arrest or activation of apoptosis (15, 16). Numerous studies have been conducted on p53 mutations found in tumors, which involve single amino acid mutations or the elimination of its expression (17). p53 expression is altered by the exposure of cells to extracellular stimuli including hypoxia, etoposide, and γ-irradiation (18, 19). p53 activity is also regulated by stabilization mediated with posttranslational modifications (i.e., phosphorylation and acetylation; ref. 20). The posttranslational regulatory mechanism of p53 involves mdm4 and mdm2 regulatory proteins. p53 function is positively controlled by mdm4, which does not drive p53 degradation (21). On the contrary, mdm2 targets p53 for nuclear export and proteasomal degradation. Therefore, mdm4 and mdm2 play antagonistic roles in controlling the cellular activity of p53 in the regulatory network.

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Requests for reprints: Sang Geon Kim, College of Pharmacy, Seoul National University, Sillim-dong, Kwanak-Gu, Seoul 151-742, Korea. Phone: 82-2880-7840; Fax: 82-2872-1795. E-mail: sgk@snu.ac.kr

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The association of activated mutation of $G\alpha_{12/13}$ with oncogenic potential might be linked with the deregulation of cell cycle control. Although many important findings on the networks downstream of cell surface receptors have provided signaling channels to the control of cell proliferation or survival, no information is available on the $G\alpha_{12/13}$-associated cell signaling pathways for the regulation of the p53-mdm4-mdm2 system. Information on the regulatory role of $G\alpha_{12/13}$ for these proteins would help us understand the central mechanism by which cell proliferation or survival is regulated. This study first investigated whether the absence of $G\alpha_{12/13}$ affected the basal expression of p53, and if so, whether the change in p53 level elicited by $G\alpha_{12/13}$ deficiency resulted from alterations in mdm4 or mdm2 expression. To identify the pathway of $G\alpha_{12/13}$ regulation of p53, we used mouse embryonic fibroblast (MEF) cells with targeted disruption of each gene, and MCF10A (human breast epithelial) cells transfected with their activated mutants (i.e., $G\alpha_{12}$QL and $G\alpha_{13}$QL). In this study, we found for the first time that $G\alpha_{12/13}$ regulates the basal level of p53 via mdm4 expression, but not that of mdm2. The possible regulatory relationship between p53 and mdm4 expression was assessed by protein overexpression and knockdown experiments. Finally, we examined the serine phosphorylations of p53, known to be responsible for apoptosis in response to DNA damage, and also evaluated the effects of genotoxic agents on mdm4 level.

Results

Down-Regulation of p53 by $G\alpha_{12}$ Deficiency

First, we investigated whether p53 expression and DNA binding activity were changed by targeted disruption of the *$G\alpha_{12}$* or *$G\alpha_{13}$* gene. To examine this, we measured the levels of p53 in lysates prepared from wild-type and knockout MEF cells; the basal level of p53 is relatively high in MEF cells (22). In wild-type MEF cells, p53 was well detected, as previously reported (22), and the levels of p53 in the lysates of these cells were identical to those of rhodopsin kinase–null (RK−/−) MEF cells (Fig. 1A). In this study, RK−/− cells were used as a knockout control in association with G protein because the physiologic function of RK was only restricted to phototransduction and was not relevant to the function of endogenous G protein in MEFs. Immunoblot analyses indicated that p53 was endogenously expressed in the RK−/− cells, but was weakly detected in the $G\alpha_{12}$−/− cells (Fig. 1A). $G\alpha_{13}$ deficiency moderately inhibited the level of p53. To further probe p53 expression, immunocytochemistry was done. p53 was detected mostly in the nuclei of wild-type or RK−/− MEF cells (Fig. 1B). It seemed that targeted disruption of the *$G\alpha_{12}$* gene decreased the staining intensity of p53 in the nuclei. Despite a decrease in p53 level in lysates due to $G\alpha_{13}$ deficiency, the immunocytochemical staining intensity of p53 was not much changed in $G\alpha_{13}$−/− cells. Next, we analyzed the interaction of p53 in nuclear extracts with the p53-binding oligonucleotide using gel shift assays. The deficiency of $G\alpha_{12}$ inhibited p53 DNA binding activity, whereas that of $G\alpha_{13}$ slightly decreased it (Fig. 1C). Competition experiments using excess quantities of unlabeled p53 or SP-1 binding oligonucleotide confirmed the specificity of p53 DNA binding.

Alterations in p53 Target or p53-Associated Proteins

To assess whether $G\alpha_{12/13}$ deficiency affected the expression of downstream target genes regulated by p53, we monitored the levels of p21, Bcl_2, and Bax in the MEF cells. The expression of p21, a protein that inhibits cell cycle progression, was repressed by the absence of $G\alpha_{12}$ or $G\alpha_{13}$. On the contrary, Bcl_2 was up-regulated by the loss of $G\alpha_{12}$ and, to a lesser extent, by that of $G\alpha_{13}$ (Fig. 2A). The expression of Bax, known to be up-regulated by p53 in certain cells (23), was unaffected by the deficiency of G proteins. Thus, the lack of $G\alpha_{12}$ or $G\alpha_{13}$ resulted in increases in the Bcl_2 to Bax ratio. Data presented here indicate that deficiency of $G\alpha_{12}$ or $G\alpha_{13}$, particularly $G\alpha_{12}$, alters p53 target protein levels.

In an effort to find the causal relationship of $G\alpha_{12}$ deficiency with low p53 levels, we examined the expression of the proteins that potentially regulate p53. The expression of PTEN and β-catenin, known to protect p53 from mdm2-mediated degradation (24, 25), was unchanged in the cells (Fig. 2B), suggesting that low p53 content might not be associated with their expression. Activated ATM and Chk2 directly phosphorylate p53 in response to genotoxic stress (26, 27). As a continuing effort to identify the proteins that regulate p53 during $G\alpha_{12}$ or $G\alpha_{13}$ deficiency, ATM activity and p-Thr^{68}Chk2 levels were determined in the cell lysates. The absence of $G\alpha_{12}$ or $G\alpha_{13}$ minimally changed ATM kinase activity and Chk2 phosphorylation, indicating that the $G\alpha_{12}$ pathway might not regulate the kinases that phosphorylate p53.

The ability of p53 to exert regulatory functions largely depends on its phosphorylations mediated by cellular mitogen-activated protein kinases (28). Given the report that ERK1/2 acts as an upstream regulator of p53 during DNA damage response (29), we asked if MEK-ERK1/2 was responsible for the alterations in p53 level. Because the loss of $G\alpha_{12}$ decreased p53 levels to a greater extent than that of $G\alpha_{13}$, we primarily used $G\alpha_{12}$−/− cells in subsequent experiments. Treatment of $G\alpha_{12}$−/− cells with U0126 or PD98059 (MEK inhibitors), failed to reverse p53 down-regulation, providing evidence that ERK might not be associated with p53 suppression (Fig. 2C, *top*). Studies have shown that activation of phosphatidylinositol-3-kinase (PI3K) or Akt promotes the nuclear entry of mdm2 and then targets p53 for proteasomal degradation (30, 31). To determine whether PI3K-Akt activity contributes to p53 degradation during the absence of $G\alpha_{12}$, we evaluated p53 expression in $G\alpha_{12}$−/− cells treated with LY294002 or wortmannin. Chemical inhibition of Akt phosphorylation failed to restore p53 content (Fig. 2C, *bottom*). Our data indicate that the pathway of ERK1/2 or Akt is unlikely to regulate p53 in the cells.

p53 Accumulation by Proteasomal Inhibition

Subsequently, we determined whether the decrease in p53 due to the absence of $G\alpha_{12}$ or $G\alpha_{13}$ resulted from transcriptional inhibition. Semiquantitative reverse transcription-PCR (RT-PCR) analysis revealed no decrease in p53 mRNA levels in the $G\alpha_{12}$−/− or $G\alpha_{13}$−/− cells compared with controls (Fig. 3A). Data suggested that p53 down-regulation due to the deficiency of $G\alpha_{12}$ or $G\alpha_{13}$ might not have resulted from either transcriptional repression or changes in the mRNA stability.

