


# Mitogenic Signaling by the *gcp* Oncogene Involves the Upregulation of S-Phase Kinase-Associated Protein 2

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## Abstract

The *gcp* oncogene, defined by the activated mutant of the  $\alpha$ -subunit of the G protein  $G_{12}$  ( $G_{\alpha_{12}Q229L}$  or  $G_{\alpha_{12}QL}$ ), potently stimulates the proliferation of many different cell types in addition to inducing neoplastic transformation of several fibroblast cell lines. While it has been demonstrated that  $G_{\alpha_{12}QL}$  accelerates G1- to S-phase cell cycle progression, the precise mechanism through which  $G_{\alpha_{12}}$  communicates to cell cycle machinery is largely unknown. In the present study, we report that the activated—mutational as well as receptor-mediated— $G_{\alpha_{12}}$  transmits its proliferative signals to cell cycle machinery by modulating the levels of the S-phase kinase-associated protein 2 (Skp2), an E3 ubiquitin ligase, involved in the regulation of the cyclin-dependent kinase inhibitor (CKI), p27<sup>Kip1</sup>. Our results show that the expression of  $G_{\alpha_{12}QL}$  leads to an increase in the levels of Skp2 with a correlatable decrease in p27<sup>Kip1</sup> levels and subsequent increase in the activities of specific CDKs. By demonstrating that the transient expression of  $G_{\alpha_{12}QL}$  induces an increase in Skp2 levels with resultant downregulation of p27<sup>Kip1</sup> in both NIH3T3 and human astrocytoma 1321N1 cells, we establish here that the effect of  $G_{\alpha_{12}}$  on Skp2/p27<sup>Kip1</sup> is cell type independent. In addition, we demonstrate that LPA-stimulated proliferation and changes in Skp2 and p27<sup>Kip1</sup> levels in 1321N1 cells could be inhibited by the expression of a dominant-negative mutant of  $G_{\alpha_{12}}$ , thereby pointing to the critical role of  $G_{\alpha_{12}}$  in LPA-mediated mitogenic signaling. Our findings also indicate that LPA as well as  $G_{\alpha_{12}}$ -mediated upregulation of Skp2 requires a yet to be characterized mechanism involving JNK. Since Skp2 has been identified as an oncogene, and it is overexpressed in many cancers, our results presented here describe for the first time that Skp2 is a novel target in the cell cycle machinery through which  $G_{\alpha_{12}}$  and its cognate receptors transmit their oncogenic signals.

## Keywords

oncogene, G12, LPA, Skp2, JNK

## Introduction

GPCR-mediated signaling pathways have recently been identified to play a major role in cancer cell growth and progression.<sup>1–5</sup> Many GPCRs have been observed to be overexpressed in various cancer types, and they appear to contribute to tumor cell growth when activated by circulating or locally produced ligands. Confirming this view, a growing list of human cancers including ovarian, breast, prostate, and pancreatic carcinomas has been shown to exhibit aberrant expressions of GPCRs and the ligands that activate them.<sup>4–10</sup> In general, ligand-activated GPCRs stimulate diverse physiological responses by catalyzing the guanine nucleotide exchange in the  $\alpha$ -subunit of different heterotrimeric G proteins.<sup>11</sup> Of the different  $\alpha$ -subunits that have been analyzed thus far, the  $\alpha$ -subunit of the G protein  $G_{12}$  ( $G_{\alpha_{12}}$ ), referred to as the *gcp* oncogene,<sup>12</sup> exhibits the most potent mitogenic and oncogenic activities.<sup>11–13</sup> Previous studies from our laboratory as well as others have identified different but complementary mechanisms through which  $G_{\alpha_{12}}$  promotes oncogenic proliferation.<sup>11–24</sup> Although these studies have indicated that  $G_{\alpha_{12}}$  promotes G1/S-phase progression<sup>23,24</sup>—similar to many oncogenes—the mechanism by which  $G_{\alpha_{12}}$  communicates to cell cycle machinery is poorly understood. In light of the recent observations that  $G_{\alpha_{12}}$  and receptors that couple to  $G_{\alpha_{12}}$  are implicated in the genesis and/or progression of several cancers,<sup>11–13</sup> defining

the mechanism(s) by which  $G_{\alpha_{12}}$  accelerates cell cycle progression may prove critically important to identify novel diagnostic, therapeutic, or prognostic targets.

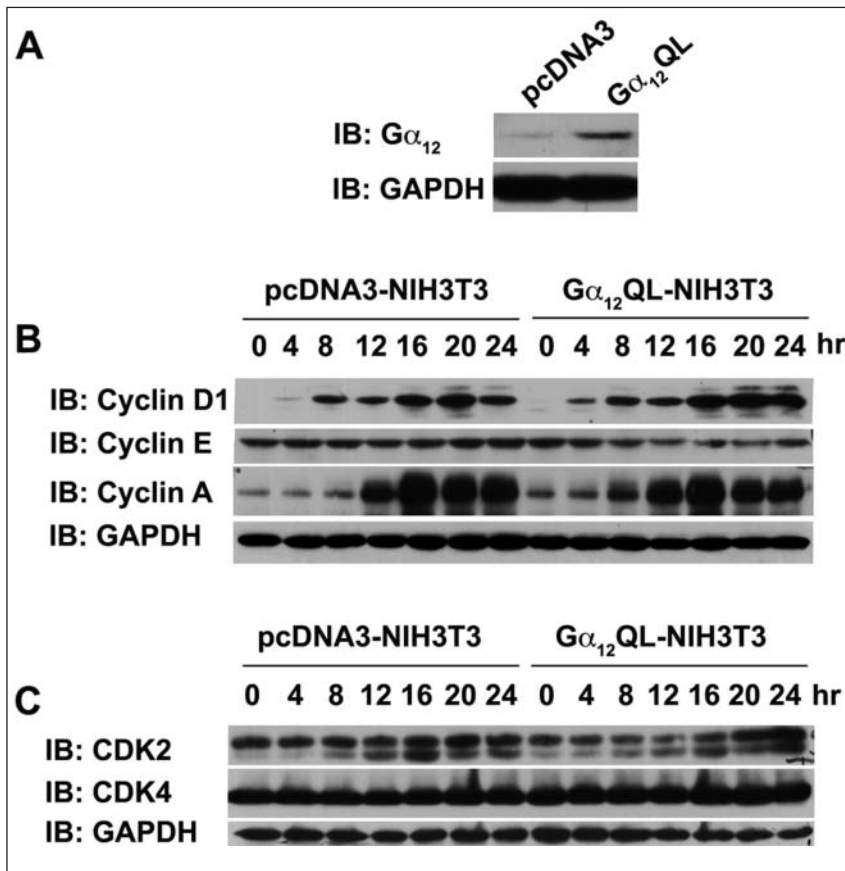
Cell cycle analyses have indicated that mitogenic signaling pathways often converge onto mid to late G1 phase of cell cycle to accelerate cell progression into S phase.<sup>25,26</sup> In brief, cell cycle progression is regulated by a coordinated series of phosphorylation events, chiefly mediated by the cyclin-dependent kinase (CDK) family of serine/threonine kinases.<sup>25–27</sup> The activities of CDKs are regulated by the stimulatory cyclins and inhibitory cyclin-dependent kinase inhibitors (CKIs).<sup>25–28</sup> Once activated, cyclin-CDK complexes drive the cell cycle through its different phases via the phosphorylation of specific downstream targets such as retinoblastoma protein (pRb).<sup>29,30</sup> Our studies presented

