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G alpha z Inhibits Serum Response Factor-Dependent Transcription by Inhibiting Rho Signaling

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RUNNING TITLE: Gαz inhibits RhoA-mediated SRF activation

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Non-standard abbreviations used: SRE, serum response element; SRF, serum response factor; GEF, guanine nucleotide exchange factor; HEK, human embryonic kidney; ROK, Rho-kinase; LPA, lysophosphatidic acid; GPCR, G protein coupled receptor; RBD, Rho binding domain; PAGE, polyacrylamide gel electrophoresis; LEF/TCF, lymphoid enhancing factor/T cell factor; TCF, ternary complex factor; GST, glutathione S-transferase; RGS, regulator of G protein signaling; AGS, activator of G protein signaling; PKA, protein kinase A.

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ABSTRACT

Gα12/13 or Gαq signals induce activation of Rho GTPase, leading to serum response factor (SRF)-mediated gene transcription and actin cytoskeletal organization. However, less is known regarding how Rho pathway signals are down-regulated. Here we report that Gαz signals inhibit serum response factor (SRF)-dependent transcription. Gαz expression inhibits Gα12/13-, Gαq-, and Rho guanine nucleotide exchange factor (GEF)-induced serum response element (SRE) reporter activation in HEK293T and PC12 cells. Expression of Gαz mutants with defective fatty acylation have no inhibitory effect. Expression of Gαz, but not Gαi, attenuates serum-induced SRE reporter activation, suggesting that Gαz can down-regulate endogenous signals leading to SRF. Whereas Gαz also blocks SRE reporter induction by the activated mutant RhoAL63, it does not affect Gα12- or Rho GEF-induced RhoA activation, or RhoAL63-GTP binding in vivo. Moreover, Gαz does not inhibit SRE reporter induction by an activated form of Rho-kinase (ROK). Since Gαz inhibits RhoAL63/A188-induced reporter activation, phosphorylation of RhoA on serine 188 does not appear to be involved; furthermore, RhoA subcellular localization was not affected. Use of pharmacologic inhibitors implies that Gαz-induced reduction of SRE reporter activation occurs via a mechanism other than adenylate cyclase modulation. These findings suggest that Gαz signals may attenuate Rho-induced stimulation of SRF-mediated transcription.

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The ubiquitous Rho small GTPase is required for multiple cellular responses including cell growth, contractility and migration (Hall 1998). At the molecular level, Rho controls a signaling pathway that regulates extracellular factor-induced actin filament assembly (Hall 1998), and serum response element (SRE)-dependent gene transcription via the transcription factor serum response factor (SRF) (Hill et al. 1995). Defective Rho pathway signaling has been implicated in several diseases including cancer and cardiovascular disease (Boettner and Van Aelst 2002; Toksoz and Merdek 2002); however, much remains to be understood regarding how this ubiquitous pathway is normally regulated.

Rho responses are induced by serum stimulation, and agonists for certain G protein coupled receptors (GPCRs such as lysophosphatidic acid (LPA) induce actin polymerization and SRE transcriptional reporter activation via Rho (Seasholtz et al. 1999; Kjoller and Hall 1999). These GPCR signals are transduced to Rho via heterotrimeric Gα12/13 and Gαq family members through partially understood mechanisms likely involving guanine nucleotide exchange factors (GEFs) (Seasholtz et al. 1999; Schmidt and Hall 2002). GTPases such as Rho bind guanine nucleotides, and their activation state is determined by whether they bind GDP in the inactive state, or GTP in the active state. Rho GEFs directly activate Rho by inducing rapid GDP/GTP exchange, resulting in activated GTP-bound Rho (Schmidt and Hall 2000). Once activated, Rho signals are translated into downstream cellular responses via specific effectors (Bishop and Hall 2002) such as Rho kinase, or ROK.

Rho is required for actin filament assembly which underlies phenotypic changes in cell shape, cell contraction, adhesion and migration in most tissues. For example, Rho stimulates formation of actin stress fibers in fibroblasts (Ridley and Hall 1992). In addition, Rho is required for extracellular factor-induced activation of SRF which binds to the SRE found in many gene promoters, leading to transcription (Hill et al., 1995). In addition to regulating immediate-early genes such as *c-fos* and *Egr-1*, SRF regulates several skeletal and smooth muscle-specific genes including α-and γ-*actin* required for differentiated tissue function, as well as ubiquitously expressed genes such as *vinculin* (reviewed in Arsenian et al., 1998). The requirement for Rho in SRF function has led to the common use of SRE transcriptional reporter assays as a measure for Rho-dependent signals in vivo.

While the requirement for Rho function in many cellular responses is now well established, there is also accumulating evidence that certain cellular responses require suppression of Rho function. For example, inhibition of Rho function has been reported to be required for processes such as dendritic outgrowth in melanocytes (Busca et al., 1996), development of neurite extensions (Jalinck et al., 1994; Li et al., 2002), axon regeneration (Lemann et al., 1999), vasopressin-mediated aquaporin-2 translocation (Klussmannet al., 2001), nitric oxide/cGMP/G-kinase pathway-mediated transcriptional modulation (Gudi etal., 2002), and somatostatin-induced inhibition of cell migration (Buchan et al., 2002). Thus, Rho inhibition likely plays an integral role in particular stages of differentiation in certain cell types, and in particular cellular responses.

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However, pathways and mechanisms involved in Rho signal down-regulation are poorly understood.

Here we investigate the potential role of Gαi family members in Rho signal down-regulation. Of the four Gα subunit families, Gα12/13, Gαq, Gαi and Gαs (Simon et al., 1991), Gαi and Gαs do not appear to be involved in induction of Rho pathway signals. Results presented here suggest that signals by the Gαi family member Gαz inhibit Rho-mediated responses. Gαz attenuates SRF-mediated transcription induced by Gα12/13/q signals, by Rho GEFs, and by serum. In addition, Gαz blocks transcriptional activation by constitutively active RhoAL63 mutant, although it appears to have no effect on transcriptional activation induced by the activated ROK downstream effector. Potential mechanisms for the observed inhibition of SRF-dependent transcription are investigated and discussed. Finally, the influence of Gαz signals on actin cytoskeletal organization is evaluated.

MATERIALS and METHODS

Cell lines: Human embryonic kidney (HEK293T) and PC12 cell lines are from American Tissue Culture Collection. HEK293T were grown in Dulbecco's modified essential medium (DMEM) (Gibco BRL) containing 10% fetal bovine serum. PC12 were grown in F12K medium (Gibco BRL CA) containing 15% heat inactivated horse serum and 2.5% fetal bovine serum (FBS). Quiescent serum-starved Swiss 3T3 fibroblasts were maintained and prepared as described (Nobes and Hall 1995).