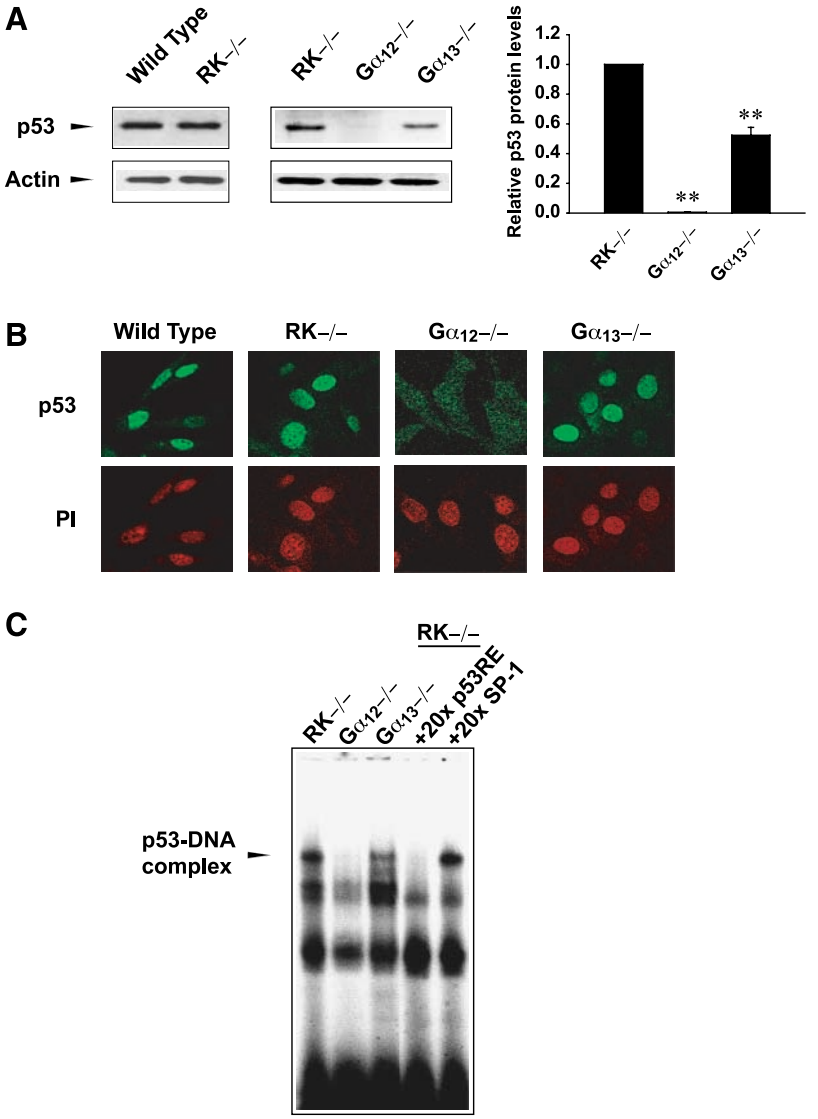

FIGURE 1. Down-regulation of p53 protein by $G\alpha_{12}$ deficiency. **A.** Immunoblot of p53. Representative immunoblot analyses show the levels of p53 in the lysates prepared from MEF cells. Each lane was loaded with 10 μg of proteins. Equal loading of proteins was verified by probing the replicate blots for actin. Columns, means of three separate experiments; bars, SE (significant compared with RK−/−; **, $P < 0.01$). **B.** Immunocytochemistry of p53. p53 was immunochemically localized using anti-p53 antibody in the cells. The same fields were counterstained with propidium iodide to verify the location and integrity of nuclei. **C.** p53 DNA binding activity. Gel shift analyses were done with the p53-binding oligonucleotide (*p53RE*) and nuclear extracts prepared from the cells. The specificity of p53 DNA binding was determined by the competition experiment using unlabeled p53 or SP-1 binding oligonucleotides. Results were confirmed by repeated experiments.

p53 was exported to the cytoplasm after ubiquitination and subjected to proteasomal degradation (32). Under normal conditions, p53 is kept at low levels by ubiquitin-mediated continuous degradation. Because low p53 content following the loss of $G\alpha_{12}$ was not due to transcriptional inactivation, we tested the possibility that the decrease in p53 resulted from a change in protein stability (but not the inability to synthesize p53). We examined whether exposure of the cells to 26S proteasome inhibitors allowed p53 levels to recover. p53 accumulated in the lysates of RK−/− cells treated with MG132 (30 min-1 h), declining from the maximum at later times (Fig. 3B, *left*). We confirmed that the same changes occurred in wild-type cells (data not shown). In $G\alpha_{12}$−/− cells, immunoreactive p53 content slowly elevated after MG132 treatment, peaked at 6 to 12 h, and then decreased from the maximum at 24 h. Slow accumulation of p53 in the cells might be due to the low basal protein content. MG132 treatment also promoted p53 accumulation in $G\alpha_{13}$−/− cells. The weak intensities at 12 to 24 h after MG132 treatment might be associated with its biphasic effects (i.e., increase in proteasomal activity because of low concentrations of MG132; ref. 33). Similar time courses in p53 accumulation were observed in those treated with lactacystin (Fig. 3B, *right*). Immunocytochemistry confirmed a clear increase in p53 fluorescence intensity after the treatment of $G\alpha_{12}$−/− cells with MG132 (Fig. 3C).

p53 Levels in $G\alpha_{12/13}$ Double-Knockout Cells and the Effect of MG132

$G\alpha_{12}$ and $G\alpha_{13}$ may exert differential functions in cell signaling. We next asked if $G\alpha_{12}$ and $G\alpha_{13}$ redundantly or antagonistically cross-talk for p53 expression. To answer this question, we measured the basal p53 expression and the levels of the proteins regulated by p53 in $G\alpha_{12/13}$ double-knockout ($G\alpha_{12/13}$−/−) cells (Fig. 4A). p53 was repressed by the deficiency of both $G\alpha_{12}$ and $G\alpha_{13}$, indicating that the two proteins might not act against each other. In parallel with a decrease in p53 content, p21 expression was inhibited in the $G\alpha_{12/13}$−/− cells, whereas Bcl_2 was up-regulated. p53