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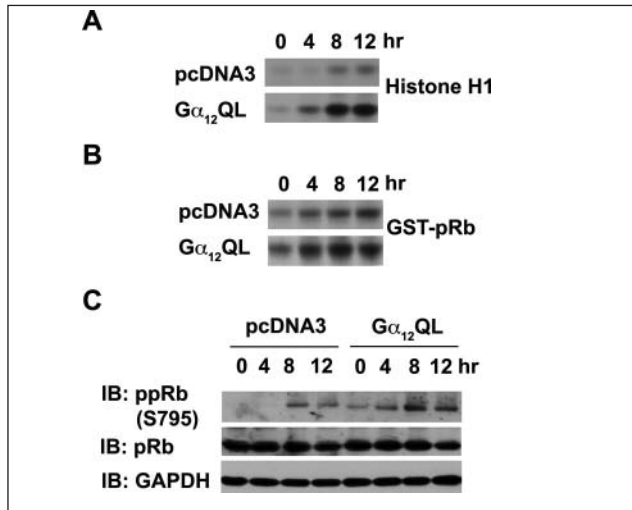
**Figure 1.** Expression levels of G1/S-phase cyclins and CDKs in  $G\alpha_{12}$ QL-expressing cells. **(A)** Lysates (50  $\mu$ g) from pcDNA3 vector control and  $G\alpha_{12}$ QL-transformed NIH3T3 cells ( $4 \times 10^5$ ) were analyzed for the expression of  $G\alpha_{12}$ QL by immunoblot analysis using antibodies to  $G\alpha_{12}$ . The blot was reprobbed with GAPDH to monitor equal loading of proteins. **(B)** Lysates (50  $\mu$ g) prepared from vector control and  $G\alpha_{12}$ QL-NIH3T3 cells serum starved for 24 hours followed by stimulation with 5% CS for varying lengths of time were separated on a 10% SDS-PAGE and subjected to immunoblot analysis using antibodies specific to cyclin D1, cyclin A, or cyclin E. The blot was reprobbed with antibodies to GAPDH to monitor equal loading of proteins. **(C)** An identical blot was probed for monitoring the levels of CDK4 and CDK2 using the respective antibodies. The blot was reprobbed with antibodies to GAPDH to monitor equal loading of proteins. These analyses were repeated at least 3 times, and the results are from a typical analysis.

here are focused on identifying the mechanism by which  $G\alpha_{12}$  communicates to this complex array of interrelated events to accelerate cell cycle progression. We demonstrate here that the expression of the constitutively activated mutant of  $G\alpha_{12}$  stimulates the expression of cyclin D1 and cyclin A along with an increase in the activities of CDK2 and CDK4 in NIH3T3 cells. We show that the expression of  $G\alpha_{12}$ QL leads to a decrease in the levels of p27<sup>Kip1</sup>, a CKI primarily involved in the inhibition of CDK2 and CDK4.<sup>26-28</sup> We establish further that the decrease in p27<sup>Kip1</sup> accompanies the upregulation of S-phase kinase associated protein-2 (Skp2), an E3 ubiquitin ligase involved in downregulating p27<sup>Kip1</sup> levels.<sup>31-34</sup> The ability of transiently expressed  $G\alpha_{12}$ QL to recapitulate similar events in 1321N1 astrocytoma cells indicates that the

effect of  $G\alpha_{12}$  on the levels of Skp1 and p27<sup>Kip1</sup> is cell type independent. Our studies presented here also demonstrate that LPA, which has been identified as an oncogenic lipid growth factor in many cancers,<sup>4,35-38</sup> stimulates the proliferation of 1321N astrocytoma cells along with an increase in the levels of Skp2 via  $G\alpha_{12}$ . Finally, our studies point to JNK<sub>12</sub> as a novel mediator in LPA/LPAR- $G\alpha_{12}$ -mediated upregulation of Skp2. Taken together with the findings that Skp2 that has been defined as an oncogene<sup>39-42</sup> is over-expressed in many different cancers<sup>43-48</sup> similar to the upregulation of LPA-LPAR signaling in many cancers,<sup>4,35-38</sup> our data presented here unravel a potential role for  $G\alpha_{12}$  and its cognate receptors in upregulating the levels of Skp2 in many cancers.

## Results

*Activated mutant of  $G\alpha_{12}$  stimulates an increase in cyclin levels.* To define  $G\alpha_{12}$ -mediated changes in cell cycle-associated proteins involved in G0-G1-S transition, we examined the profiles of different cyclins in NIH3T3 cells expressing  $G\alpha_{12}$ QL during G1-S progression. After verifying the expression of  $G\alpha_{12}$ QL (Fig. 1A), the levels of G1/S cyclins such as cyclin D1, cyclin E, and cyclin A were monitored by immunoblot analyses using lysates from NIH3T3 cells expressing  $G\alpha_{12}$ QL ( $G\alpha_{12}$ QL-NIH3T3) or the empty vector. Results from this analysis indicated that upon stimulation of cell proliferation by 5% serum, an increased expression of cyclin D1 can be observed in cells expressing  $G\alpha_{12}$ QL by 4 hours following serum stimulation, whereas such an increase can be seen in control cells only from 8 hours onwards (Fig. 1A). In contrast, an increase in cyclin A levels can be seen in  $G\alpha_{12}$ QL-NIH3T3 cells at all the time points compared to the vector control cells (Fig. 1B). In the case of cyclin E,  $G\alpha_{12}$ QL cells showed an increase—albeit small but consistent—in the basal levels compared to the vector controls. Thus, cells expressing  $G\alpha_{12}$ QL primarily showed an increase in the expression profiles of the G1/S-phase cyclins, cyclin D1 and cyclin A. An immunoblot analysis of the G1/S phase-associated CDKs, namely, CDK2