Plasmids. Wild-type Gαz, and activated mutant Gαz Q205L, Gαi1 Q204L, Gαi2 Q205L, GαoA Q205L, Gαq Q209L, Gα12 Q231L, Gα13 Q226L, and Gαs Q213L in pcDNA3.1

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vector were obtained from Guthrie cDNA Resource (PA). Plasmids for pcDNA:GαzG2A, pcDNA:GαzG2A3A, RhoAL63/A188, SRE.L and TOPFLASH luciferase reporters, pEF C3 transferase, pGEX2T Rhotekin RBD, dominant active ROK, and p115 RhoGEF were gifts. pSR:proto-Lbc Rho GEF is described in Sterpetti et al., 1999.

Antibodies and reagents. Anti-RhoA, anti-Gα12 and anti-myc antibodies were obtained from Santa Cruz Biotechnology (CA). Protein kinase A inhibitor H-89 and adenylate cyclase inhibitor MDL-12 were obtained from Calbiochem (CA).

Cell transfection. 6 well dishes for transcriptional reporter assays, or 100 mm dishes for RBD assays at 80% confluence were transfected with plasmid DNA using Lipofectamine Plus (GIBCO BRL CA) according to manufacturer's recommendation for 5 hours. Cells were serum-starved overnight, and lysed the following day.

Immunoblotting. Cellular material was resolved by 10% SDS/PAGE. Immunoblotting was carried out as described in Sterpetti et al., 1999.

Dual Luciferase reporter assay. SRE.L luciferase reporter plasmid, which encodes a mutant SRE that encodes functional SRF binding sites, but eliminates the ternary complex factor (TCF) binding site (Hill et al., 1995) was used with the Dual-Luciferase Reporter Assay System (Promega) as recommended. Inducible firefly luciferase results obtained were normalized to internal control *Renilla* luciferase values expressed from pTK-RL plasmid (Promega) following sequential measurement of the two luciferase activities. Each point was in triplicate; experiments were repeated more than twice.

RBD Assay. In vivo GTP-Rho pull-down was carried out using GST-Rhotekin Rho binding domain (RBD) fusion protein (Ren et al., 1999) as previously described in Dutt et al., 2002; experiments were repeated more than twice.

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Subcellular fractionation. HEK293T cell lysates were fractionated into S-100 soluble and P-100 particulate fractions as described in Sterpetti et al., 1999.

Microinjection. To prepare confluent quiescent, serum-starved Swiss 3T3 cells for microinjection, cells were seeded onto acid-washed coverslips at a density of 5 X 10^4, in DMEM containing 5% serum. After the cells became quiescent (approximately 7-10 days after seeding), they were serum-starved for 16 h in DMEM containing 2 μg/liter $NaHCO_3$. Eukaryotic expression vectors (0.1 μg/μl) together with biotin-dextran were injected into the nucleus of approximately 50 cells over a period of 15 min. Cells were returned to the incubator for 2-3 h for optimal expression, fixed in 4% paraformaldehyde for 10 minutes at room temperature, and stained for the epitope tag, injection marker, and actin, as described in Nobes and Hall, 1995. Fluorescence images were recorded on a CCD camera and processed using Openlab software.

Statistical analysis. Data was analyzed using Student's *t* test; a p value < 0.05 was considered to indicate significance.

RESULTS

Effects of Gα subunit family members on Rho pathway signals were tested by an in vivo transcriptional reporter assay based on the SRE.L luciferase reporter which encodes a mutant SRE that contains Rho-dependent SRF binding sites, but eliminates the Ras-responsive ternary complex factor (TCF) binding site (Hill et al., 1995). A dual luciferase reporter system was utilized, which allows normalization of inducible luciferase activity readings against an internal *Renilla* luciferase standard provided by the co-transfected pTK-RL plasmid. As shown in Figure 1A, expression of activated mutant Gαs, Gαi1,

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Gαi2, Gαo or Gαz forms alone had little or no inductive effect on SRE luciferase reporter activity. Next we investigated potential inhibitory effects of Gαi family members on inductive signals mediated by Gα12, Gα13, and Gαq. As shown in Figure 1A, co-expression of activated Gαi1, Gαi2, Gαo or Gαz separately by co-transfection of modest plasmid amounts (100 nanograms) each had a substantial reducing effect on Gα12/13 and Gαq-induced reporter activity. Inhibition was observed in the ranges of ~40-60% by Gαi1, ~50-77% by Gαi2, ~37-54% by Gαo and ~70-85% by Gαz. In contrast, co-expression of comparable amounts of GαsQL had no inhibitory effect, suggesting that the effect is not due to non-specific Gα subunit co-expression. The inhibitory effects of Gαi family members was not due to cytotoxicity, as the internal *Renilla* luciferase control value levels were comparable to those of the inductive Gα subunit levels alone (not shown). Immunoblotting of total cell lysates shown in Figure 1B revealed that levels of the stimulatory Gα subunit such as Gα12QL are not altered by co-expression of Gαi family members, indicating that the inhibitory effect is unlikely to be due to decreased Gα subunit expression. In addition, immunoblotting with anti-Glu-antibody showed that levels of expression of Glu-tagged activated Gαi family members are comparable. Moreover, immunoblotting for endogenous RhoA showed that levels of total cellular RhoA were unchanged. In addition, we evaluated the effects of Gαi2QL and GαzQL expression on Gα12QL-induced activation of endogenous Rho by carrying out Rho "pull-down" experiments by affinity purification with Rhotekin RBD which preferentially binds GTP-RhoA. As shown in Figure 1C, Gαi2QL co-expression had no effect on levels of Gα12QL-induced GTP-RhoA, and GαzQL co-expression with

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Gα12QL led to only a marginal reduction which was not statistically significant ($p>0.05$).

Next the effect of Gαi family expression on Rho pathway components was tested. As expected and shown in Figure 2A, expression of two different Rho GEFs, p115 and Lbc Rho GEF, led to SRE reporter induction via endogenous Rho. Co-expression of increasing levels of activated GαzQL with Lbc or p115 Rho GEF caused inhibition of Rho GEF-induced SRE reporter activation even at 50 ng dosage of GαzQL plasmid. As a control for the observed Gαz effect, we used a Gαz mutant, GαzG2A, which has defective fatty acylation due to a point mutation that destroys the myristoylation site, and thus abolishes Gαz signaling ability (Morales et al., 1998). GαzG2A co-expression had no inhibitory effect on Rho GEF-induced reporter activation even at 200 ng dosage (although wild-type Gαz is inhibitory; see Figure 4A). In contrast to its effect on Gα12/13/q-induced signals, expression of activated Gαi2 had no blocking effect on Rho GEF-induced reporter activity, and the remaining Gαi members also did not affect Rho-GEF signals (not shown). We next tested whether GαzQL inhibits downstream Rho effectors such as ROK, which readily induces SRF-mediated responses (Sotiropoulos et al., 1999). Figure 2A shows that in contrast to its effect on Rho GEFs, co-expression of increasing amounts of GαzQL with activated ROK had little effect on ROK-induced SRE reporter activation. In addition, we evaluated the effect of GαzQL expression on Lbc Rho GEF-induced activation of endogenous Rho by carrying out Rho "pull-down" experiments with Rhotekin RBD to preferentially affinity purify GTP-RhoA. As shown

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in Figure 2B, coexpression of either Gαi2QL or GαzQL with Lbc Rho GEF did not have a noticable effect on levels of Lbc-induced GTP-RhoA in vivo.