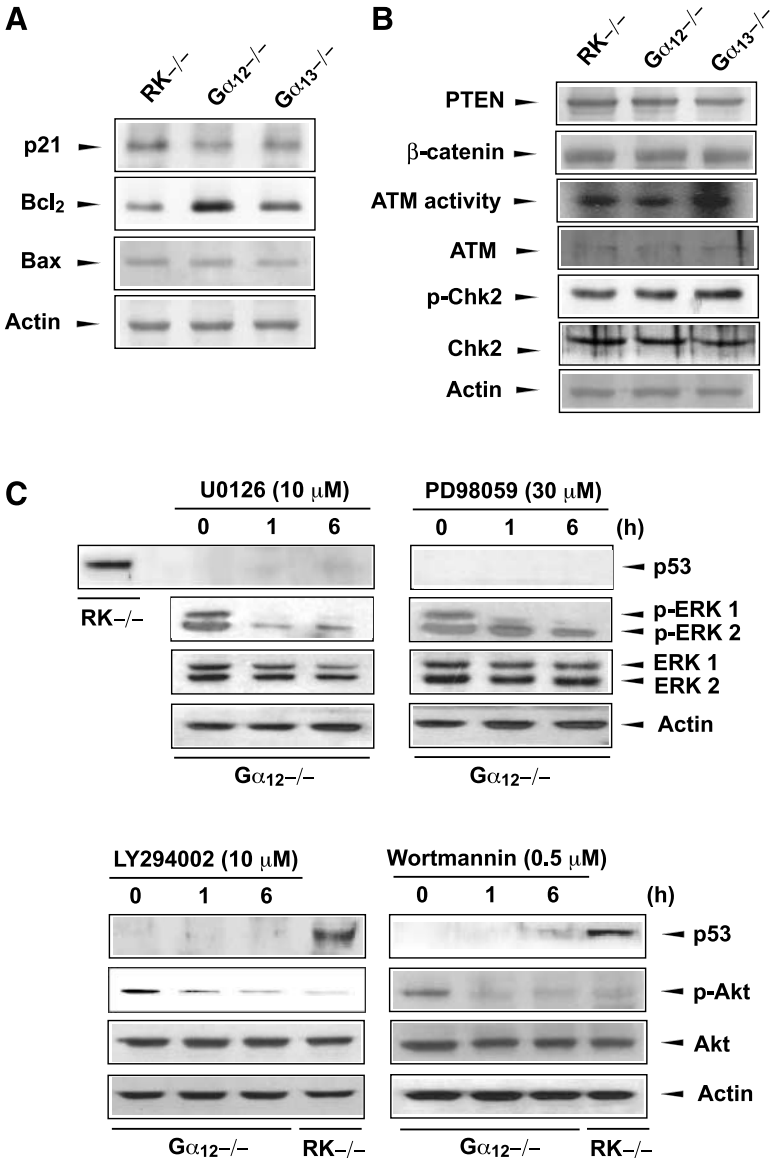

FIGURE 2. The effect of $G\alpha_{12}$ or $G\alpha_{13}$ deficiency on the expression of the proteins regulated by p53. **A.** The expression of p53 target gene products. p21, Bcl_2, and Bax, the gene transcription of which was regulated by p53, were immunoblotted in cell lysates. Each lane contained 20 μg of lysate proteins. Equal loading of proteins was verified by probing the replicate blots for actin. **B.** Immunoblot analyses. The levels of PTEN, β-catenin, and p-Thr^{68}Chk2 were determined in cell lysates as the proteins that potentially regulate p53 expression. ATM immunocomplex kinase assays were carried out by monitoring PHAS-1 phosphorylation in ATM immunoprecipitates. **C.** The role of ERK1/2 or PI3K for the low p53 protein level by $G\alpha_{12}$ or $G\alpha_{13}$ deficiency. Immunoblot analyses were done in the lysates of cells treated with vehicle, MEK1, or PI3K inhibitor. Results were confirmed by repeated experiments.

gradually accumulated in the cells after MG132 treatment (Fig. 4B), suggesting that *de novo* synthesis of p53 was intact in the cells and that the G proteins have overlapping specificities.

Regulation of Mdm4 by $G\alpha_{12/13}$

Mdm4 is a p53-binding protein that antagonizes the activity of mdm2, stabilizing p53 (34, 35). To test whether the low levels of p53 following $G\alpha_{12}$ loss resulted from the inhibition of mdm4, we examined mdm4 levels in the cells. Mdm4 levels were decreased notably by the deficiency of $G\alpha_{12}$, and moderately by that of $G\alpha_{13}$, compared with controls (Fig. 5A, *top*). Loss of both $G\alpha_{12}$ and $G\alpha_{13}$ further repressed mdm4 as well as p53 levels (data not shown). We found that the expression of mdm2, which targets p53 for degradation by the ubiquitin-dependent pathway (36), was unaffected by $G\alpha_{12}$ or $G\alpha_{13}$ deficiency. Semiquantitative RT-PCR analyses indicated no changes in the mdm4 mRNA levels during the absence of $G\alpha_{12}$ or $G\alpha_{13}$, suggesting that the G proteins posttranscriptionally regulate mdm4. Studies have shown that p53 regulates mdm4 expression in certain cells (37). To exclude the possibility that the lack of p53 causes mdm4 repression, we determined mdm4 content in RK−/− or $G\alpha_{12}$−/− cells infected with a p53-encoding adenoviral vector. Mdm4 was not induced by p53 overexpression in either RK−/− or $G\alpha_{12}$−/− cells (Fig. 5A, *bottom*).

To verify the role of $G\alpha_{12}$ or $G\alpha_{13}$ in mdm4 expression in another cell line, we established the clones of MCF10A cells stably transfected with a $G\alpha_{12}$QL or a $G\alpha_{13}$QL construct. As anticipated, mdm4 was induced by the stable expression of $G\alpha_{12}$QL or $G\alpha_{13}$QL (Fig. 5B). Consistently, $G\alpha_{12}$QL or $G\alpha_{13}$QL promoted p53 expression. Mdm2 expression levels were unchanged in the cells. To confirm the stabilizing effect of mdm4 on p53, p53 levels were assessed in MCF10A cells transfected with the plasmid encoding mdm4. Figure 5C shows that ectopic expression of mdm4 enhanced p53 level in the

cells. In the mdm4-overexpressed MCF10A cells, mdm2 levels were slightly decreased, which is consistent with the previous observation (38). We then carried out additional experiments using small interfering RNAs (siRNA) directed against human $G\alpha_{12/13}$. Preliminary studies showed that siRNA knockdown of either $G\alpha_{12}$ or $G\alpha_{13}$ alone was insufficient to alter mdm4 or p53 levels presumably due to cross-talk of the G proteins. Therefore, we used both siRNAs simultaneously. Knockdown of both $G\alpha_{12}$ and $G\alpha_{13}$ in MCF10A cells stably transfected with $G\alpha_{12}$QL or $G\alpha_{13}$QL decreased mdm4 content (Fig. 5D), corroborating the role of $G\alpha_{12}$ members in mdm4 regulation. In parallel with this, we observed decreases in p53 content. Exposure of RK−/− cells to the siRNA against $G\alpha_{12/13}$ resulted in a similar repressing effect. Collectively, the observations presented here provide evidence that $G\alpha_{12}$ members regulate p53, which may result from changes in the level of mdm4.

p53 Induction by Genotoxic Stress

Genotoxic stress activates p53 (18). To differentiate the $G\alpha_{12/13}$-mediated p53 regulatory pathway from p53 activation by genotoxicants, the effect of doxorubicin treatment on p53 expression was monitored in wild-type and knockout MEF cells. As expected, doxorubicin treatment (1 μmol/L) increased p53 expression in wild-type or RK−/− cells (Fig. 6A). Immunoblot analyses also indicated that the levels of mdm2, but not mdm4, were enhanced by doxorubicin, which paralleled those of p53. In $G\alpha_{12}$−/− or $G\alpha_{13}$−/− cells, p53 and mdm2 were also induced by anticancer agents. Mdm4 levels were not affected by the deficiency of $G\alpha_{12}$ or $G\alpha_{13}$. We next examined whether p53 binding to mdm4 or mdm2 was altered by the absence of $G\alpha_{12}$ or $G\alpha_{13}$. The association of p53 with mdm4 was notably decreased by the deficiency of $G\alpha_{12}$, moderately by that of $G\alpha_{13}$, whereas the interaction between p53 and mdm2 was rather slightly enhanced by the lack of $G\alpha_{12}$ (Fig. 6B, *left*). Our data showing the decrease in p53 binding to mdm4 with its increased affinity to mdm2 was consistent with the concept that the loss of $G\alpha_{12}$ decreased p53 stability. When RK−/− cells were treated with doxorubicin, the association of p53 with mdm4 seemed to be decreased, as previously reported (39). In $G\alpha_{12}$−/− cells, p53 binding to mdm2 seemed to be decreased by doxorubicin treatment (Fig. 6B, *right*).

To analyze the functional aspect of p53 induction by genotoxic agents, we measured the fractions of pre-G_1 phase cells using flow cytometric analysis. As expected, cells in the sub-G_1 phase were accumulated by doxorubicin treatment in $G\alpha_{12}$−/− or $G\alpha_{12/13}$−/− cells as well as RK−/− cells