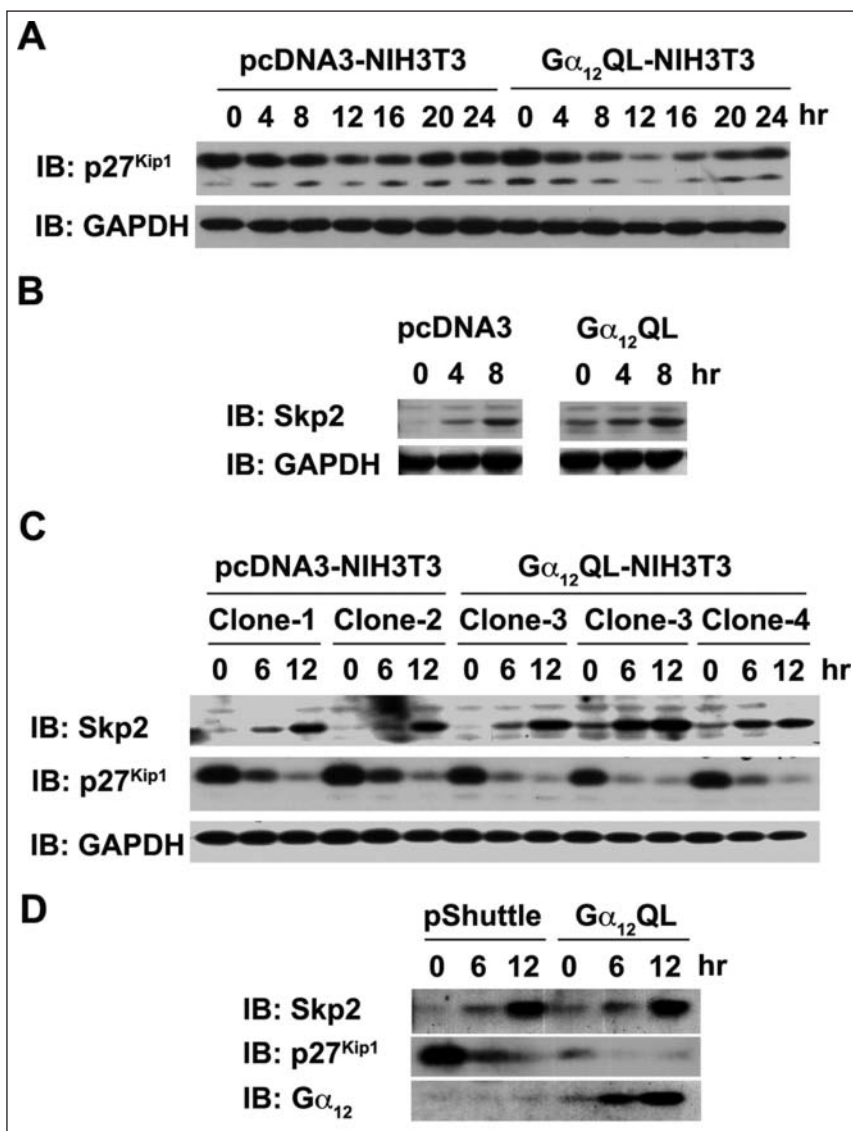
**Figure 2.** G $\alpha_{12}$ QL stimulates the activation of CDKs. **(A)** CDK2 was immunoprecipitated from the lysates (100  $\mu$ g) prepared from serum-deprived pcDNA3- and G $\alpha_{12}$ QL-NIH3T3 that were stimulated with 5% calf serum for 0, 4, 8, and 12 hours using antibodies to CDK2. Immune complex kinase assay was carried out using purified histone H1 as a substrate. The phosphorylated histone H1 was separated by SDS-PAGE and visualized by autoradiography. **(B)** From the lysates (100  $\mu$ g) as prepared above, CDK4 was immunoprecipitated with antibodies to CDK4, and immune complex kinase assay was carried out using purified recombinant GST-pRb as a substrate. The phosphorylated GST-pRb was separated by SDS-PAGE and visualized by autoradiography. **(C)** These lysates (50  $\mu$ g) were separated on a 10% SDS-PAGE, and immunoblot analysis was carried out using antibodies specific to Ser795-phosphorylated pRb. The blot was stripped and reprobed with antibodies to pRb to monitor the expression levels. The blot was further probed with antibodies to GAPDH to monitor equal loading of proteins. The results presented are from a typical set of experiments that were repeated at least 3 times.

and CDK4, showed no changes in their expression levels during these time points (Fig. 1C).

**G $\alpha_{12}$  stimulates an increase in CDK activity.** Since increased levels of cyclin D1 and cyclin A are often associated with a corresponding increase in CDK4 and CDK2 activities rather than their levels of expression,<sup>25-27</sup> we examined whether the increased levels of cyclins in G $\alpha_{12}$ QL-expressing cells could be correlated with a corresponding increase in the activities of their catalytic partners CDK2 or CDK4. Lysates prepared from pcDNA3- and G $\alpha_{12}$ QL-NIH3T3 cells that were stimulated with serum for different lengths of time were subjected to immune complex kinase assays using antibodies specific for CDK2 or CDK4.<sup>49</sup> CDK2 immune complex kinase assay was carried out using histone H1 as a substrate,<sup>49</sup> whereas CDK4 immune complex kinase assay was carried out using GST-Rb as the substrate.<sup>49</sup> As compared to the vector control, cells expressing G $\alpha_{12}$ QL show a rapid and more potent increase in CDK2 as well as CDK4 activities (Fig. 2A and 2B), while their levels remain unaltered (Fig. 1B). Based on the findings that

mitogenic pathways stimulate the rapid phosphorylation of Ser-795 of pRb through the cyclin D1-CDK4 and cyclin A-CDK2 complexes,<sup>50,51</sup> the phosphorylation status of Ser795 of pRb has been used as an index of the increased activities of CDK2/4.<sup>52</sup> Therefore, we analyzed the phosphorylation of Ser795 of pRb in G $\alpha_{12}$ QL-expressing cells to further assess and confirm the increase in the activities of CDK2/4. Results from such an immunoblot analysis indicated a rapid increase in the phosphorylated form of pRb in G $\alpha_{12}$ QL-NIH3T3 cells upon serum stimulation compared to vector control cells (Fig. 2C).

**G $\alpha_{12}$  stimulates prometogenic changes in the levels of p27<sup>Kip1</sup> and Skp2.** It has been well established that the activities of specific cyclin-CDK complexes are finely and dynamically regulated by distinct CDK inhibitors (CKIs).<sup>25-28</sup> Of the different CKIs, p27<sup>Kip1</sup> has been observed to play a major role in the regulation of CDK2/4 activities.<sup>25-28</sup> Therefore, we monitored the levels of p27<sup>Kip1</sup> in response to the expression of G $\alpha_{12}$ QL. Vector control and G $\alpha_{12}$ QL-NIH3T3 cells that were made quiescent by 24 hours of serum starvation were stimulated with 5% serum, and the lysates prepared from these cells at different time points were subjected to immunoblot analysis using antibodies to p27<sup>Kip1</sup>. The results indicated that the cells expressing G $\alpha_{12}$ QL showed an accelerated decrease in p27<sup>Kip1</sup> levels (from 8 hours) compared to vector control cells upon growth stimulation by serum (Fig. 3A). Since the levels of p27<sup>Kip1</sup> have been shown to be dynamically regulated via ubiquitin-mediated degradation of p27<sup>Kip1</sup> by the E3 ubiquitin ligase Skp2,<sup>31-34</sup> we investigated whether the observed decrease in p27<sup>Kip1</sup> levels in G $\alpha_{12}$ QL-NIH3T3 cells is associated with an increase in the expression levels of Skp2. Results from such an analysis indicated an increase in Skp2 levels—during unstimulated as well as serum-stimulated conditions—in G $\alpha_{12}$ QL cells compared to the vector control cells (Fig. 3B). To establish that the expression of Skp2 is not due to the clonal variation of a specific G $\alpha_{12}$ QL-expressing clone of NIH3T3 cells, the expression levels of Skp2 and p27 were monitored in 3 different G $\alpha_{12}$ QL-NIH3T3 clones. Results from such an experiment indicated that an increase in Skp2 levels along with a concomitant decrease in the levels of p27 could be observed in all the G $\alpha_{12}$ QL-NIH3T3 clones (Fig. 3C), thus establishing that the observed decrease in Skp2 levels is not due to clonal variation. To further confirm that the increased levels of Skp2 are directly in response to G $\alpha_{12}$ QL, the expression of Skp2 in response to transiently expressed G $\alpha_{12}$ QL was monitored. Results from this analysis indicated that the transient expression of G $\alpha_{12}$ QL stimulated the expression of Skp2 with a concomitant decrease in p27 levels (Fig. 3D), thereby confirming that the changes in Skp2-p27<sup>Kip1</sup> levels are directly in response to the expression of G $\alpha_{12}$ QL.