We next tested whether Gαz can modulate endogenous Rho pathway signals induced by extracellular stimuli. As shown in Figure 2C, stimulation of HEK293T cells by 15% serum treatment leads to SRE.L reporter activation; and this induction is Rho-dependent as determined by inhibition upon C3 transferase expression (not shown; Hill et al., 1995). Interestingly, expression of increasing amounts of GαzQL (50-400 ngs) in serum-stimulated cells led to a ~40% reduction in SRE reporter activity which was significant. In contrast, expression of Gαi2QL did not inhibit serum-induced reporter activity. When the effect of GαzQL expression on serum-induced GTP-RhoA levels was measured, a significant reduction was not observed (result not shown).

We next assessed whether the suppressive effect of Gαz extends to other cell types by carrying out the same experiments in the PC12 cell line which is derived from adrenal pheochromocytoma tissue which normally expresses endogenous Gαz (Ho and Wong 2001). While the transfection efficiency of PC12 cells was lower than in HEK293T, Figure 3A shows that GαzQL expression had the same potent blocking effect on Gα12/13 and Lbc Rho GEF-induced SRE reporter activation in PC12 cells as observed in HEK293T. Similar results were also obtained in 3T3 fibroblasts (not shown). To further investigate the Gαz effect, we used a different transcriptional luciferase reporter in the form of the TOPFLASH luciferase reporter construct, which encodes binding sites for LEF/TCF transcription factor which is induced by activated β-catenin (Morin et al., 1997), but not by Rho (not shown). As shown in Figure 3B, expression of the activated

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β-catenin Δ45 form (Morin et al., 1997) in HEK293T cells led to robust induction of TOPFLASH luciferase reporter which was unaffected by co-expression of GαzQL at two different doses (100 ng and 400 ng).

As Gαz inhibits signals to SRF/SRE by Rho GEFs, but not by a downstream effector such as activated ROK, we next investigated potential effects on activated GTPase-deficient RhoAL63 mutant. Figure 4A shows that co-expression of activated Gαi1, Gαi2 or Gαo with RhoAL63 had no inhibitory effect on SRE reporter activation. In contrast, co-expression of wild-type Gαz or activated GαzQL potently blocked RhoAL63-induced SRE reporter activation. As controls for the observed effect, we used two Gαz mutants, GzG2A and GzG2A3CA, which have defective fatty acylation due to point mutations that destroy the myristoylation, and myristoylation plus palmitoylation sites, respectively, and thus abolish Gαz signaling ability (Morales et al., 1998). As shown in Figure 4, co-expression of Gαz acylation mutants had no negative effect on RhoAL63-induced reporter activation. Immunoblotting of total cell lysates shown in Figure 4B revealed that RhoAL63 protein levels were comparable in Gαi, Gαo or Gαz co-expressing cells, indicating that the inhibitory effect of Gαz was unlikely to be due to decreased RhoAL63 expression. As expected for a constitutively GTPase-deficient mutant, GTP-Rho "pull-down" assay indicated that relative levels of GTP-RhoAL63 in vivo were not significantly reduced upon GαzQL co-expression (not shown).

In light of these findings, we investigated potential mechanisms for the observed Gαz-induced down-regulation. The downstream target of pertussis toxin (PTX)-sensitive Gαi family members is inhibition of adenylate cyclase activity (Wong et al., 1992), leading to

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reduced protein kinase A (PKA) activity. Although not a strong candidate target of (PTX)-insensitive Gαz, we nevertheless tested whether inhibition of adenylate cyclase or PKA activity by cell-permeable selective inhibitors leads to reduced Rho signals. Cells expressing RhoAL63, Lbc Rho GEF, GαqQL, or Gα12QL were treated separately with the adenylate cyclase inhibitor MDL-12 or PKA inhibitor H-89. Figure 5A shows that neither of these pharmacologic agents significantly reduced SRE reporter activity induced by any of the stimuli used, although they blocked Gαs-induced activation of a luciferase reporter encoding a CREB site (not shown). Serine phosphorylation of RhoA at S188 blocks RhoA signaling function (Lang et al., 1996), and the RhoAL63/A188 mutant is resistant to this inhibitory modification. Figure 5B shows that co-expression of GαzQL with RhoAL63/A188 led to a decrease in RhoAL63/A188-induced reporter activity, similar to its effect on RhoAL63/S188 signals.

To determine whether Gαz signals may lead to altered Rho subcellular localization, RhoAL63 was co-expressed with GαzQL or GαzG2GA in HEK293T, and cell lysates separated into cytosolic (soluble) and membrane-rich (pellet) fractions by high-speed fractionation. Figure 6 shows that in keeping with its activated state, a larger proportion of RhoAL63 localized to the pellet versus the soluble fraction when co-transfected with vector alone (top panel). Co-expression of GαzQL (middle panel) or GαzG2GA (lower panel) with RhoAL63 did not appear to alter the relative proportion of Rho in these fractions, as quantified by the graph. Additional experiments yielded the same outcome. Moreover, no changes in endogenous RhoA localization was observed under the same conditions (not shown).

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We next evaluated whether Gαz expression affects actin stress fiber formation, a cytoskeletal process which requires Rho function. For this purpose, quiescent Swiss 3T3 fibroblasts which contain few stress fibers were microinjected with a plasmid encoding an activated form of Net1 Rho GEF, Net1ΔN (Alberts and Treisman 1998), along with either vector, GαzQL or GαzG2A. After 2-4 hours, cells were fixed and stained for phalloidin to visualize actin. Figure 7B shows that Net1 Rho GEF microinjection led to increased stress fiber formation; moreover, this was not affected by co-expression of GαzQL or GαzG2A forms (C and D), and these results are quantified by the graph in Figure 7A. Similar results were obtained upon co-microinjection of RhoAL63 and GαzQL (not shown).