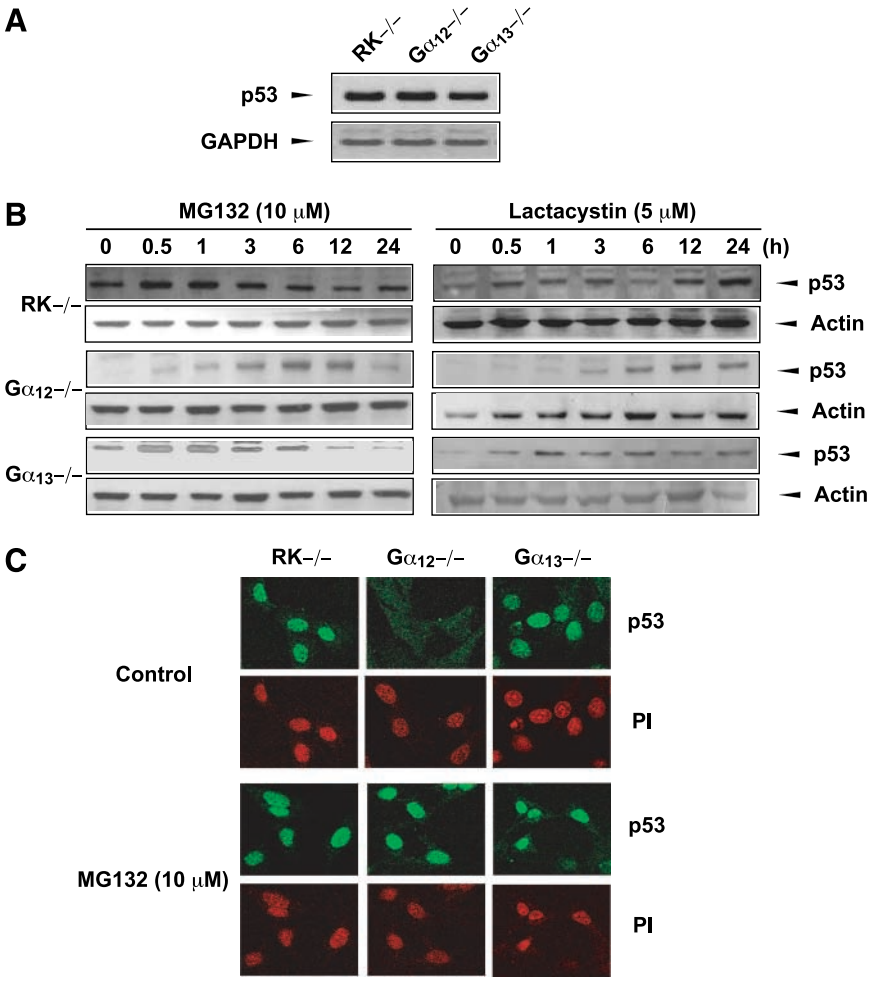

FIGURE 3. p53 accumulation by proteasomal inhibition. **A.** Representative RT-PCR analysis of p53 mRNA. The effect of $G\alpha_{12}$ or $G\alpha_{13}$ deficiency on p53 mRNA transcripts was assessed by semiquantitative RT-PCR analysis. GAPDH mRNA was measured as loading control. **B.** Immunoblot analyses of p53. p53 was immunoblotted in the lysates of cells treated with MG132 (10 μmol/L) or lactacystin (5 μmol/L) for 0.5 to 24 h. Each lane contained 10 μg of proteins. **C.** Immunocytochemistry of p53. Cells treated with vehicle or MG132 (10 μmol/L, 12 h) were incubated with anti-p53 antibody (1:100, for 1 h) and further treated with FITC-conjugated goat anti-rabbit IgG. The same fields were counterstained with propidium iodide to locate nuclei. Results were confirmed by repeated experiments.

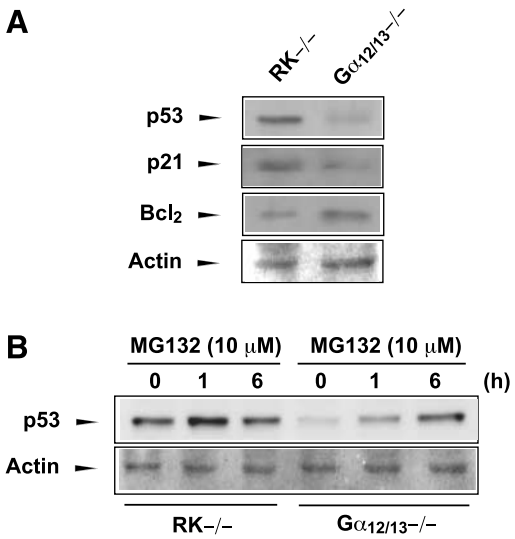

FIGURE 4. The effects of dual deficiency of $G\alpha_{12/13}$ on p53 and its target protein expression. **A.** The expression of p53 and p53 target proteins. Immunoblots, levels of p53, p21, and Bcl_2. Each lane was loaded with 10 μg of lysate proteins. **B.** Accumulation of p53 by MG132 treatment. p53 was immunoblotted in the lysates of cells treated with vehicle or MG132. Equal loading of proteins was verified by probing the replicate blots for actin. The results were confirmed by repeated experiments.

(Fig. 7A, *left*). These cells also exhibited susceptibility to etoposide (Fig. 7A, *right*). Previous studies have shown that p53 stability was enhanced by specific phosphorylations of p53 at serine residues, which inhibited mdm2-mediated p53 degradation via changes in the protein conformation (40). Next, we sought to determine p53 phosphorylations at the residues of Ser^{15}, Ser^{392}, or Ser^{20} known to be responsible for the protein stability (40-42). Phosphorylation at Ser^{15} was enhanced by doxorubicin or etoposide treatment in RK−/− cells (Fig. 7B). Interestingly, DNA damage greatly stimulated p53 phosphorylation at Ser^{15} in the $G\alpha_{12}$−/− or $G\alpha_{12/13}$−/− cells. Similarly, specific phosphorylation of p53 at Ser^{392} was increased by the agents in these cells, whereas Ser^{20} phosphorylation of p53 was unaffected (Fig. 7B). Therefore, it is highly likely that increases in the level of phosphorylated p53 caused by genotoxic agents lead to apoptosis apart from $G\alpha_{12}$ or $G\alpha_{13}$ deficiency. We then verified the effect of etoposide treatment on mdm4 expression in these cells. In spite of p53 phosphorylations, mdm4 expression, which was much lower in $G\alpha_{12}$−/− or $G\alpha_{12/13}$−/− cells than in RK−/− cells, was not promoted by etoposide (Fig. 7C). Our data support the notion that genotoxic stress promotes apoptosis, and also increases specific phosphorylations of p53, which leads to its accumulation presumably as a consequence of the inhibition of mdm2-dependent p53 degradation (40). Because doxorubicin treatment increased p53 phosphorylations in all of the MEF cells, we further examined the phosphorylation status of Chk2, a p53-phosphorylating kinase downstream of ATM, in doxorubicin-treated cells. Doxorubicin treatment caused increases in Chk2 phosphorylation in all of the MEF cells (Fig. 7D), which supports the contention that genotoxic stress activates the pathway responsible for p53 phosphorylations apart from $G\alpha_{12}$ or $G\alpha_{13}$ regulation.

Given the mdm4-mediated p53 induction caused by activated mutants of $G\alpha_{12}$ or $G\alpha_{13}$, we finally determined the effects of $G\alpha_{12}$QL and $G\alpha_{13}$QL on p53 phosphorylations at serines. Stable transfection of MCF10A cells with $G\alpha_{12}$QL or $G\alpha_{13}$QL did not induce p53 phosphorylations at Ser^{15} or Ser^{392} (Fig. 7E), identifying the aspect that the signaling pathway of $G\alpha_{12/13}$ for p53 regulation is distinct from p53 activation by genotoxic agents (Fig. 8).

Discussion

G protein–coupled receptors (GPCR) transmit signals through conformational changes on ligand activation and interaction with heterotrimeric G proteins. GPCR activation initiates intracellular signaling responses by the exchange of GDP for GTP bound to the Gα subunit and its dissociation from the βγ heterodimers. PI3K and Akt lie at the crossroads of GPCR and other membrane receptor signaling pathways in response to a variety of extracellular stimulation. Thus, Gα members function as the molecular switches between the effectors of GPCRs and the cell survival pathway. Among Gα family members that serve as the effector molecules of GPCRs, $G\alpha_{12/13}$ may exert distinct and/or overlapping cell signal pathways.

Studies have shown that $G\alpha_{12}$, also referred to as the *gep* oncogene, might be involved in the mitogenic pathways in multiple cell types, inducing the neoplastic transformation of cells (e.g., fibroblast cells; refs. 11, 43). This hypothesis is supported by the observation that $G\alpha_{12}$ regulates lysophosphatidic acid–mediated mitogenic pathways (44). $G\alpha_{12}$ members regulate the activities of small Rho, which may participate in the regulation of cell differentiation (45), cell cycle progression (46), and malignant transformation. Because activated mutants of $G\alpha_{12}$ or $G\alpha_{13}$ are involved in oncogenic transformation (4, 10-12), we intended to unveil the physiologic role of $G\alpha_{12/13}$ in the expression of cell cycle–associated proteins, which would be of assistance in understanding GPCR-mediated signaling for cell proliferation, survival, or apoptosis. Our results presented in this study identify the regulatory pathway coupled with $G\alpha_{12/13}$ for p53 stabilization, which may account in part for the $G\alpha_{12}$-dependent alterations in the cell physiology. Our finding showing the role of $G\alpha_{12}$ or $G\alpha_{13}$ in p53 and its associated proteins was functionally supported by alterations in the expression of p53 target proteins such as p21, a representative inhibitory protein in cell cycle progression (47). The expression of Bcl_2, a signal representing cell survival (48), was reciprocally up-regulated as p53 levels decreased because of the loss of $G\alpha_{12}$. The observations that the deficiency of $G\alpha_{13}$, unlike that of $G\alpha_{12}$, only partially inhibited p21 as well as p53 suggest that $G\alpha_{13}$ exerts some overlapping specificity with $G\alpha_{12}$ in regulating p53. The comparable change of Bcl_2 expression in $G\alpha_{13}$−/− cells further confirmed the partial effect of $G\alpha_{13}$ and its overlapping function with $G\alpha_{12}$.