**Figure 3.**  $G\alpha_{12}$  modulates the levels of p27<sup>Kip1</sup> and Skp2. **(A)** Lysates (50  $\mu$ g) from pcDNA3- and  $G\alpha_{12}$ QL-NIH3T3 cells, serum starved for 24 hours and stimulated with 5% CS for the indicated lengths of time, were separated on a 10% SDS-PAGE and subjected to immunoblot analysis using antibodies specific to p27<sup>Kip1</sup>. The blot was reprobed with GAPDH antibodies to monitor equal loading of proteins. **(B)** Lysates (50  $\mu$ g) from pcDNA3- and  $G\alpha_{12}$ QL-NIH3T3 cells that were serum starved for 24 hours and stimulated with 5% CS for 0, 4, and 8 hours were subjected to immunoblot analysis using Skp2-specific antibodies. The blot was probed with antibodies to GAPDH to monitor equal loading of proteins. **(C)** Lysates (50  $\mu$ g) were prepared from 2 independent pcDNA3- and 3  $G\alpha_{12}$ QL-NIH3T3 cell clones that were serum starved for 24 hours and then stimulated with 5% CS for 0, 6, and 12 hours. Immunoblot analysis was carried out using Skp2-, p27<sup>Kip1</sup>-, or GAPDH-specific antibodies. The experiment was repeated 3 times; results from a typical experiment are presented. **(D)** NIH3T3 cells that were infected with 600 MOI of  $G\alpha_{12}$ QL expressing adenovirus or empty pShuttle adenovirus were serum starved for 24 hours and then stimulated with 5% CS for the indicated time points (0, 6, and 12 hours). The blot prepared using the lysates (50  $\mu$ g) from these cells were sequentially probed with antibodies specific to Skp2, p27<sup>Kip1</sup>, and  $G\alpha_{12}$ .

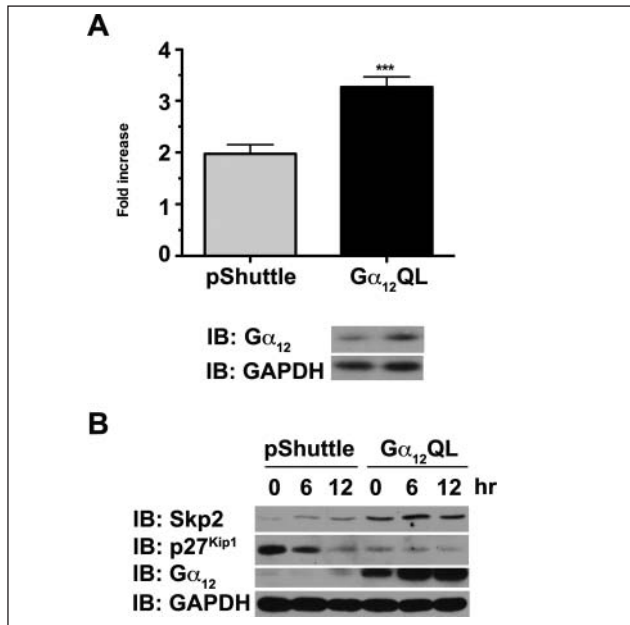
*Regulation of Skp2 by  $G\alpha_{12}$  is cell type independent.*  $G\alpha_{12}$  has been shown to stimulate mitogenic signaling pathways in many different cell types including the astrocytoma cell line 1321N1.<sup>12,22</sup> Therefore, we investigated whether

activated  $G\alpha_{12}$  could induce changes in Skp2 levels in 1321N1 cells. Activated mutant of  $G\alpha_{12}$  was transiently expressed in 1321N1 cells by infecting them with adenoviral vectors encoding  $G\alpha_{12}$ QL for 24 hours, and the expression levels of Skp2 were monitored using immunoblot analysis. Results indicated that the expression of activated mutant of  $G\alpha_{12}$  stimulated the proliferation of 1321N1 cells, confirming previous studies (Fig. 4A). When lysates from these cells were subjected to immunoblot analyses to monitor the expressions of Skp2 and p27<sup>Kip1</sup>, the results indicated that the cells expressing the activated mutant of  $G\alpha_{12}$  showed an upregulation of Skp2 along with a concomitant decrease in p27<sup>Kip1</sup> (Fig. 4B). Together, these findings, for the first time, clearly establish the ability of  $G\alpha_{12}$ QL to increase the expression levels of Skp2 in 2 distinctly different cell types.

*$G\alpha_{12}$  mediates LPA-stimulated cell proliferation and Skp2 upregulation in 1321N astrocytoma cells.* The findings that the mutationally activated  $G\alpha_{12}$  stimulated the upregulation of Skp2 raised an interesting question whether receptor activation of endogenous  $G\alpha_{12}$  would lead to similar changes in the levels of Skp2 and p27<sup>Kip1</sup>. This was analyzed with the use of LPA, an oncogenic lipid growth factor, which is known to stimulate cell proliferation via  $G\alpha_{12}$ . After confirming that LPA (60  $\mu$ M) stimulated the proliferation of 1321N1 cells (Fig. 5A), the expression profiles of Skp2 and p27<sup>Kip1</sup> in LPA-stimulated cells were monitored by immunoblot analysis. Results indicated that LPA stimulated a strong increase in the levels of Skp2 along with a drastic decrease in p27<sup>Kip1</sup> levels by 24 hours compared to the unstimulated controls (Fig. 5B). To

test whether the effects of LPA on Skp2 and p27<sup>Kip1</sup> involve  $G\alpha_{12}$ , we analyzed the effect of expressing the dominant-negative mutant of  $G\alpha_{12}$  on LPA-stimulated changes in Skp2/p27<sup>Kip1</sup> levels. Based on the findings that the C-termini of G





