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

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Abstract

G\textsubscript{12/13}, which belongs to the G\textsubscript{12} family, participates in the regulation of diverse physiologic processes. In view of the control of G\textsubscript{12/13} in cell proliferation, this study investigated the role of G\textsubscript{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\textsubscript{12} deficiency decreased p53 levels and its DNA binding activity, accompanying p21 repression with Bcl2 induction, whereas G\textsubscript{12} deficiency did not change p53 mRNA expression. ERK1/2 or Akt was not responsible for p53 repression due to G\textsubscript{12} deficiency. Mdm4, a p53-stabilizing protein, was repressed by G\textsubscript{12} and to a lesser extent by G\textsubscript{13} deficiency, whereas mdm2, PTEN, \beta-catenin, ATM, and Chk2 were unaffected. p53 accumulation by proteasomal inhibition during G\textsubscript{12} deficiency suggested the role of G\textsubscript{12} in p53 stabilization. Constitutively active G\textsubscript{12} (G\textsubscript{12QL}) or G\textsubscript{13} (G\textsubscript{13QL}) 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\textsubscript{12/13}. In control or G\textsubscript{12/13}-deficient cells, genotoxic stress led to p53 accumulation. At concentrations increasing the flow cytometric pre-G\textsubscript{1} phase, doxorubicin or etoposide treatment caused serine phosphorylations in G\textsubscript{12}+/− or G\textsubscript{12/13}+/− cells, but did not induce mdm4. G\textsubscript{12/13}QL transfection failed to phosphorylate p53 at serines. Our results indicate that G\textsubscript{12/13} regulate basal p53 levels via mdm4, which constitutes a cell signaling pathway distinct from p53 phosphorylations elicited by genotoxic stress.

Introduction

Heterotrimeric GTP-binding proteins (G proteins) participate in cell signaling for the regulation of a variety of physiologic processes. The G\alpha subunits which define G proteins are divided into G\alpha\textsubscript{16}, G\alpha\textsubscript{11}, G\alpha\textsubscript{14}, and G\alpha\textsubscript{13} family members. Among the G\alpha families, G\alpha\textsubscript{12} members are activated by the stimulation of thrombin, TXA\textsubscript{2}, lysophosphatidic acid, and thyroid-stimulating hormone receptors (1). The G\alpha\textsubscript{12} family members, which consist of G\alpha\textsubscript{12} and G\alpha\textsubscript{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\textsubscript{12} and G\alpha\textsubscript{13}, the proteins sometimes exhibit differential abilities to recruit signaling pathways for physiologic effectors (6). G\alpha\textsubscript{13} gene knockout resulted in impaired angiogenesis and intra-uterine death, whereas mice deficient in the G\alpha\textsubscript{12} gene were alive (7). G proteins and their associated proteins (e.g., \beta-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\textsubscript{12} 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\textsubscript{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 post-translational 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.
The association of activated mutation of \( G_{12/13} \) 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 \( G_{12/13} \)-associated cell signaling pathways for the regulation of the p53-mdm4-mdm2 system. Information on the regulatory role of Gp53 has been found for the first time that Gp53 is up-regulated in response to genotoxic stress (26, 27). As a result, Gp53 accumulation by proteasomal inhibition might be reversed by the absence of \( G_{12} \) or \( G_{13} \). On the contrary, Bcl2 was up-regulated by the loss of \( G_{12} \) and, to a lesser extent, by that of \( G_{13} \) (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 \( G_{12} \) or \( G_{13} \) resulted in increases in the Bcl2 to Bax ratio. Data presented here indicate that deficiency of \( G_{12} \) or \( G_{13} \), particularly \( G_{12} \), alters p53 target protein levels.

In an effort to find the causal relationship of \( G_{12/13} \) deficiency with low p53 levels, we examined the expression of the proteins that potentially regulate p53. The expression of PTEN and \( \beta \)-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 \( G_{12} \) or \( G_{13} \) deficiency, ATM activity and p-Thr68-Chk2 levels were determined in the cell lysates. The absence of \( G_{12} \) or \( G_{13} \) minimally changed ATM kinase activity and Chk2 phosphorylation, indicating that the \( G_{12} \) 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). 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 \( G_{12} \) decreased p53 levels to a greater extent than that of \( G_{13} \), we primarily used \( G_{12}^{-/-} \) cells in subsequent experiments. Treatment of \( G_{12}^{-/-} \) 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 \( G_{12} \), we evaluated p53 expression in \( G_{12}^{-/-} \) 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.