It has been reported that ERK activation by cisplatin, a cancer chemotherapeutic agent, caused p53 accumulation, implying that p53 might act as a potential target of ERK (29). The current finding that chemical inhibition of ERK activity did not change p53 expression provides evidence that the pathway of $G\alpha_{12}$ regulates p53 irrespective of MEK1-ERK

activity. Also, we initially anticipated that PI3K-Akt activation might lead to p53 down-regulation because the pathway phosphorylates key intermediate signaling molecules for cell metabolism, growth, and survival. Our observation, showing that inhibition of the PI3K-Akt pathway failed to restore p53 levels during the absence of $G\alpha_{12}$, supports no functional role in the pathway for the decrease of p53 content.

PTEN antagonizes PI3K-Akt signaling. Sensitization of tumor cells to chemotherapeutic agents by PTEN depends on p53 function, which in turn, may induce the *PTEN* gene (18, 25). PTEN may protect p53 from mdm2-mediated degradation (49). The absence of any changes in PTEN expression due to $G\alpha_{12}$ deficiency (shown in this study) may reflect the compensatory adaptation of the cells for survival in the absence of $G\alpha_{12}$. Lysophosphatidic acid receptor activation stimulates cell proliferation through the β-catenin–mediated pathway, and thus, deregulation in β-catenin may lead to p53-dependent growth arrest (25, 50). Studies from other laboratories have shown that $G\alpha_{12/13}$ specifically interact with the cytoplasmic domains of the cadherin family members, which comprise β-catenin (51, 52). The present demonstration that β-catenin levels were unaffected by $G\alpha_{12}$ deficiency rules out the possibility that p53 inhibition by the loss of $G\alpha_{12}$ results from β-catenin repression. Nevertheless, we could not exclude the aspect that the low levels of p53 might be associated with the lack of β-catenin interaction with $G\alpha_{12}$. In addition, our data, showing the absence of $G\alpha_{12}$ or $G\alpha_{13}$, did not affect ATM kinase activity, whereas Chk2 phosphorylation rules out the possibility that the G proteins regulated the upstream kinases of p53.

We found that the p53 mRNA levels were unchanged by $G\alpha_{12}$ or $G\alpha_{13}$ deficiency, suggesting that the decreased content of p53 due to the absence of the G proteins could be attributable to protein turnover but not transcriptional inhibition or mRNA instability. Furthermore, *de novo* p53 synthesis in the absence of $G\alpha_{12}$ was evidenced by gradual accumulation of p53 after proteasomal inhibition. Differences in the time courses for p53 accumulation among the MEF cells rendered us to propose the hypothesis that $G\alpha_{12/13}$ regulates p53 stability. Delayed accumulation of p53 by MG132 in $G\alpha_{12}-/-$ cells might have reflected alterations in p53 stability and degradation. Our findings showing that dual deficiency of $G\alpha_{12/13}$ repressed both p53 and p21 to a greater extent with reciprocal induction of Bcl_2, and that proteasomal inhibition caused p53 accumulation

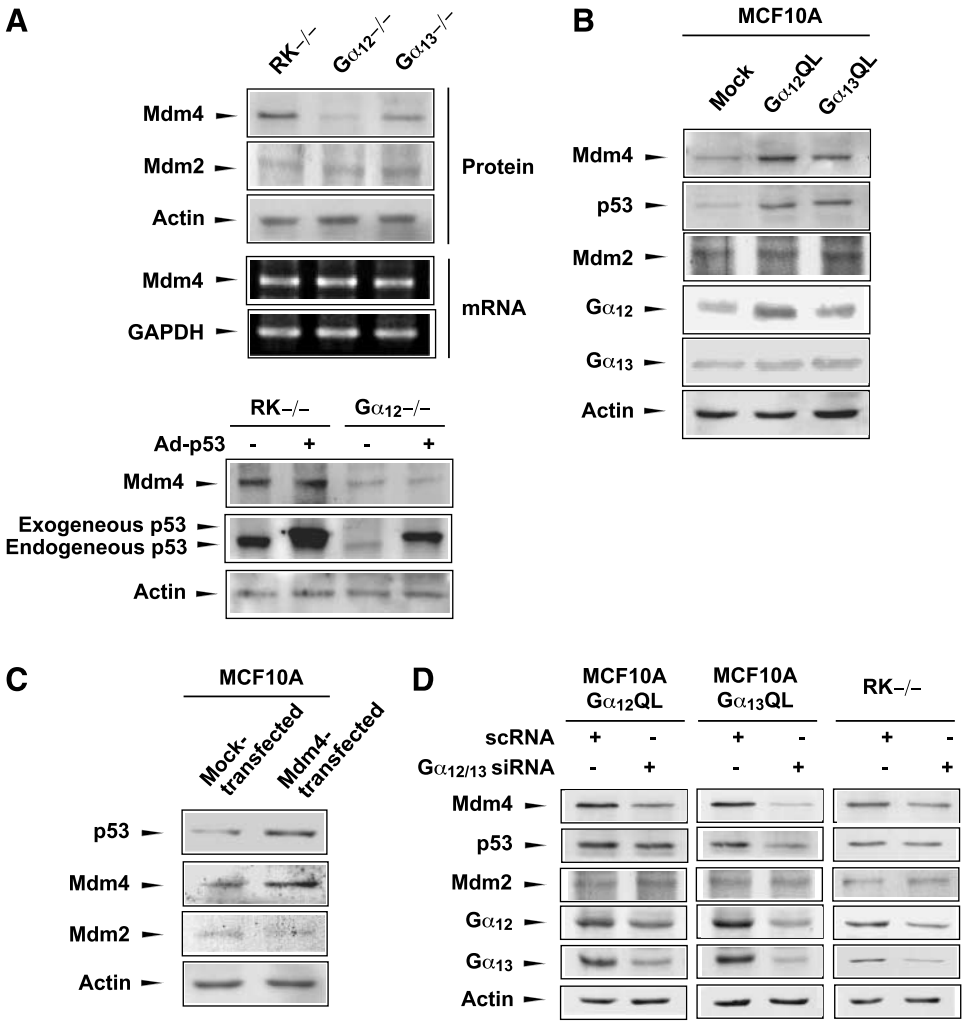

FIGURE 5. Mdm4-mediated p53 expression downstream of $G\alpha_{12/13}$. **A.** Mdm4 repression by $G\alpha_{12}$ or $G\alpha_{13}$ deficiency. Mdm4 and mdm2 proteins were immunoblotted in the lysates of untreated MEF cells. The mdm4 and GAPDH mRNA levels were determined by semiquantitative RT-PCR analyses (*top*). Mdm4 was immunoblotted in the lysates of MEF cells infected with the adenoviral construct (Ad-p53) comprising the region encoding for p53 (*bottom*). **B.** Induction of mdm4 and p53 by activated mutants of $G\alpha_{12}$ or $G\alpha_{13}$ ($G\alpha_{12}$QL or $G\alpha_{13}$QL). p53, mdm4, and mdm2 levels were assessed in MCF10A cells stably transfected with the plasmid encoding $G\alpha_{12}$QL or $G\alpha_{13}$QL. **C.** p53 induction by mdm4. p53 was immunoblotted in the lysates of MCF10A cells transfected with the plasmid encoding mdm4. Mdm4 overexpression was confirmed by immunoblottings. **D.** Mdm4 repression by siRNA knockdown. MCF10A cells stably transfected with $G\alpha_{12}$QL or $G\alpha_{13}$QL were transfected with siRNAs directed against human $G\alpha_{12/13}$ or scRNA (RNA with scrambled sequences), incubated for 24 h, and then subjected to immunoblot analysis for mdm4. Knockdown of $G\alpha_{12/13}$ was confirmed by immunoblotting. The results were confirmed by at least two repeated experiments.