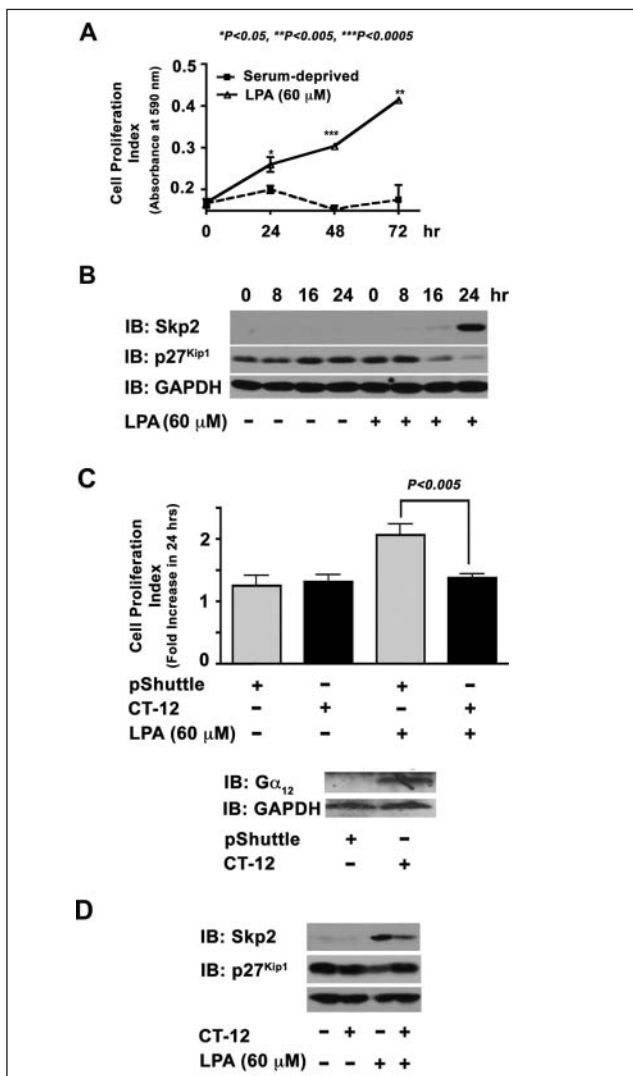
**Figure 4.**  $G\alpha_{12}$  QL upregulates Skp2 in 1321N1 astrocytoma cells. **(A)** 1321N1 astrocytoma cells ( $5 \times 10^3$  cells/well) were infected with 600 MOI of either  $G\alpha_{12}$  QL expressing adenovirus or empty pShuttle adenovirus. Proliferation of cells growing under low serum (2% FCS) was determined using an XTT-based cell proliferation kit as described in Materials and Methods. Fold increase was calculated by determining the increase in the absorbance of formazan dye at 492 nm over a period of 24 hours. The expression of  $G\alpha_{12}$  QL was monitored by immunoblot analysis of lysates prepared from an identical set of these cells using  $G\alpha_{12}$ -specific antibodies (**lower panel**). **(B)** 1321N1 astrocytoma cells infected with 600 MOI of  $G\alpha_{12}$  QL expressing adenovirus or the empty pShuttle adenovirus were stimulated with 5% FBS for 0, 6, and 12 hours. Lysates (50  $\mu$ g) from these cells were analyzed for the expression of Skp2, p27<sup>Kip1</sup>, and  $G\alpha_{12}$  by immunoblot analysis using respective antibodies. The blot was reprobbed with antibodies to GAPDH to ascertain equal loading of proteins.

protein  $\alpha$ -subunits are critical for binding to their cognate receptors,<sup>53</sup> an adenoviral vector that encodes the C-terminal 11 amino acids of  $G\alpha_{12}$  (CT-12) has been successfully used in 1321N1 astrocytoma cells to inhibit  $G\alpha_{12}$ -specific responses.<sup>54</sup> Using this adenoviral construct, we analyzed the role of  $G\alpha_{12}$  in LPA-mediated cell proliferation and associated changes in Skp2-p27<sup>Kip1</sup> levels. Results from these analyses indicated that the proliferation of 1321N1 cells stimulated by LPA could be effectively inhibited by the expression of the dominant-negative mutant of  $G\alpha_{12}$  (Fig. 5C). Similarly, the expression of CT-12 attenuated the LPA-stimulated upregulation of Skp2 along with the associated increase in p27<sup>Kip1</sup> levels (Fig. 5D). Taken together, these results clearly establish that the LPA-stimulated increase in Skp2, associated downregulation of p27<sup>Kip1</sup>, and the resultant cell proliferation in 1321N1 cells require  $G\alpha_{12}$ , the inhibition of which attenuates all of these events.

$G\alpha_{12}$  regulation of Skp2 is mediated by JNK. Being a critical player in G0-G1-S-phase transition, Skp2 is regulated by multiple signaling pathways involving ERKs,<sup>55</sup> AKT,<sup>56</sup> and FAK.<sup>57</sup> However, it is significant to note here that  $G\alpha_{12}$ -mediated mitogenic signaling critically involves the potent activation of JNKs.<sup>12,14-16</sup> Therefore, we examined whether LPA-mediated increase in Skp2 levels involves JNK, the dominant signaling pathway utilized by  $G\alpha_{12}$ . As shown in Figure 6A, inhibition of JNKs using SB600125 potentially attenuated the increase in Skp2 levels stimulated by LPA in 1321N1 cells. Consistent with this observation, treating NIH3T3 cells expressing  $G\alpha_{12}$  QL with SB600125 resulted in a similar inhibition in the upregulation of Skp2 compared to vehicle-treated  $G\alpha_{12}$  QL-NIH3T3 cells (Fig. 6B). Previous studies have shown that  $G\alpha_{12}$ -mediated activation of JNK involves the small GTPase CDC42.<sup>15</sup> Consistent with this observation, inhibition of CDC42 by expressing CDC42 N17, a dominant-negative mutant of CDC42, attenuated  $G\alpha_{12}$  QL-mediated upregulation of Skp2 levels in 1321N1 cells (Fig. 6C). These results pointing out a role for JNK in LPA- and  $G\alpha_{12}$ -mediated upregulation of Skp2 are quite novel and hitherto unreported.

## Discussion

$G\alpha_{12}$ , the  $\alpha$ -subunit of  $G_{12}$  that constitutes the *gpc* oncogene, has been shown to be the most potent  $\alpha$ -subunit in promoting cell proliferation and neoplastic transformation.<sup>11-13,58,59</sup> While studies describing the diversity of signals generated from  $G\alpha_{12}$  in inducing proliferation have been extensively documented, the mechanism by which mitogenic signaling cues from  $G\alpha_{12}$  are integrated in to the G1 to S phase of the cell cycle is relatively unknown. The results presented here identify for the first time a multiparallel, but interrelated, mechanism(s) through which  $G\alpha_{12}$  communicates to cell cycle machinery. An overview of these pathways establishes a paradigm in which the multiple targets involved in  $G\alpha_{12}$ -mediated mitogenic signaling converge towards the stimulation and potentiation of CDK activities. The first target of  $G\alpha_{12}$  signaling appears to be the upregulation of the levels of different G0/G1/S-phase stimulatory cyclins. The second set of targets involves the stimulation of the respective CDKs via p27<sup>Kip1</sup> and Skp2. As can be seen in Figure 1, the expression of  $G\alpha_{12}$  promotes an increase in the levels of cyclin D1, A, and E. Although the mechanism by which  $G\alpha_{12}$  upregulates these levels was not investigated in the present study, the ability of  $G\alpha_{12}$  to stimulate Akt signaling in these cells as previously demonstrated by us<sup>21</sup> and the subsequent Akt-mTOR-mediated stimulation of cellular translational apparatus appears to play a major role in this process (Ha and Dhanasekaran, unpublished data). Activation of the cyclin D1-CDK4 complex has been shown to play a critical role in inducing