DISCUSSION

Our findings indicate that of the Gi alpha subunit family, Gαz expression has a potent inhibitory effect on Rho-induced signals to SRF/SRE. Whereas Gαz inhibits Gα12/13- and Gαq-induced SRE reporter activation, it has no effect on Gα12-induced Rho activation as determined by measuring GTP-Rho levels in vivo (Figure 1C). This indicates that Gαz expression does not interfere with the signaling ability of Gα12 *per se*. Hence it is unlikely that the observed inhibition is due to Gαz competition with other G alpha subunits for regulators of Gα signaling such as regulator of G protein signaling (RGS) proteins (Ross and Wilkie 2000), activators of G protein signaling (AGS) proteins and/or beta/gamma subunits (Blumer and Lanier 2003). Moreover, this finding implies

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that Gαz-induced inhibition occurs downstream of Gα12/13/q. The Gαi1 and Gαi2 family members tested here also attenuated Gα12/13 and Gαq-induced transcriptional signals to SRF, and in this case, competition with other G alpha subunits for G protein regulators cannot be ruled out. However, in contrast to Gαz, Gαi1 and Gαi2 had no substantial effect on Rho GEF- or RhoAL63-induced reporter acivation. The basis for this difference between Gαi isotypes and Gαz is not known at present, although such differences are in keeping with other reports (Ho and Wong 2001) indicating a distinct function for Gαz compared to other Gαi family members. Gαi is ubiquitously expressed, whereas Gαz expression is more restricted, and is found in adrenal medulla, hypothalamus, retina, neural tissues, and platelets, (Ho and Wong 2001), although recent reports indicate that Gαz may be expressed in a wider variety of tissues than previously thought (Nagahama et al. 2002; Hendry et al., 2000), and its precise role in signaling is poorly understood.

Our finding that Rho GEF-induced GTP-Rho levels in vivo is unaltered by Gαz makes it unlikely that the observed block of Rho GEF-induced reporter activation by Gαz is due to inhibition of Rho GEF function, a notion compatible with the idea that the target of Gαz action lies further downstream. The finding that Gαz did not effectively block SRE reporter induction by activated ROK suggests that the Gαz inhibitory effect may not extend to Rho effectors. However, while ROK induces SRF-mediated transcription under experimental conditions, it is not considered to be the main physiologic Rho effector that mediates SRF responses. The finding that Gαz, but not Gαi2 expression partially blocks serum-induced SRE reporter activity suggests that Gαz signals can attenuate endogenous signaling pathways to Rho, and implies that the endogenous

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effectors of Gαz are present in HEK293T. Use of Gαz fatty acylation mutants indicates that the observed inhibition requires correct plasma membrane localization of Gαz, a prerequisite for Gαz signal transduction (Morales et al., 1998). Serum-induced signals leading to Rho-dependent responses are transduced by Gα12/13 and Gαq (Seasholtz and Brown 1999); however, serum-induced GTP-RhoA formation was not affected by Gαz signals (not shown). This further supports the notion that Gα signaling function *per se* is not affected by Gαz, and suggests that Gαz targets a subsequent step on the signaling pathway. GPCR(s) which may inhibit signaling to SRF/SRE via Gαz are not known at present. However, recently for the first time, stimulation of a GPCR (somatostatin GPCR), has been shown to inhibit Rho-dependent responses (Buchan et al.,2002). This indicates the existence of GPCR-linked pathways which inhibit Rho, and is in keeping with our findings of Gαz detailed here, although whether Gαz is specifically involved in somatostatin-induced responses remains to be determined. The finding that Gαz inhibits Gα12/13 and Rho GEF-induced SRE reporter in PC12 neuronal cells which resemble Gαz–expressing tissue, suggests that the observed effect may also occur in Gαz-rich tissues, and is not restricted to HEK293T cells. The lack of effect of Gαz signals on the LEF/TCF transcriptional reporter makes it unlikely that Gαz signals block a common event required for transcriptional activation.

The observed inhibition of RhoAL63-induced SRE reporter activation by Gαz co-expression is unlikely to be due to modulation of a Rho regulator such as a Rho GEF or Rho GAP, since RhoAL63 activity is largely independent of these regulators. The lack of

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effect of GαzQL on GTP-RhoAL63 levels supports the notion that Gaz signals target the pathway at a point subsequent to Rho activation. The finding that wt Gαz is nearly as active as GαzQL in inhibiting RhoAL63 signals is notable though not unique, since transfected wild-type versions of Gα12/13 are also highly active in signaling to SRE luciferase reporter in HEK293T cells (Mao et al. 1998; Dutt et al., 2003). This likely reflects the ability of the sensitive luciferase reporter assay to detect some portion of the transfected wild-type Gα subunit which subsequently becomes activated in vivo.

The lack of effect of the adenylate cyclase and PKA inhibitors tested here on SRE reporter activity suggests that these signaling components (Wong et al., 1992) are not involved in the observed effect, and is consistent with existing data showing that these components mainly transduce signals by Gαi, rather than Gαz. Reports indicate the existence of Gi signaling pathways which involve effectors other than adenylate cyclase (Yang et al., 2002), and a number of alternate Gαz effectors and mediators have been proposed (Ho and Wong 2001). These include Rap1Gap (Meng et al., 1999), Rap1 GTPase (Woulfe et al, 2002), and GRIN (G protein regulated inducer of neurite outgrowth) (Chen et al., 1999), and the potential involvement of these components in the modulation described here warrants further investigation.

A possible basis for the Gαz-mediated effect is an affect on localization of a Rho signaling complex, and whereas altered RhoA subcellular localization in response to Gαz was not detected here, it is conceivable that localization of other components of a RhoA signaling complex may be altered. In addition, Gαz signals may lead to altered posttranslational modification(s) of Rho. Phosphorylation of RhoA on Ser^{188} by cAMP or

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cGMP-dependent kinases inhibits Rho activity (Lang et al., 1996) by promoting Rho binding to cytosolic Rho guanine–dissociation inhibitor (Rho GDI) (Ellerbroek et al., 2003). However, our finding that Gαz inhibits RhoAL63/A188 signals shows that this modification is not responsible for the effect, and together with the RhoAL63/S188 result, implies a block downstream of Rho. Yet another possible basis for the observed results is inhibitory modification of other components of a Rho signaling complex.