### Results

#### Down-Regulation of p53 by \( G_{12} \) Deficiency

First, we investigated whether p53 expression and DNA binding activity were changed by targeted disruption of the \( G_{12} \) or \( G_{13} \) 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 \( G_{12}^{-/-} \) cells (Fig. 1A). \( G_{13} \) 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 \( G_{12} \) gene decreased the staining intensity of p53 in the nuclei. Despite a decrease in p53 level in lysates due to \( G_{13} \), the immunocytochemical staining intensity of p53 was not much changed in \( G_{13}^{-/-} \) cells. Next, we analyzed the interaction of p53 in nuclear extracts with the p53-binding oligonucleotide using gel shift assays. The deficiency of \( G_{12} \) inhibited p53 DNA binding activity, whereas that of \( G_{13} \) 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 \( G_{12/13} \) deficiency affected the expression of downstream target genes regulated by p53, we monitored the levels of p21, Bcl2, and Bax in the MEF cells. The expression of p21, a protein that inhibits cell cycle progression, was repressed by the absence of \( G_{12} \) or \( G_{13} \). On the contrary, Bcl2 was up-regulated by the loss of \( G_{12} \) and, to a lesser extent, by that of \( G_{13} \) (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 \( G_{12} \) or \( G_{13} \) resulted in increases in the Bcl2 to Bax ratio. Data presented here indicate that deficiency of \( G_{12} \) or \( G_{13} \), particularly \( G_{12} \), alters p53 target protein levels.

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#### p53 Accumulation by Proteasomal Inhibition

Subsequently, we determined whether the decrease in p53 due to the absence of \( G_{12} \) or \( G_{13} \) resulted from transcriptional inhibition. Semiquantitative reverse transcription-PCR (RT-PCR) analysis revealed no decrease in p53 mRNA levels in the \( G_{12}^{-/-} \) or \( G_{13}^{-/-} \) cells compared with controls (Fig. 3A). Data suggested that p53 down-regulation due to the deficiency of \( G_{12} \) or \( G_{13} \) might not have resulted from either transcriptional repression or changes in the mRNA stability.
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 Ga12 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/C0/C0 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 Ga12/C0/C0 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 Ga13/C0/C0 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 Ga12/C0/C0 cells with MG132 (Fig. 3C).

p53 Levels in Ga12/13 Double-Knockout Cells and the Effect of MG132

Ga12 and Ga13 may exert differential functions in cell signaling. We next asked if Ga12 and Ga13 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 Ga12/13 double-knockout (Ga12/13/C0) cells (Fig. 4A). p53 was repressed by the deficiency of both Ga12 and Ga13, 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 Ga12/13/C0 cells, whereas Bcl2 was up-regulated. p53

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**FIGURE 1.** Down-regulation of p53 protein by Ga12 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 immunocytochemically 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.
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 Gα12/13.**

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 Gα12 loss resulted from the inhibition of mdm4, we examined mdm4 levels in the cells. Mdm4 levels were decreased notably by the deficiency of Gα12, and moderately by that of Gα13, compared with controls (Fig. 5A, top). Loss of both Gα12 and Gα13 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 Gα12 or Gα13 deficiency. Semiquantitative RT-PCR analyses indicated no changes in the mdm4 mRNA levels during the absence of Gα12 or Gα13, 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/C0/Gα12/C0 cells infected with a p53-encoding adenoviral vector. Mdm4 was not induced by p53 overexpression in either RK/C0/Gα12/C0 or Gα12/C0/Gα13/C0 cells (Fig. 5A, bottom).

To verify the role of Gα12 or Gα13 in mdm4 expression in another cell line, we established the clones of MCF10A cells stably transfected with a Gα12QL or a Gα13QL construct. As anticipated, mdm4 was induced by the stable expression of Gα12QL or Gα13QL (Fig. 5B). Consistently, Gα12QL or Gα13QL 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

**FIGURE 2.** The effect of Gα12 or Gα13 deficiency on the expression of the proteins regulated by p53. A. The expression of p53 target gene products, p21, Bcl2, 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-Thr68Chk2 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 Gα12 or Gα13 deficiency. Immunoblot analyses were done in the lysates of cells treated with vehicle, MEK1, or PI3K inhibitor. Results were confirmed by repeated experiments.
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 Ga12/13. Preliminary studies showed that siRNA knockdown of either Ga12 or Ga13 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 Ga12 and Ga13 in MCF10A cells stably transfected with Ga12QL or Ga13QL decreased mdm4 content (Fig. 5D), corroborating the role of Ga12 members in mdm4 regulation. In parallel with this, we observed decreases in p53 content. Exposure of RK−/− cells to the siRNA against Ga12/13 resulted in a similar repressing effect. Collectively, the observations presented here provide evidence that Ga12 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 Ga12/13-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 Ga12−/− or Ga13−/− cells, p53 and mdm2 were also induced by anticancer agents. Mdm4 levels were not affected by the deficiency of Ga12 or Ga13. We next examined whether p53 binding to mdm4 or mdm2 was altered by the absence of Ga12 or Ga13. The association of p53 with mdm4 was notably decreased by the deficiency of Ga12, whereas the interaction between p53 and mdm2 was rather slightly enhanced by the lack of Ga12. When RK−/− cells were treated with doxorubicin, the association of p53 with mdm2 was further increased (Fig. 6B, right).