**Figure 5.** LPA upregulates Skp2 via  $G\alpha_{12}$ . **(A)** Serum-deprived 1321NI astrocytoma cells ( $5 \times 10^3$  cells/well) were stimulated with 60  $\mu$ M of LPA for 24 hours along with the unstimulated control group. The increase in cell numbers at 24, 48, and 72 hours was monitored using crystal violet-based dye binding assay as described in Materials and Methods. A fold increase at 24 hours was calculated to the absorbance at 0 hours. **(B)** Serum-deprived 1321NI astrocytoma cells were stimulated with 60  $\mu$ M of LPA for varying lengths of time (0, 8, 16, and 24 hours) along with unstimulated controls. Lysates (50  $\mu$ g) from these cells were analyzed for the expression levels of Skp2 and p27<sup>Kip1</sup> by immunoblot analysis using respective antibodies. The blot was reprobed with GAPDH antibodies to monitor equal protein loading. **(C)** Quiescent 1321NI astrocytoma cells ( $5 \times 10^3$  cells/well) infected with 150 MOI of CT-12 encoding adenovirus or empty pShuttle adenovirus (24 hours) were stimulated with LPA (60  $\mu$ M) for 24 hours. Cell proliferation was measured using the XTT-based cell proliferation assay as described in Materials and Methods. Fold increase in cell proliferation was calculated by monitoring the increase in the absorbance of the formazan dye at 490 nm over a period of 24 hours. The expression of CT-12 was monitored by immunoblot analysis of lysates prepared from identical set-up experimental samples, using the  $G\alpha_{12}$  antibody that recognizes the CT-12 (lower panel). **(D)** Lysates (50  $\mu$ g) from these cells were subjected to immunoblot analysis using Skp2, p27<sup>Kip1</sup>, or GAPDH. The results are from a representative set of experiments ( $n = 3$ ).

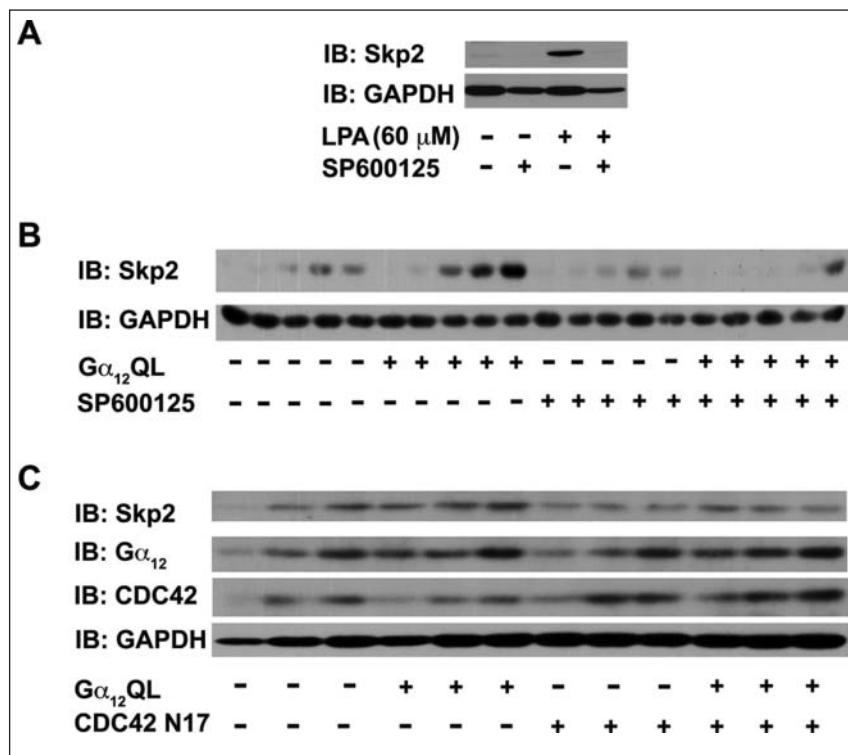
G1- to S-phase progression.<sup>26-30</sup> Similarly, cyclin A-CDK2 has been established as a key promoter of the S phase of the cell cycle.<sup>25,26</sup> By stimulating an increase in the levels of the respective cyclins and their associated CDKs,  $G\alpha_{12}$  signaling appears to target 2 complementary critical components of cell cycle machinery to ensure accelerated G1/S-phase progression. This is further substantiated by the observed increase in the phosphorylation of Ser795 of pRb in  $G\alpha_{12}$ -QL-expressing cells (Fig. 2D) since an increase in the activities of both cyclin D-CDK4 and cyclin A-CDK2 activity has been shown to contribute to the efficient phosphorylation of pRb, thereby relieving its inhibitory effect on S-phase entry. Thus, in addition to upregulating the levels of cyclins,  $G\alpha_{12}$  is also involved in stimulating the activities of the respective CDKs. The answer to the question as to how  $G\alpha_{12}$  stimulates the activities of CDK2/4 appears to lie, at least in part, within the ability of  $G\alpha_{12}$  to modulate the levels of p27<sup>Kip1</sup> through Skp2.

It should be noted here that during G1/S-phase progression, p27<sup>Kip1</sup> plays a dominant role in negatively regulating CDK2/4.<sup>26-28</sup> In turn, p27<sup>Kip1</sup> levels are dynamically regulated by the degradation of p27<sup>Kip1</sup> by ubiquitin proteasome machinery involving Skp2.<sup>31-34</sup> Our results presented here show that  $G\alpha_{12}$ -QL stimulates the upregulation of Skp2 along with a concomitant decrease in p27<sup>Kip1</sup> levels (Fig. 3A and 3B). Thus, the increase in CDK activities seen in  $G\alpha_{12}$ -QL cells possibly results from the decreased levels of p27<sup>Kip1</sup> brought out by the upregulated Skp2. Modulation of Skp2 levels appears to be one of the major mechanisms through which mitogens regulate the activities of CDKs in stimulating cell cycle progression.<sup>26-34</sup> In this context, our observation that  $G\alpha_{12}$  signaling stimulates an increase in Skp2 levels along with the downregulation of p27<sup>Kip1</sup> in 2 different cell types (Figs. 3 and 4) in which  $G\alpha_{12}$  promotes proliferation strongly identifies Skp2 as a major signaling target—in addition to cyclins—by which  $G\alpha_{12}$  promotes G1- to S-phase progression. It should be noted here that Skp2 is also involved in maintaining the optimal levels of cyclin E during G1/S transition.<sup>34</sup> Thus, it is likely that the observed decrease in cyclin E levels upon growth stimulation (Fig. 1B) is in response to the elevated Skp2 levels in  $G\alpha_{12}$ -QL cells (Fig. 3). Given the primary role of Skp2 in regulating CKIs and other key players in cell cycle progression, the results presented here that  $G\alpha_{12}$  upregulates Skp2 are highly significant. In addition, considering the potent mitogenic activity of  $G\alpha_{12}$ , our findings unravel Skp2 as the novel target in the cell cycle machinery through which  $G\alpha_{12}$  accelerates G1- to S-phase cell cycle progression and subsequent cell proliferation.