Since we did not observe any effect on Rho GEF-induced stress fiber formation when we co-microinjected Gαz in fibroblasts, this suggests that Gαz interferes at a point in the SRF activation pathway which is independent of actin rearrangement. However, the possibility that the Gαz expression level was insufficient to induce inhibition of stress fibers in this system cannot be ruled out. Moreover, additional assessment in cells other than fibroblasts, as well as on potentially more subtle effects on the actin cytoskeleton, is warranted. One potential target for the negative regulation of SRF/SRE by Gαz is MAL, recently shown to be an SRF coactivator that responds to changes in levels of G-actin, but has not been reported to regulate actin dynamics (Miralles et al., 2003). Another potential target is hCNK1, which is involved in Rho activation of SRF in an actin-independent manner (Jaffe et al., 2004). Interestingly, G-kinase induces a similar effect in that it inhibits SRE/SRF transcription, but does not appear to affect Rho-dependent cytokeletal responses (Gudi et al., 2002). The Gαz–induced transcriptional inhibition described here may play a role during certain stages of cell growth/differentiation which are accompanied by down-regulation of SRF-dependent genes. Thus, Gαz signals may target a component(s) which selectively mediates SRF-dependent transcriptional responses, and

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elucidation of such signaling events would further contribute to our understanding of how this important transcriptional target is regulated.

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FIGURE LEGENDS

Figure 1. Gαz expression inhibits Gα12/13- and Gαq-induced SRE.L reporter activity in HEK293T cells. A. Activated mutant cDNA forms of indicated Gαi family members or Gαs (100 ng) were co-expressed with either pcDNA vector (V) or Gα12QL, Gα13QL, or GαqQL in HEK293T cells in the presence of SRE.L luciferase reporter (100 ng). Dual luciferase activity was measured after 24 hrs. RLU=relative luciferase units. (*, $p< 0.003$ for the comparison of Gα12, Gα13 in the absence versus presence of Gαz; $p< 0.01$ for the comparison of Gαq in the absence versus presence of Gαz). C3 = C3 exoenzyme transferase. B. Top panel anti-Gα12 immunoblot (IB) shows Gα12QL (Gα12*) expression in cell lysates co-transfected with either pcDNA vector or indicated Gαi family members. Middle panel anti-Glu immunoblot shows expression of designated Glu-Glu-tagged activated Gαi family members in transfected cell lysates. Bottom panel anti-RhoA immunoblot shows endogenous RhoA expression in cell lysates expressing the indicated Gαi family members. C. Gα12QL (Gα12*) was co-expressed with either vector (V), Gαi2QL (Gαi2*) or GαzQL (Gαz*) in HEK293T, and GTP-RhoA pull-downs were carried out using Rhotekin RBD as detailed in Methods. Graph shows mean fold-change in GTP-RhoA densitometric values based on RhoA immunoblots of GTP-RhoA pull-down and total cellular RhoA from three experiments. Values were normalized to vector which was assigned the value of 1. *GTPase indicates the activated mutant form as described under Plasmids. Results are mean $\pm$ S.D.

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Figure 2. Gαz expression inhibits SRE.L reporter activation induced by Rho GEFs and serum, but not by ROK. A. Increasing amounts (50-200 ng) of GαzQL (Gαz*) or Gαz G2A (200 ng) were separately co-expressed with p115 RhoGEF, proto-Lbc Rho GEF or activated ROK in HEK293T cells, and dual luciferase levels measured after 24 hrs. ng=nanograms of transfected plasmid. RLU=relative luciferase units. Results are mean ± S.D. B. proto-Lbc Rho GEF was co-expressed with either pcDNA vector (V), Gαi2QL (Gαi2*) or GαzQL (Gαz*) in HEK293T, and GTP-RhoA pull-downs were carried out using Rhotekin RBD as detailed in Methods. Graph shows mean fold-change in GTP-RhoA densitometric values based on RhoA immunoblots of GTP-RhoA pull-down and total cellular RhoA from three experiments. Values were normalized to the vector alone group which was assigned the value of 1. C. Gαz expression attenuates serum-induced SRE.L reporter activity. HEK293T cells were transfected with increasing amounts (50, 100, 200 and 400 ngs) of activated GαzQL (Gαz*) or Gαi2QL (Gαi2*) along with 100 ngs SRE.L luciferase reporter. After overnight incubation in media lacking serum, cells were stimulated with 15% serum for 6 hours, and dual luciferase activity measured (*, $p<$ 0.03 for the comparison of serum-treated values in the absence versus presence of Gαz*).

Figure 3. Gαz inhibits SRE.L reporter induction in PC12 cells, but has no effect on a transcriptional induction of a Rho-insensitive reporter such as LEF/TCF luciferase reporter. A. Gα12QL (Gα12*), Gα13QL (Gα13*) or proto-Lbc Rho GEF cDNAs (2 ug) were co-expressed with either pcDNA vector or GαzQL (Gαz*) plasmid (2 ug) in PC12 cells in the presence of 300 ng SRE.L luciferase reporter, and dual luciferase activity measured. B. Activated β-cateninΔ45 (400 ng) was co-expressed with two different

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amounts (100 - 400 ng) of GαzQL (Gαz*) in the presence of the LEF/TCF TOPFLASH luciferase reporter, and dual luciferase activity was measured after 24 hrs. RLU=relative luciferase units. ng=nanograms of transfected plasmid. Results are mean ± S.D. from three experiments.

Figure 4. Inhibition of RhoAL63-induced SRE.L reporter activation by Gαz. A. RhoAL63 was expressed with either pcDNA vector (V), activated Gαi family subunits (Gα∗), wild-type Gαz (GαzWT) or Gαz fatty acylation mutants (GzG2A, GzG2AC3A) in HEK293T cells (100 ngs) in the presence of 100 ngs SRE.L luciferase reporter. Dual luciferase activity was measured after 24 hrs. RLU=relative luciferase units. . Results are mean ± S.D. from three experiments. B. Anti-myc immunoblot of myc:RhoAL63 from total cell lysates of a representative experiment as in (A) is shown. Gα∗ indicates activated mutant forms as described under Plasmids

Figure 5. A. Effect of pharmacologic inhibitors on SRE.L reporter activity, and Rho serine 188 phosphorylation on Gαz-mediated inhibition of SRE.L reporter activity. A. HEK293T were transfected with the indicated plasmids along with 100 ng SRE.L luciferase reporter, and incubated overnight. Gα* indicates activated mutant forms as described under Plasmids. Following treatment with the adenylate cyclase inhibitor MDL-12 (100 μM) or the PKA inhibitor H-89 (400 nM) for 6 h, dual luciferase levels were assayed. B. 100 ng RhoAL63/S188 or RhoAL63/A188 was co-expressed with 100 ng or 200 ng of GαzQL (Gαz*) in the presence of SRE.L reporter, and dual luciferase activity assayed after 24 hrs. (*, $p < 0.01$ for the comparison of RhoAL63/S188 or