To analyze the functional aspect of p53 induction by genotoxic agents, we measured the fractions of pre-G1 phase cells using flow cytometric analysis. As expected, cells in the sub-G1 phase were accumulated by doxorubicin treatment in Ga12−/− or Ga12/13−/− cells as well as RK−/− cells.

**FIGURE 3.** p53 accumulation by proteasomal inhibition. A. Representative RT-PCR analysis of p53 mRNA. The effect of Ga12 or Ga13 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.
Given the mdm4-mediated p53 induction caused by activated mutants of G\textsubscript{12} or G\textsubscript{13}, we finally determined the effects of G\textsubscript{12}QL and G\textsubscript{13}QL on p53 phosphorylations at serines. Stable transfection of MCF10A cells with G\textsubscript{12}QL or G\textsubscript{13}QL did not induce p53 phosphorylations at Ser\textsuperscript{15} or Ser\textsuperscript{392} (Fig. 7E), identifying the aspect that the signaling pathway of G\textsubscript{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 Go subunit and its dissociation from the \( \beta \gamma \) 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, Gs family members as the molecular switches between the effectors of GPCRs and the cell survival pathway. Among Gq family members that serve as the effector molecules of GPCRs, G\textsubscript{12/13} may exert distinct and/or overlapping cell signal pathways.

Studies have shown that G\textsubscript{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\textsubscript{12} regulates lysophosphaticid acid–mediated mitogenic pathways (44). G\textsubscript{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\textsubscript{12} or G\textsubscript{13} are involved in oncogenic transformation (4, 10-12), we intended to unveil the physiologic role of G\textsubscript{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\textsubscript{12/13} for p53 stabilization, which may account in part for the G\textsubscript{12}-dependent alterations in the cell physiology. Our finding showing the role of G\textsubscript{12} or G\textsubscript{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 Bcl2, a signal representing cell survival (48), was reciprocally up-regulated as p53 levels decreased because of the loss of G\textsubscript{12}. The observations that the deficiency of G\textsubscript{13}, unlike that of G\textsubscript{12}, only partially inhibited p21 as well as p53 suggest that G\textsubscript{13} exerts some overlapping specificity with G\textsubscript{12} in regulating p53. The comparable change of Bcl2 expression in G\textsubscript{13}−/− cells further confirmed the partial effect of G\textsubscript{13} and its overlapping function with G\textsubscript{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\textsubscript{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\textsubscript{a12}, 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\textsubscript{a12} deficiency (shown in this study) may reflect the compensatory adaptation of the cells for survival in the absence of G\textsubscript{a12}. Lysophosphatidic acid receptor activation stimulates cell proliferation through the \(\beta\)-catenin–mediated pathway, and thus, deregulation in \(\beta\)-catenin may lead to p53-dependent growth arrest (25, 50). Studies from other laboratories have shown that G\textsubscript{a12} deficiency specifically interact with the cytoplasmic domains of the cadherin family members, which comprise \(\beta\)-catenin (51, 52). The present demonstration that \(\beta\)-catenin levels were unaffected by G\textsubscript{a12} deficiency rules out the possibility that p53 inhibition by the loss of G\textsubscript{a12} results from \(\beta\)-catenin repression. Nevertheless, we could not exclude the aspect that the low levels of p53 might be associated with the lack of \(\beta\)-catenin interaction with G\textsubscript{a12}. In addition, our data, showing the absence of G\textsubscript{a12}, 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\textsubscript{a12} or G\textsubscript{a13} 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, \textit{de novo} p53 synthesis in the absence of G\textsubscript{a12} 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\textsubscript{a12a13} regulates p53 stability. Delayed accumulation of p53 by MG132 in G\textsubscript{a12}+/− cells might have reflected alterations in p53 stability and degradation. Our findings showing that dual deficiency of G\textsubscript{a12a13} repressed both p53 and p21 to a greater extent with reciprocal induction of Bcl2, and that proteasomal inhibition caused p53 accumulation