Our studies presented here also identify 2 newer correlates on LPA-mediated mitogenic signaling: 1) LPA stimulates proliferation along with promitogenic changes in Skp2/p27<sup>Kip1</sup> levels via  $G\alpha_{12}$ , and 2) LPA- $G\alpha_{12}$ -mediated upregulation of Skp2 involves JNK. While the increase in LPA levels and LPA-LPAR-mediated signaling have been

implicated in the growth and progression of many tumors, the mechanisms through which it promotes such oncogenic activities are largely unknown. Our results demonstrating that LPA stimulates proliferation of 1321N1 astrocytoma cells along with the upregulation of Skp2 with the resultant downregulation of p27<sup>Kip1</sup> that can promote cell cycle progression (Fig. 5A and 5B), both of which can be inhibited by the dominant-negative mutant of G $\alpha_{12}$  (Fig. 5C and 5D), constitute the first demonstration of a cell cycle-based mechanism through which the oncogenic growth factor LPA and G $\alpha_{12}$ , the *gcp* oncogene, stimulate the mitogenic pathway. Another interesting aspect of LPA-G $\alpha_{12}$ -mediated upregulation of Skp2 relates to our finding that JNK is involved in this process. The expression of Skp2 is known to be regulated by different kinases including ERK, AKT, and FAK.<sup>49,55-57</sup> Until now, JNK is not known as one of the kinases involved in the regulation of Skp2. In this context, our results indicating a role for JNK in LPA- and G $\alpha_{12}$ -mediated upregulation of Skp2 (Fig. 6) are quite important as they identified a so far unknown JNK-dependent mechanism underlying the regulation of Skp2. Although the mechanism(s) by which JNK mediates an increase in Skp2 levels is not clear at present, further studies should clarify its precise role in G $\alpha_{12}$ -mediated upregulation of Skp2.

Taken together, our results support a model in which G $\alpha_{12}$ -mediated increase in the levels of stimulatory cyclins as well as the decrease in the levels of p27<sup>Kip1</sup> through the upregulation of Skp2 cumulatively contribute to the overall increase in the activities of cyclin-CDK complexes, thereby accelerating G1/S progression. A simplistic working model integrating LPA-LPAR-G $\alpha_{12}$  signaling to cell cycle machinery is presented in Figure 7. In light of the observations that 1) Skp2 levels are increased in glioblastomas<sup>46</sup> and astrocytic gliomas,<sup>47</sup> 2) LPA is locally produced in many different cancers including glioblastomas,<sup>4,38</sup> 3) LPA-LPAR signaling can promote cell growth and cancer progression through the facilitation of autocrine and paracrine signaling loops,<sup>4,7,35-39</sup> and 4) G $\alpha_{12}$  transmits signals from LPA-LPAR to intracellular effector molecules through JNK,<sup>11,58</sup> our identification of



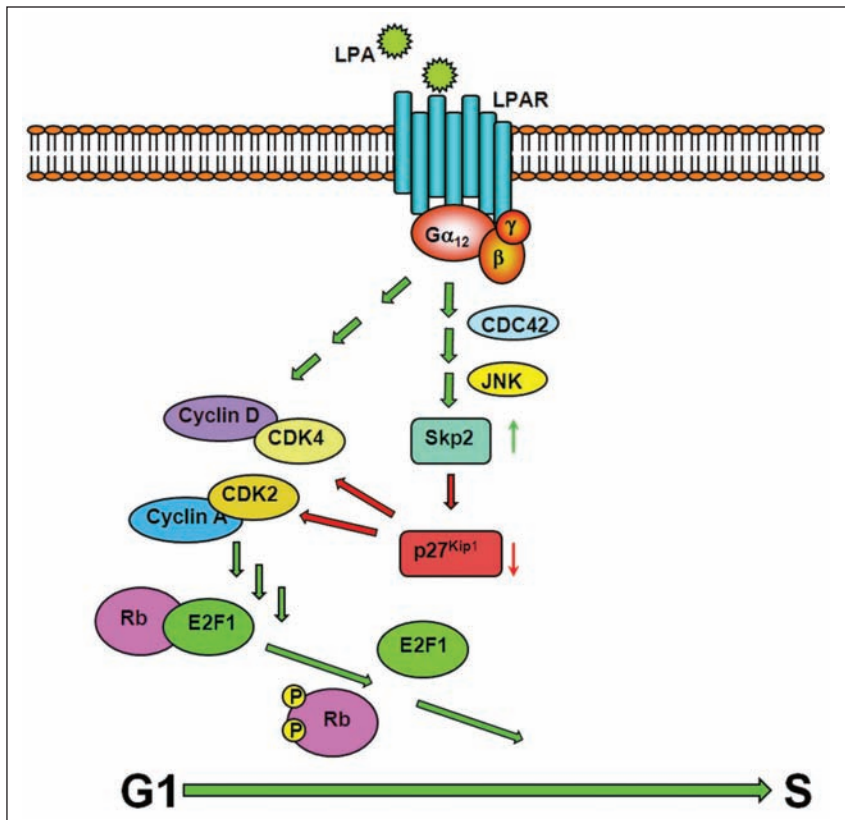
**Figure 6.** LPA-G $\alpha_{12}$ -mediated upregulation of Skp2 involves JNK. **(A)** 1321N1 astrocytoma cells ( $0.5 \times 10^6$  cells/60-mm plate) were treated with SP600125 (50  $\mu$ M) or DMSO 2 hours prior to stimulation with 60  $\mu$ M for 24 hours. Lysates (50  $\mu$ g) from these cells were subjected to immunoblot analysis using antibodies to Skp2. The blot was stripped and reprobed with GAPDH antibodies to monitor equal loading of proteins. **(B)** Lysates (50  $\mu$ g) from pcDNA3- and G $\alpha_{12}$ QL-NIH3T3 cells that were treated with SP600125 (50  $\mu$ M) or DMSO 2 hours prior to stimulation with 5% CS for 0, 4, 8, 12, and 16 hours and analyzed for Skp2 levels by immunoblot analysis using Skp2 antibodies. The blot was reprobed with GAPDH antibodies to monitor equal loading of proteins. Results are from a typical experiment ( $n = 3$ ). **(C)** 1321N1 astrocytoma cells ( $5 \times 10^3$  cells/well) were infected with 600 MOI of G $\alpha_{12}$ QL-encoding adenoviral vector singly or in combination with CDC42 N17-encoding adenoviral vectors along with appropriate control vectors. After 24 hours, the cells were stimulated with LPA (60  $\mu$ M) for 24 hours. Lysates (50  $\mu$ g) from these cells were subjected to immunoblot analysis using antibodies to Skp2, G $\alpha_{12}$ , CDC42, or GAPDH. The results are from a representative set of experiments ( $n = 3$ ).

Skp2 as the target protein regulated by G $\alpha_{12}$  is highly significant. Although finer details of this working model remain to be filled in, with the identification of Skp2 as a major protein being regulated by LPA-LPAR-G $\alpha_{12}$  signaling, further studies should define the role of this signaling nexus as well as other critical players involved in this signaling pathway in the genesis and progression of various cancers.

## Materials and Methods

**Cell lines.** Parental NIH3T3, 1321N1 astrocytoma cells as well as previously described pcDNA3-NIH3T3 and G $\alpha_{12}$ Q229L-NIH3T3 cell lines were maintained as described earlier<sup>21</sup> in Dulbecco's modified Eagle's medium (DMEM) (Cellgro, Mediatech Inc., Manassas, VA) containing 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin, and 5% calf serum





**Figure 7.** Schematic model for  $G\alpha_{12}$ -mediated cell cycle progression. Receptor or mutationally activated  $G\alpha_{12}$  accelerates G1-S progression by upregulating the levels of specific cyclins and Skp2, both of which contribute to the increased activities of CDKs. While cyclins stimulate their respective CDKs, CDK activities could be further potentiated by Skp2, which downregulates the levels of p27<sup>Kip1</sup>, an inhibitory CKI, via the ubiquitin-proteasome degradation system.  $G\alpha_{12}$ -mediated upregulation of Skp2 involves a novel signaling node involving CDC42 and JNK.