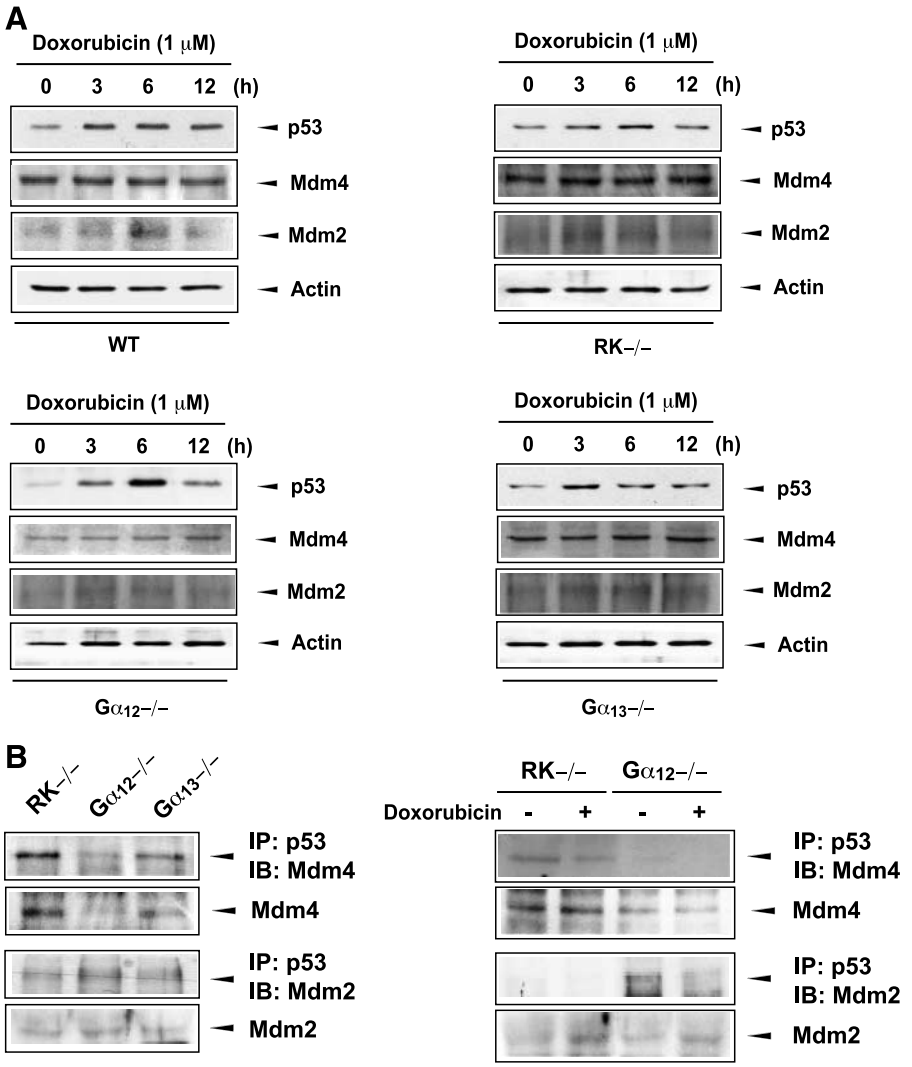

FIGURE 6. p53 induction by doxorubicin and p53 association with mdm4/mdm2. **A.** The effects of doxorubicin on p53, mdm4, and mdm2. Cells grown in the medium containing 10% fetal bovine serum for 24 h were exposed to doxorubicin (1 μmol/L) for 3 to 12 h. Immunoblot analyses for p53, mdm4, and mdm2 were conducted in cell lysates. Each lane was loaded with 10 μg of proteins. The results were confirmed by repeated experiments. **B.** Association of p53 with mdm4 or mdm2. Mdm4 or mdm2 levels were immunoblotted in p53 immunoprecipitates prepared from the cell lysates (*left*). p53 binding to mdm4 or mdm2 was also examined in RK−/− or $G\alpha_{12}$−/− cells treated with 1 μmol/L of doxorubicin for 3 h (*right*).

during $G\alpha_{12/13}$ deficiency, supporting the contention of their posttranslational p53 regulation, and excluding the possibility that the G proteins antagonistically regulate p53. Mdm2 targets p53 for degradation by the proteasomal system (40). Thus, mdm2 sequestration decreases p53 stabilization. It has been shown that mdm2 binds and ubiquitinylates p53 in the nucleus and that mdm2 binding to ubiquitinylated p53 unmasks p53 NES, resulting in the export of p53 from the nucleus for degradation (40). p53 degradation is then activated in the cytoplasm (53). These aspects, along with our data showing the nuclear accumulation of p53 by MG132 in $G\alpha_{12}$−/− cells, render us to speculate that the accumulated p53 might be functionally active with its NLS moiety being intact. Our observations that the expression of mdm2, a transcriptional target of p53, was unchanged by $G\alpha_{12}$ deficiency also supported the possibility that a decrease in p53 stability was attributable to other molecules which control an autoregulatory p53 feedback loop.

Mdm4, the biological function of which is antagonistic to mdm2, binds to p53 (34). Mdm4 present within the nucleus heterodimerizes with mdm2 through their ring finger domains (54), inhibiting p53 degradation. Hence, mdm4 is an important target for p53 regulation. A significant aspect of the present finding is the identification of mdm4 repression by $G\alpha_{12}$ deficiency, and to a lesser degree, by $G\alpha_{13}$ deficiency. Decreases in mdm4 level due to the absence of $G\alpha_{12}$ or $G\alpha_{13}$ correlated well with p53 down-regulation, which is also in line with the current finding that the association of p53 with mdm4 was decreased by $G\alpha_{12}$ deficiency, whereas that with mdm2 was increased.

In the present study, the absence of alterations in mdm2 expression supports the notion that the low p53 content was due to the level of mdm4, but not mdm2. Regulation of the p53-mdm4 system downstream of $G\alpha_{12/13}$ was consistent with our experimental results showing that $G\alpha_{12/13}$ deficiency did not change p53 mRNA levels. A previous report that mdm4 overexpression enhanced p53 stabilization and apoptosis (35) parallels our contention that mdm4 repression due to $G\alpha_{12/13}$ deficiency causes p53 inhibition. The identification of mdm4 as a target regulated by $G\alpha_{12/13}$ was further supported by reversal experiments showing that transfection of the $G\alpha_{12}$−/− cells with $G\alpha_{12}$QL or $G\alpha_{13}$QL restored the levels of mdm4 as well

as p53. In addition, the ectopic expression of mdm4 resulted in the accumulation of p53 in MCF10A cells, whereas that of p53 did not induce mdm4. The essential role of $G\alpha_{12}$ or $G\alpha_{13}$ in mdm4 regulation was also confirmed by our siRNA knockdown experiments, in which we had to use the siRNAs degrading both $G\alpha_{12}$ and $G\alpha_{13}$ simultaneously due to the overlapping specificities of $G\alpha_{12}$ and $G\alpha_{13}$ in mdm4 regulation. The decrease in p53 levels following siRNA knockdown was not very distinct, presumably because the inhibitory effect of the siRNAs for mdm4 was partial. Taken together, the results shown here provide strong evidence that $G\alpha_{12/13}$ posttranslationally regulates the basal level of p53 and that the p53 regulation by $G\alpha_{12/13}$ was mediated with mdm4. Accumulation of p53 by proteasomal inhibition, despite the lack of mdm4 during the absence of $G\alpha_{12/13}$, also supports our identification of $G\alpha_{12/13}$-mdm4–mediated p53 regulation.