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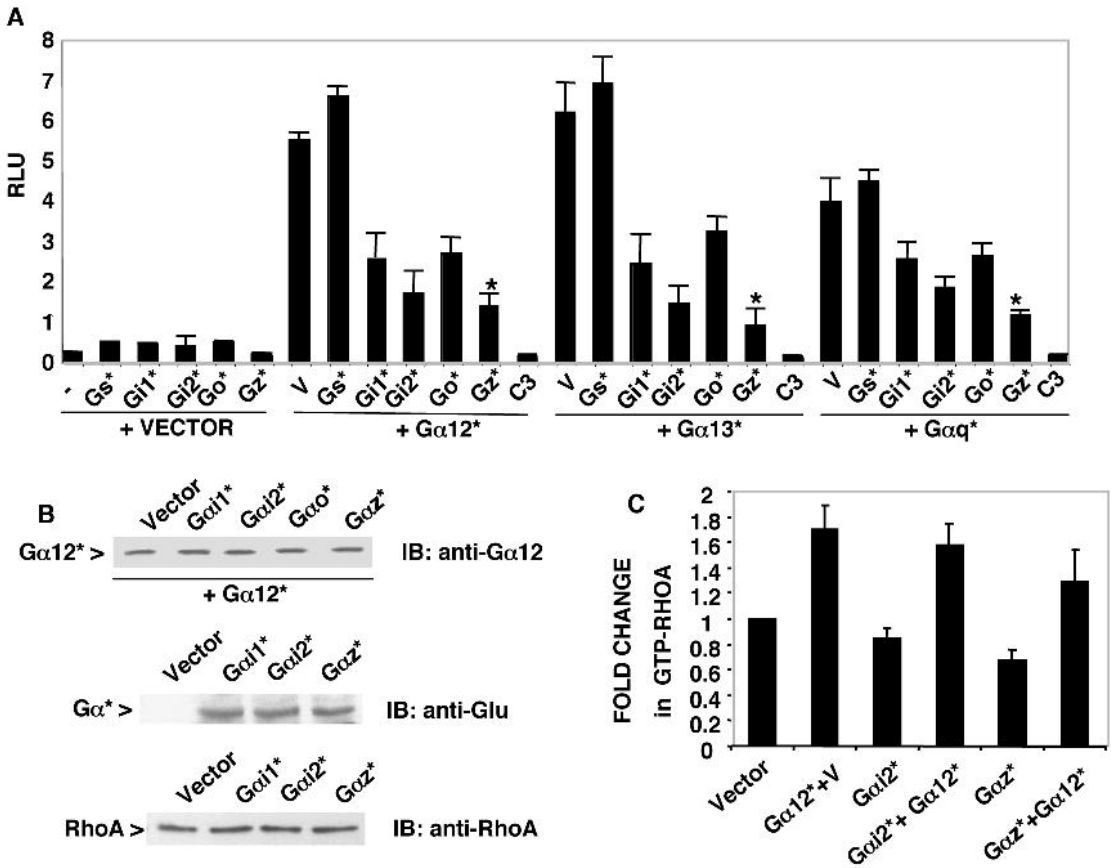
RhoAL63/A188 in the absence versus presence of GαzQL). ng = nanograms; RLU=relative luciferase units; V=pcDNA vector. Results are mean ± S.D.

Figure 6. Gαz expression does not alter RhoAL63 subcellular localization. In this representative experiment, HEK293T cells were co-transfected with 2 μg of pcDNA:Myc:RhoAL63 plus 2 μg of either pcDNA vector, pcDNA:Gαq Q209L, or pcDNA:GαzG2A as indicated. Twenty-four hours post-transfection, cells were collected and fractionated into P100 soluble and pellet fractions as previously described (19). Equal volumes of soluble (S) and pellet (P) fractions were separated by 10% SDS PAGE and immunoblotted for Myc:RhoAL63 using an anti-Myc epitope antibody. Graph shows relative RhoAL63 band densities from immunoblots as quantified using an Alpha Innotech IS-2200 Digital Imaging System and software. The ratio of the RhoAL63 soluble fraction band density:pellet fraction band density was calculated for each transfection group and plotted.

Figure 7. Gαz expression does not influence Rho-GEF-induced actin stress fiber formation. A. Quantitation of the effect of activated and inactive forms of Gαz on stress fiber formation induced by the oncogenic form of the Net1 RhoGEF, Net1ΔN. Data are expressed as mean ± SD of at least three experiments, in which a minimum of 40 expressing cells were counted per experiment. (B-D). Representative images of confluent quiescent, serum-starved Swiss 3T3 fibroblasts injected with expression constructs for Net1ΔN, together with empty vector (B), GαzQL (C), or Gαz G2A (D). Injected cells are marked with an arrowhead.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Gα12* >
IB: anti-Gα12
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IB: anti-Glu
RhoA >
IB: anti-RhoA
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Gαz*
Gαz*+Gα12*