(NIH3T3 cells) or 5% fetal bovine serum (1321N1 cells) at 37°C in a 5% CO<sub>2</sub> incubator. The sera were obtained from Life Technologies Inc. (Gaithersburg, MD).

**Adenoviral constructs.** Construction of adenoviral vector-expressing  $G\alpha_{12}$  QL was carried out using the cDNA insert encoding  $G\alpha_{12}$  QL (1,800 bp) excised from the pcDNA3- $G\alpha_{12}$  QL vector<sup>14</sup> using the restriction nucleases *KpnI* and *XbaI*. The cohesive ends were blunted and cloned in the *EcoRV* site of the pShuttle-IRES-GFP2 vector (Stratagene, La Jolla, CA). The resultant plasmid was linearized using *PmeI* before it was transformed into *Escherichia coli* BJ5183-AD-1 cells (pretransformed with pAdEasy-1 plasmid) in which the homologous recombination event with the pAdEasy-1 plasmid containing adenoviral backbone could take place. The recombinant clones were selected by analysis of the *PacI*-digested DNA from these clones. The positive recombinant DNA was amplified by transforming it on a suitable *E. coli* strain. The recombinant DNA was cut with *PacI* and then purified before transfection onto AD293 cells

for the virus production. Isolation of the virus was carried out following the standard freeze-thaw procedure. If needed, further amplification of the virus was carried out using AD293 cells. These recombinant adenoviruses were titrated by plaque assay before target cells were infected. Adenoviral vectors expressing the dominant-negative mutant of CDC42 were constructed following identical procedures, using the Bam I–Xho I insert of CDC42 N17.<sup>61</sup> Recombinant adenoviral vector expressing the dominant-negative mutant of  $G\alpha_{12}$ , CT-12,<sup>54</sup> was kindly provided by Dr. Yoh Takuwa (Kanazawa University Graduate School of Medicine, Kanazawa, Japan). This virus was amplified by infecting AD293 cells. These recombinant adenoviruses were titrated by plaque assay before using them for infecting the target cells.

**Determining the optimal multiplicity of infection (MOI).** Based on the titer obtained from the plaque assay, the volume of virus that corresponds to the particular MOI to be infected was determined. An equal number of NIH3T3 and 1321N1 astrocytoma cells ( $1 \times 10^6$  cells/plate) were plated on 100-mm culture plates and infected with different MOIs (0, 25, 100, 300, and 600) of the adenovirus. Media containing serum were removed from the plates and washed with PBS and then replaced with 2 mL of serum-free medium containing the adenovirus. The plates were kept in the rocker inside the incubator for 2 hours for the cells to get infected. Following this, the plates were replaced with growth medium containing serum and incubated for 24 hours. Lysates were prepared from these cells and analyzed for the expression of proteins encoded by the adenovirus. Based on the expression profile, the optimum MOI to be used for the experiment was determined. The optimal MOI was deduced as the one at which the transgene showed the highest expression with little or no cytotoxic effects. Based on these results, an MOI of 600 was used for both NIH3T3 cells and 1321N1 astrocytoma cells. In experiments in which low serum growth conditions were used, the MOI was reduced to 150.

**Cell proliferation assay.** The growth profile of 1321N cells in response to LPA was monitored using an assay based on crystal violet staining of live cells following previously



published procedures.<sup>60</sup> An equal number of 1321N1 cells ( $5 \times 10^3$ ) grown on 12-well plates were serum starved for 24 hours, after which they were stimulated with 60  $\mu$ M of LPA for 24, 48, and 72 hours, respectively. At specific time points, cells were fixed using 10% formalin (Fisher Scientific, Pittsburgh, PA) dissolved in PBS for 10 minutes, following which the fixed samples were stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO) for 6 hours. The samples were then washed extensively to remove excess dye and dried overnight. The cell-associated dye was then extracted by incubation with 1 mL acetic acid (Fisher Scientific) for 60 seconds. The optical density of each sample was quantified at 590 nm. Proliferation of 1321N1 astrocytoma cells transiently infected with adenovirus encoding CT-12 or empty pShuttle adenovirus was monitored using the XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide]-based cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN). An equal number of cells ( $5 \times 10^3$  cells/well) grown in 96-well plates were serum starved for 24 hours. These cells were infected with an MOI of 150 of either pShuttle or CT-12 adenovirus in freshly replaced serum starvation medium supplemented with 0.2% fetal bovine serum followed by stimulation with LPA (60  $\mu$ M) for 24 hours. The absorbance of formazan dye at 490 nm was monitored as an index of actively growing cells using a microplate reader.

**Immunoblot analysis.** Immunoblot analyses were carried out following the previously published procedures.<sup>21</sup> Antibodies to G $\alpha_{12}$  (sc-409), cyclin A (sc-596), cyclin D1 (sc-8396), cyclin E (sc-481), CDK4 (sc-601), CDK2 (sc-163), p27<sup>Kip1</sup> (sc-528), Skp2 (sc-7164), JNK1 (sc-474), and JNK2 (scv-827) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to phospho-pRb (Ser795; #9301), control Rb (#9302), and phospho-JNK (#9251) were purchased from Cell Signaling Technology Inc. (Danvers, MA). Antibodies to GAPDH (#4300) were purchased from Ambion Inc. (Austin, TX). Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK) and Promega (Madison, WI), respectively.

**Immune complex kinase assays.** Immune complex kinase assays to monitor the kinase activities of CDK2 and CDK4 were carried out using previously published procedures with appropriate modifications.<sup>45</sup> Cells were lysed with kinase lysis buffer (200  $\mu$ L) containing 20 mM HEPES (pH 7.4), 50 mM  $\beta$ -glycerophosphate, 0.5% Triton X-100, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 2  $\mu$ g/mL leupeptin, 4  $\mu$ g/mL aprotinin, 100  $\mu$ M PMSF, and 1 mM benzamide. CDK2 or CDK4 in the lysates was immunoprecipitated by incubating 200  $\mu$ g of protein lysate with the polyclonal antibodies specific to CDK2 (sc-163; 2  $\mu$ g) or CDK4 (sc-601; 2  $\mu$ g),

respectively, for 1 hour. This was followed by an additional incubation with 20  $\mu$ L of protein A-Sepharose (Amersham Biosciences Corp., Piscataway, NJ) for 1 hour. The immune complex-bound protein A-Sepharose beads were washed 3 times with lysis buffer. CDK2 immune complex assay was carried out with protein A-Sepharose-bound CDK2 using 5  $\mu$ g of histone H1 (Roche Diagnostics). CDK4 immune complex assay was carried out with protein A-Sepharose-bound CDK2 using 5  $\mu$ g of GST-Rb fusion protein (a generous gift from Dr. Fang Liu, Rutgers University, New Brunswick, NJ). The kinase reactions were carried out by resuspending the protein A-Sepharose beads with bound immune complexes containing CDK2 or CDK4 along with appropriate substrates in 40  $\mu$ L of kinase reaction buffer containing 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P] ATP (5,000 cpm/pmol) and incubating at 30°C for 30 minutes. The reaction was stopped by the addition of Laemmli's sample buffer followed by boiling the sample for 3 minutes. The proteins were resolved by 12% SDS-PAGE, and the dried gels were analyzed by autoradiography.

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### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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