Our observation that the mdm4 mRNA levels were comparable among the MEF cells supports the notion that

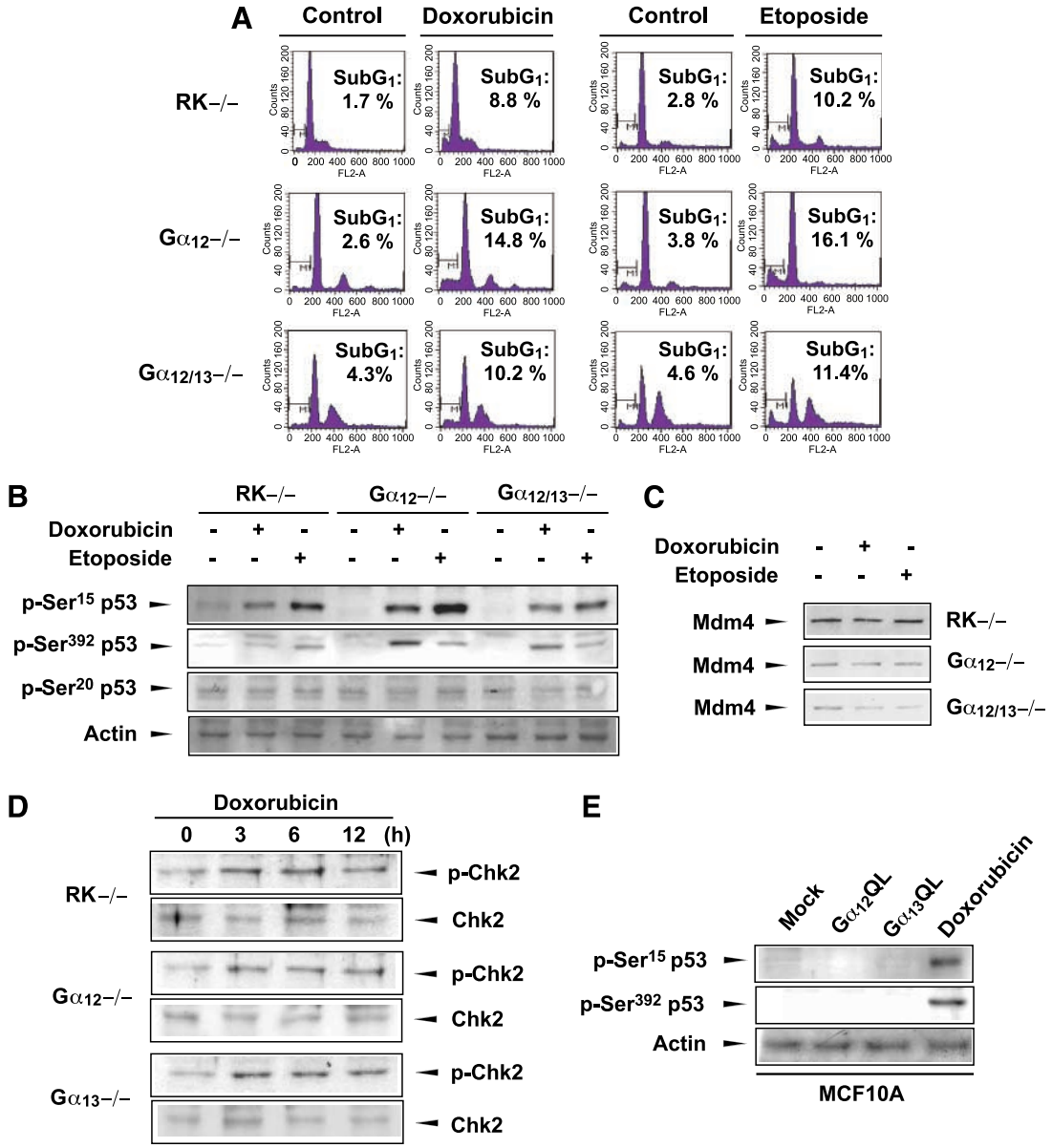

FIGURE 7. Functional aspect of p53 induction by genotoxic agents. **A.** Fluorescence-activated cell sorting analyses. MEF cells were treated with 1 μmol/L of doxorubicin for 24 h or 500 μmol/L of etoposide for 10 h. In floating and trypsin-digested cells, the percentage of cell numbers in the sub-G_1 phase was monitored by the flow cytometric analysis of propidium iodide–stained DNA. Flow cytometric histograms, in which the nuclear DNA content (*X*-axis) is plotted against the number of nuclei, indicate the percentages of cell numbers in the sub-G_1 phase of cells. Results were confirmed at least by three separate experiments. **B.** p53 phosphorylations. Immunoblot analyses were conducted in the lysates of cells treated with 1 μmol/L of doxorubicin for 24 h or 500 μmol/L of etoposide for 6 h. Each lane was loaded with 10 μg of proteins. **C.** Mdm4 expression. The cells treated as described in **B** were subjected to immunoblot analyses for mdm4. **D.** Chk2 phosphorylations. The MEF cells treated with 1 μmol/L of doxorubicin for 3 to 12 h were immunoblotted for phosphorylated Chk2 (p-Chk2 at Thr^{68}). **E.** The effect of $G\alpha_{12}$QL or $G\alpha_{13}$QL on the levels of p53 phosphorylated at Ser^{15} or Ser^{392} in MCF10A cells. Immunoblot analyses were conducted in the lysates of MCF10A cells stably transfected with $G\alpha_{12}$QL or $G\alpha_{13}$QL. The results were confirmed by repeated experiments.

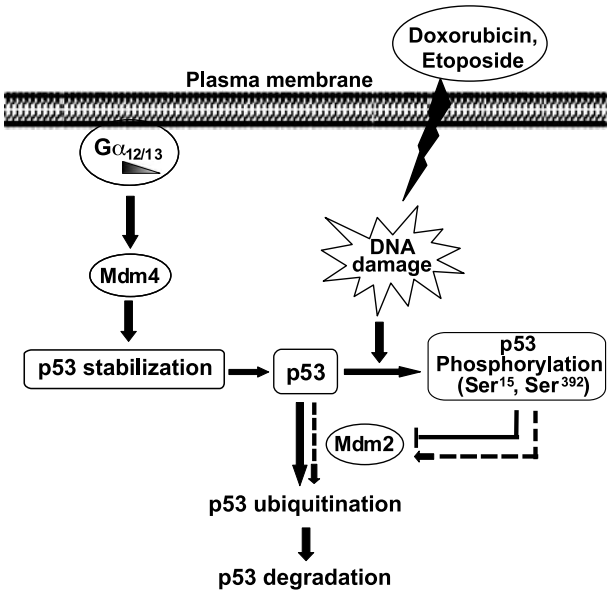

FIGURE 8. A schematic diagram illustrating the possible mechanism by which $G\alpha_{12/13}$ regulates the basal level of p53. $G\alpha_{12}$ members regulate p53 level via mdm4, whereas genotoxic agents activate p53 via its serine phosphorylations and thereby accumulate p53 through the inhibition of mdm2 (*solid lines*). Also, phosphorylated p53 transcriptionally activates mdm2 and thereby enhances mdm2 levels, leading to the feedback regulation of p53 (*dashed lines*).

$G\alpha_{12/13}$ posttranslationally regulates mdm4. Recently, we reported that $G\alpha_{12}$ deficiency does not allow JNK to be activated in response to S1P, and consequently decreases IκBα ubiquitination (55). In an additional preliminary experiment, we observed that etoposide treatment failed to activate JNK during $G\alpha_{12}$ deficiency (S.H. Ki and S.G. Kim, unpublished data). The lack of JNK activation during the absence of $G\alpha_{12/13}$ would decrease JNK-dependent protein ubiquitination, as observed in the case of nuclear factor κB. Alterations in the p53-mdm4-mdm2 system during $G\alpha_{12/13}$ deficiency may be indirectly associated with altered JNK activity, which needs to be studied in the future. In another preliminary experiment, we found that $G\alpha_{12}$ and $G\alpha_{13}$ deficiency decreased proteasomal activities by 61% and 28%, respectively, compared with controls (S.J. Lee and S.G. Kim, unpublished data). Changes in the proteasomal activities during $G\alpha_{12/13}$ deficiency may account for the mechanism by which $G\alpha_{12/13}$ pathway regulates mdm4-p53 expression, which remains to be established.

To differentiate the signal initiated by genotoxic stress from that of $G\alpha_{12/13}$, we determined the activation of p53 in response to cancer chemotherapeutic agents and its functional relevance to apoptosis. Genotoxic stress increased p53 phosphorylations at the residues of Ser^{15} and Ser^{392} for p53 up-regulation, presumably as a consequence of mdm2 inhibition (40). Our data verifies the phosphorylation and activation of p53, and the activation of ATM-Chk2 signaling, a kinase responsible for p53 phosphorylation, by genotoxic agents in the knockout cells. The results presented here show that mdm4 regulation might not be the preferential factor responding to DNA damage for p53 regulation. The lack of mdm4 induction by genotoxic stress during the absence of $G\alpha_{12/13}$ supports the hypothesis that the p53 regulatory pathway activated by genotoxic stress differs from that controlled by $G\alpha_{12/13}$ (Fig. 8). p53 and Chk2 phosphorylations by genotoxic agents functionally correlated with cell apoptosis, as shown by the increase in pre-G_1 phase fraction. In particular, increases in p53 Ser^{15} phosphorylation in $G\alpha_{12}-/-$ cells matched with its higher fraction of pre-G_1 phase. Our finding that Ser^{15} and Ser^{392} phosphorylations of p53 were not observed in MCF10A cells stably transfected with $G\alpha_{12}$QL or $G\alpha_{13}$QL lends support to the conclusion that the $G\alpha_{12}$ regulatory mechanism of p53 does not involve the serine phosphorylations, whereas genotoxic stress activates p53 independent of the pathway involving $G\alpha_{12}$ or mdm4. The proposed aspect is consistent with the earlier observations that serine phosphorylations of p53 change p53 conformation and thus increase p53 stability by inhibiting mdm2-mediated p53 degradation (20, 40, 56).