![FIGURE 5. Mdm4-mediated p53 expression downstream of G\textsubscript{a12a13}. A. Mdm4 repression by G\textsubscript{a12} or G\textsubscript{a13} deficiency. Mdm4 and mdm2 proteins were immunoblotted in the lysates of untreated MEF cells. The mdm4 and GAPDH mRNAs 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\textsubscript{a12} or G\textsubscript{a13} (G\textsubscript{a12QL} or G\textsubscript{a13QL}), p53, mdm4, and mdm2 levels were assessed in MCF10A cells stably transfected with the plasmid encoding G\textsubscript{a12QL} or G\textsubscript{a13QL}. 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\textsubscript{a12QL} or G\textsubscript{a13QL} were transfected with siRNAs directed against human G\textsubscript{a12} or scRNA (RNA with scrambled sequences), incubated for 24 h, and then subjected to immunoblot analysis for mdm4. Knockdown of G\textsubscript{a12} was confirmed by immunoblotting. The results were confirmed by at least two repeated experiments.](image-url)
during Gα12/13 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 Gα12/C0/C0 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 Gα12 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 Gα12 deficiency, and to a lesser degree, by Gα13 deficiency. Decreases in mdm4 level due to the absence of Gα12 or Gα13 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 Gα12 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 Gα12/13 was consistent with our experimental results showing that Gα12/13 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 Gα12/13 deficiency causes p53 inhibition. The identification of mdm4 as a target regulated by Gα12/13 was further supported by reversal experiments showing that transfection of the Gα12/C0/C0 cells with Gα13QL or Gα13QL 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

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**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-G1 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-G1 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 Thr68). E. The effect of G\(\alpha_{12}\)QL or G\(\alpha_{13}\)QL on the levels of p53 phosphorylated at Ser15 or Ser392 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 Go_{12/13} regulates the basal level of p53. Go_{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 mdm4 and thereby enhances mdm2 levels, leading to the feedback regulation of p53 (dashed lines).

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

To differentiate the signal initiated by genotoxic stress from that of Go_{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 Go_{12/13} supports the hypothesis that the p53 regulatory pathway activated by genotoxic stress differs from that controlled by Go_{12/13} (Fig. 8). p53 and Chk2 phosphorylations by genotoxic agents functionally correlated with cell apoptosis, as shown by the increase in pre-G1 phase fraction. In particular, increases in p53 Ser^{15} phosphorylation in Go_{12}-/- cells matched with its higher fraction of pre-G1 phase. Our finding that Ser^{15} and Ser^{392} phosphorylations of p53 were not observed in MCF10A cells stably transfected with Go_{12QL} or Go_{13QL} lends support to the conclusion that the Go_{12} regulatory mechanism of p53 does not involve the serine phosphorylations, whereas genotoxic stress activates p53 independent of the pathway involving Go_{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 Go_{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 Go_{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 Go_{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-Bcl2, anti-β-catenin, anti-actin, anti-mdm4, anti-Go_{12}, anti-Go_{13}, anti-pChk2, anti-Chk2, anti-ATM antibodies, and human Go_{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. Go_{12QL} or Go_{13QL} 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 × 10^4 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).

Immunocytchemistry

Cells were grown on Lab-TEK chamber slides (Nalge Nunc International) and incubated in serum-free DMEM for 6 h at 37°C. Standard immunocytchemical 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′-TACAGAACATGCTAACATTGCAGGGG-3′) end-labeled with [γ-32P]ATP and T4 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 MgCl2, 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-32P]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′-CCGAGCCCGCTCAGGATACCCACCATC-3′; antisense, 5′-CTCATTCACTCCGGAGAACATCTC-GAAGCG-3′), mdm4 (sense, 5′-TGGGATGATGCTGACA-CAGA-3′; antisense, 5′-GTGCTGAGGTAGCAGG-3′), and GAPDH (sense, 5′-TCGTTGAGGTCTACTGCGT-3′; antisense, 5′-GCTGCTTTCACACCTTCT-3′) amplified products of 396 and 510 bp, respectively. PCRs were done for 30 cycles using the following conditions: denaturation at 95°C for 1.5 min, annealing at 54°C for 0.5 min, and elongation at 72°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 BJS183 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-shaking procedures.

Stable Transfection of Gα12QL and Gα13QL

MCF10A cells were transfected with the plasmid encoding for Gα12QL or Gα13QL 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 Gα12/13, the clones of MCF10A cells stably transfected with Gα12QL or Gα13QL were simultaneously transfected with the siRNA against human Gα12 and Gα13, 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-G1 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-G1 DNA content.

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