Figure 1

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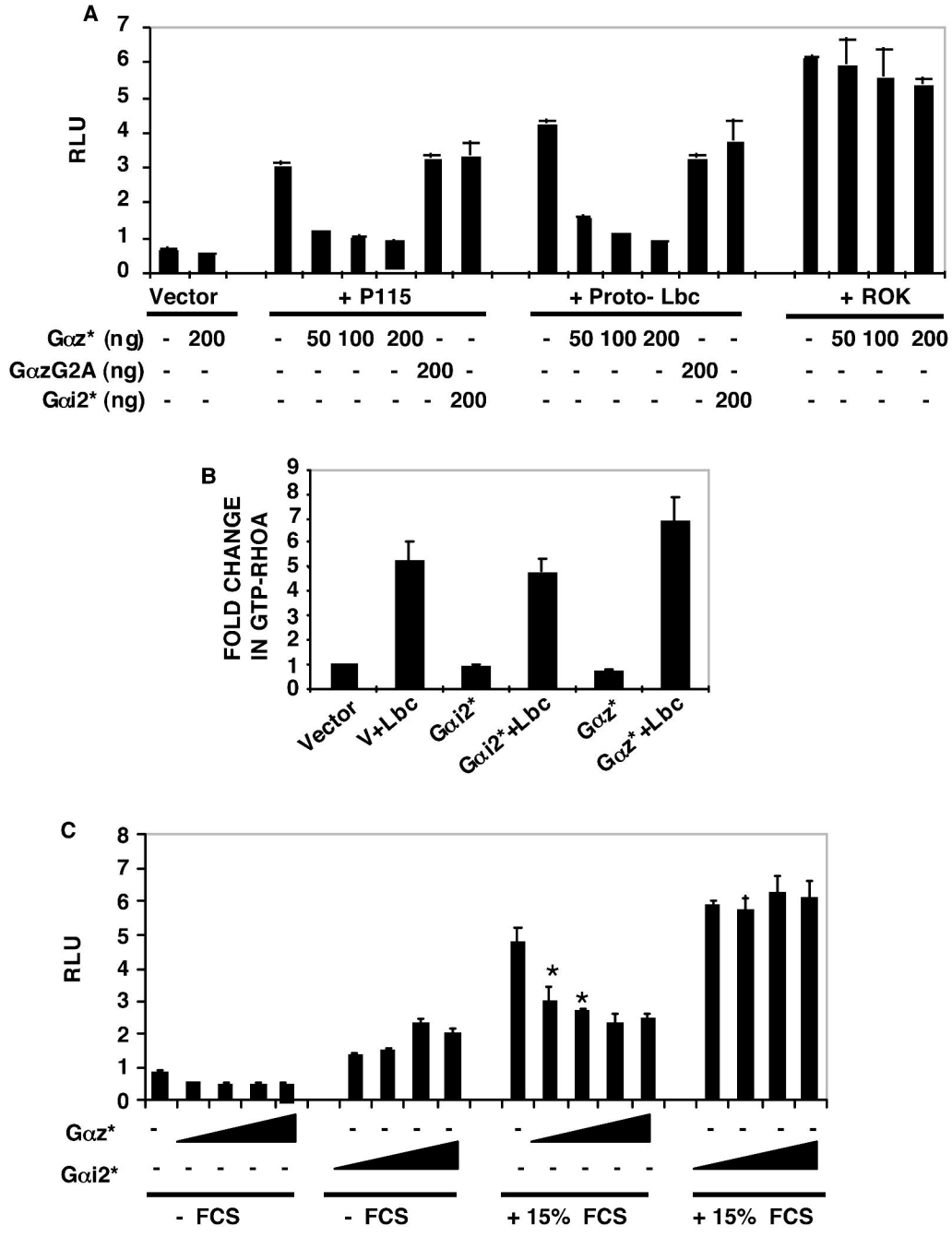
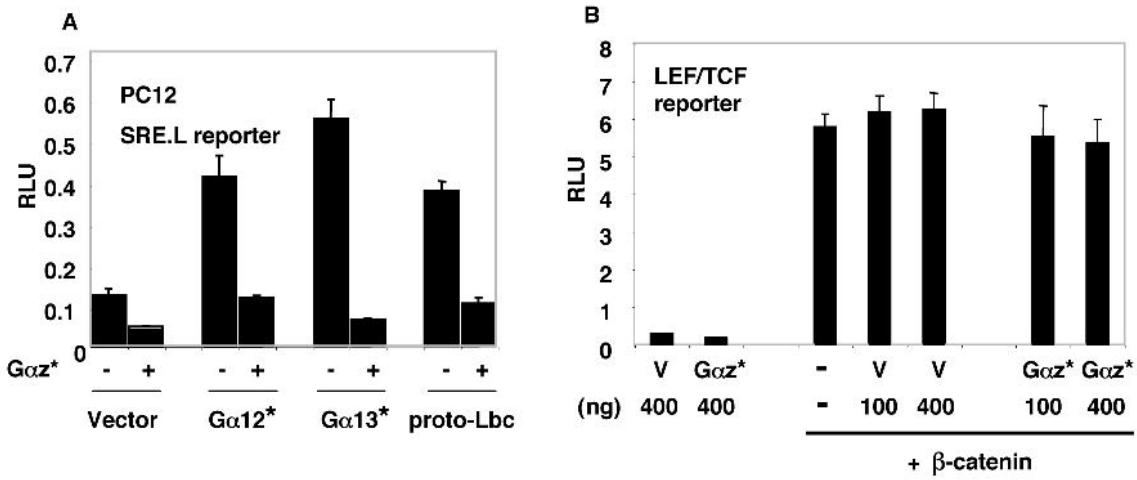


Figure 2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Vector
Gα12*
Gα13*
proto-Lbc
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LEF/TCF
reporter
RLU
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(ng) 400 400 - 100 400 100 400
+ β-catenin

Figure 3

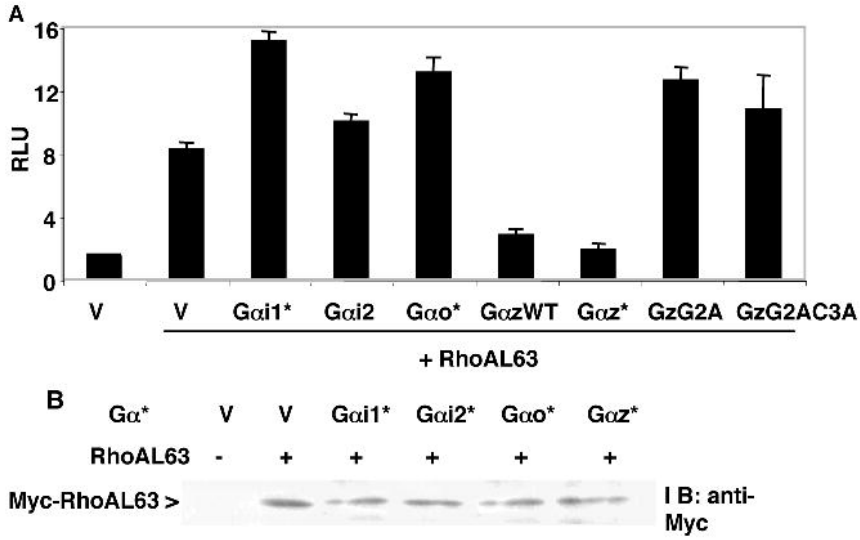
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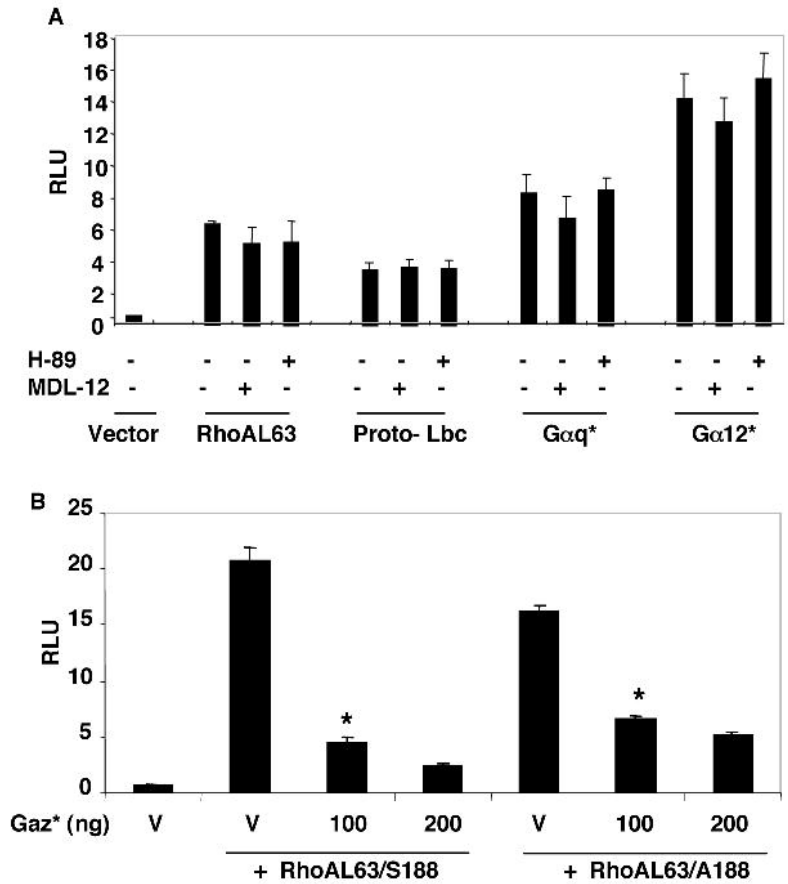
Figure 4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RhoAL63
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+ RhoAL63/S188
+ RhoAL63/A188