In summary, our results show that $G\alpha_{12/13}$ regulate the signaling pathway for the expression of mdm4, which stabilizes p53 under nongenotoxic conditions, and that the pathway differs from that of p53 stabilization mediated by serine phosphorylations in response to DNA damage (Fig. 8). The finding that $G\alpha_{12/13}$ controls the cell signaling for the mdm4-p53 system brings insights into the GPCR-mediated p53 regulatory pathway for cell cycle control and viability. Significant changes in p53 expression and its target gene expression by the inhibition or activation of $G\alpha_{12/13}$ highlight the need to consider the G protein pathway as one of the important p53 regulatory controls.

Materials and Methods

Reagents

Anti-p53, anti-p21, anti-Bax, anti-Bcl_2, anti-β-catenin, anti-actin, anti-mdm4, anti-$G\alpha_{12}$, anti-$G\alpha_{13}$, anti-pChk2, anti-Chk2, anti-ATM antibodies, and human $G\alpha_{12/13}$ siRNA were supplied by Santa Cruz Biotechnology. Anti-mdm2 antibody was obtained from Calbiochem. Anti-pERK1/2, anti-pp53, and anti-pAkt antibodies were supplied by Cell Signaling. Anti-PTEN antibody was purchased from Upstate. U0126 was obtained from Alexis. PD98059, lactacystin, and LY294002 were provided by Calbiochem. Etoposide and other reagents were supplied by Sigma Chemical. $G\alpha_{12}$QL or $G\alpha_{13}$QL plasmids were gifts from Dr. D.N. Dhanasekaran (Temple University, Philadelphia, PA). The plasmid encoding mdm4 and the adenoviral vector comprising the encoding region of p53 were kindly provided by Dr. S.J. Berberich (Wright State University, Dayton, OH) and Dr. K.W. Kang (Chosun University, Gwangju, Korea), respectively.

Cell Culture

The gene knockout MEF cell lines were supplied by Dr. M.I. Simon (California Institute of Technology, Pasadena, CA; ref. 6), and maintained in DMEM containing 10% fetal bovine serum, 50 units/mL of penicillin, and 50 μg/mL of streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Cells were plated at a density of 5×10^6 per dish and preincubated for 24 h at 37°C. MCF10A cells were cultured as described previously (57). For all experiments, cells were grown to 80% to 90% confluency.

Subcellular Fractionations

Total cell lysates and nuclear extracts were prepared according to previously published methods (58). The samples were stored at −70°C until use.

Immunoblot Analyses

SDS-PAGE and immunoblot analyses were done according to previously published procedures (58).

Immunocytochemistry

Cells were grown on Lab-TEK chamber slides (Nalge Nunc International) and incubated in serum-free DMEM for 6 h at 37°C. Standard immunocytochemical methods were used for p53 immunostaining (59). Stained cells were examined using a laser-scanning confocal microscope (Leica TCS NT).

Gel Shift Assay

A double-stranded DNA probe of p53-binding oligonucleotides (5′-TACAGAACATGTCTAAGCATGCTGGGG-3′) end-labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase was used for gel shift analysis (60). In some analyses, the specificity of protein DNA binding was determined by competition assays with a 20-fold molar excess of unlabeled oligonucleotide.

ATM Activity

ATM activities were measured by immunocomplex kinase assays. ATM in cell lysates (200 μg) was immunoprecipitated using a specific antibody (1 μg), and the precipitate was washed with the kinase reaction buffer comprising 40 mmol/L of HEPES (pH 7.0), 80 mmol/L of NaCl, 5 mmol/L of $MgCl_2$, 800 μmol/L of EDTA, 200 μmol/L of AMP, 200 μmol/L of ATP, and 1 mmol/L of DTT. To the immunoprecipitates, 20 μL of the kinase reaction buffer containing 1 μg of PHAS-1 (Calbiochem) as a substrate and 1 μCi of [γ-^{32}P]ATP were added, and the samples were incubated for 30 min at 30°C. The proteins were suspended in SDS loading buffer, resolved by SDS-PAGE, and detected by autoradiography.

RT-PCR

RT-PCR was done with total RNA obtained from cells using selective primers for murine p53 or the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Primers specific for p53 (sense, 5′-CCGAGGCCGGCTCTGAGTATACCACCATC-3′; antisense, 5′-CTCATTCAGCTCCCGGAACATCTC-GAAGCG-3′), mdm4 (sense, 5′-TGGGATGATGCTGACA-CAGA-3′; antisense, 5′-GTCGTGAGGTAGGCAG-3′), and GAPDH (sense, 5′-TCGTGGAGTCTACTGGCGT-3′; anti-sense, 5′-GCCTGCTTCACCACCTTCT-3′) amplified products of 396 and 510 bp, respectively. PCRs were done for 30 cycles using the following conditions: denaturation at 95°C for 0.5 min, annealing at 54°C for 0.5 min, and elongation at 68°C for 1.5 min.

Immunoprecipitation

To assess the interaction of p53 with mdm2 or mdm4, a fraction of the cell lysates (200 μg proteins in 250 μL) was incubated overnight with anti-p53 antibody (Santa Cruz Biotechnology) at 4°C. The antigen-antibody complex was immunoprecipitated following incubation for 2 h at 4°C with protein G-agarose. Immunocomplexes were solubilized in 2× Laemmli buffer and boiled for 5 min. Proteins in the samples were resolved using 7.5% SDS-PAGE and transferred to nitrocellulose membranes. The samples were immunoblotted with anti-mdm4 or anti-mdm2 antibody. Blots were developed using an enhanced chemiluminescence detection kit.

Construction of Recombinant p53-Expressing Adenoviral Vector

The AdEasy adenoviral system (Stratagene) was used for vector construction. The human p53 cDNA was subcloned into the shuttle vector, pShuttle-CMV. The resultant pShuttle-p53 was used to generate adenoviral recombinants through homologous recombination with the adenoviral backbone vector, pAdEasy-1, in BJ5183 bacterial cells (Stratagene). The adenoviral vector was then transfected into HEK293 cells to prepare transducing viruses. After infecting p53-expressing adenoviral vectors for 72 h, the cells were suspended in PBS buffer. The stock mixture of p53-expressing adenoviral vectors was prepared by repeated freezing-thawing procedures.

Stable Transfection of $G\alpha_{12}QL$ and $G\alpha_{13}QL$

MCF10A cells were transfected with the plasmid encoding for $G\alpha_{12}QL$ or $G\alpha_{13}QL$ using LipofectAMINE reagent (Invitrogen). Stable transfectants were selected by incubating the cells in culture medium containing 400 μg/mL of G418 (Invitrogen; ref. 47). At least 100 NeoR colonies were pooled together.

siRNA Knockdown

To knock down $G\alpha_{12/13}$, the clones of MCF10A cells stably transfected with $G\alpha_{12}QL$ or $G\alpha_{13}QL$ were simultaneously transfected with the siRNA against human $G\alpha_{12}$ and $G\alpha_{13}$, or a nonspecific scRNA (100 pmol/mL) using LipofectAMINE 2000. Twenty-four hours after transfection, lysates were used for immunoblotting.

Flow Cytometry

After the treatment of cells with vehicle, doxorubicin, or etoposide, dead cell–containing supernatants were collected and pooled with trypsinized living cells. The cells were permeabilized with 70% ethanol, washed and treated with RNase-A, and then with propidium iodide. Cell cycle status and pre-G_1 apoptotic cell populations were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Apoptosis was scored after doxorubicin or etoposide treatment by assessing the fraction of cells with sub-G_1 DNA content.

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