Figure 5

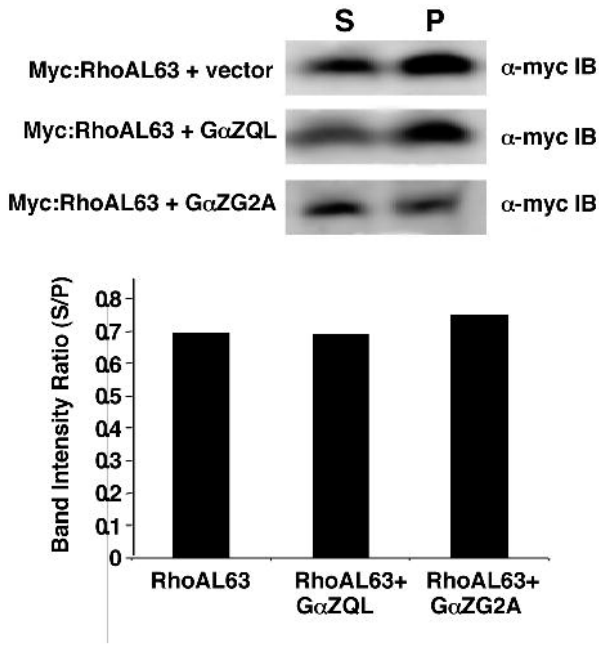
S
P
Myc:RhoAL63 + vector
α-myc IB
Myc:RhoAL63 + GαZQL
α-myc IB
Myc:RhoAL63 + GαZG2A
α-myc IB
Band Intensity Ratio (S/P)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
RhoAL63
RhoAL63+ GαZQL
RhoAL63+ GαZG2A

Figure 6

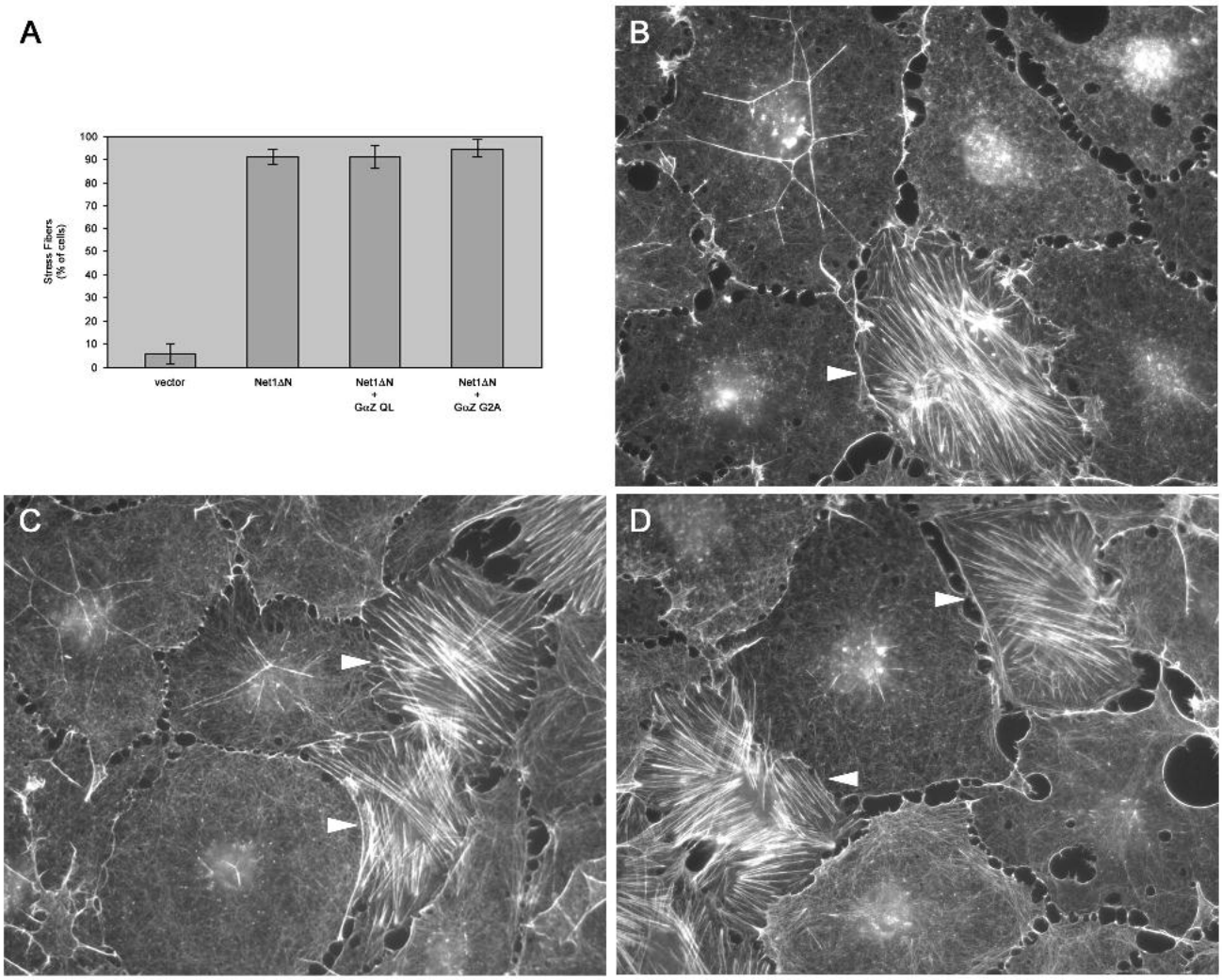
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Figure 7