The α Subunit of the G Protein G_{13} Regulates Activity of One or More Gli Transcription Factors Independently of Smoothened*‡§

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Smoothened (Smo) is a seven-transmembrane (7-TM) receptor that is essential to most actions of the Hedgehog family of morphogens. We found previously that Smo couples to members of the G_{i} family of heterotrimeric G proteins, which in some cases are integral although alone insufficient in the activation of Gli transcription factors through Hedgehog signaling. In response to a report that the G_{12/13} family is relevant to Hedgehog signaling as well, we re-evaluated the coupling of Smo to one member of this family, G_{13}, and investigated the capacity of this and other G proteins to activate one or more forms of Gli. We found no evidence that Smo couples directly to G_{13}. We found nonetheless that G_{13} and to some extent G_{o} and G_{12} are able to affect activation of Gli(s). This capacity is realized in some cells, e.g. C3H10T1/2, MC3T3, and pancreatic cancer cells, but not all cells. The mechanism employed is distinct from that achieved through canonical Hedgehog signaling, as the activation does not involve autocrine signaling or in any other way require active Smo and does not necessarily involve enhanced transcription of Gli1. The activation by G_{13} can be replicated through a G_{q}/G_{12/13}-coupled receptor, CCK_{A}, and is attenuated by inhibitors of p38 mitogen-activated protein kinase and Tec tyrosine kinases. We posit that G proteins, and perhaps G_{13} in particular, provide access to Gli that is independent of Smo and that they thus establish a basis for control of at least some forms of Gli-mediated transcription apart from Hedgehogs.

The Hedgehog family of secreted proteins is essential to cell proliferation and differentiation in an array of developmental phenomena. Among the most actively studied of these in vertebrates are induction of ventral cell fates in the central nervous system and patterning of the anterior-posterior axis of the developing limb (1–3). The Hedgehog family also assumes homeostatic roles in postembryonic tissues, for example in the maintenance of certain stem cell populations (4–7). Deficits in one or more components of signaling translate into developmental syndromes and malformations (8, 9), whereas unrepressed signaling underlies several forms of cancer (10–12).

Hedgehogs in mammals exert their actions primarily through modulation of the Gli family of zinc finger transcription factors (13, 14). Here, the seven-transmembrane (7-TM)² protein Smoothened (Smo) occupies a central position. Hedgehogs activate Smo through binding to Ptc1 (PATCHED 1), a 12-transmembrane protein at the cell surface that in some fashion normally holds Smo in a repressed conformation. Hedgehogs remove the inhibitory constraints of Ptc1, a process coupled with recruitment of Smo to the primary cilium. Activated Smo stabilizes Gli1 and Gli3, causing derepression of some genes and frank activation of others. Among the latter is that encoding Gli1, the third member of the Gli family.

Most efforts to understand forms of transduction employed by Smo have focused on transport and scaffolding (14). We contend that the interaction of Smo with heterotrimeric G proteins in relation to or apart from modulation of the Gli transcription factors is relevant as well (15–17). We demonstrated in studies with [35S]GTPγS binding in heterologous expression systems that Smo activates members of the G_{i} family, and that one or more forms of G_{i} are required in the course of Shh (Sonic hedgehog) signaling to Gli in NIH3T3 cells (15). The data regarding coupling of Smo to G_{i} are in accord with effects of a pertussis toxin on Hedgehog-induced pigment aggregation (18), capillary morphogenesis (19), and (in zebrafish) selected aspects of eye, brain, and somite patterning (20). They are also consistent with the effects of dsRNA-mediated knockdown of G_{o} on levels of CAMP and activation of Cubitus interruptus in Drosophila (21).

The coupling of Smo to G proteins evaluated through [35S]GTPγS binding using membranes of SF9 (a clonal cell line derived from Spodoptera frugiperda) cells expressing these proteins was specific for members of the G_{i} family. Smo did not, in these studies, affect activation of G_{o}, G_{q}, G_{12}, or G_{13}. We were
interested therefore in a report by Kasai et al. (22) that the activation of Gli in HEK293 cells by Shh is inhibited by the RGS (regulator of G protein signaling) domain of p115RhoGEF, a domain that can inhibit G_{12} and G_{13} signaling, and by inhibitors of Rho, a monomeric G protein downstream of G_{12} and G_{13}. G_{12} and G_{13} have received considerable attention in a number of phenomena relevant to developmental and oncogenic events (23).

The relevance of the G_{12/13} family to modulation of Gli activity by and apart from Smo was explored in this study. We find no evidence for the coupling of Smo to G_{13} or G_{13} in HEK293 cells in affirmation of our previous work with insect cells. We find, nevertheless, that the α subunit of G_{13}, and to some extent those of G_{12} and G_{12}, are capable of activating one or more forms of Gli. The activation does not involve autocrine signaling, occurs in some but not all cells, and can be recapitated by a 7-TM receptor coupled to endogenous G_{12}, G_{13}, and G_{12}. It is attenuated by inhibitors of p38 mitogen-activated protein kinase and Tec tyrosine kinases. We posit that G_{13} and to some extent G_{12} and G_{12} provide an access to Gli that is independent of Smo and thus a basis for control of at least some forms of Gli transcription apart from Hedgehogs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclopamine, purmorphamine, KN-92, KN-93, SP600125, Y27632, diltiazam, nifedipine, and verapamil were obtained from EMD Biosciences (San Diego, CA). 8-Hydroxy-2-(dipropylamino)tetratin hydrobromide (DPAT), pertussis toxin, and a rabbit antibody specific for Gli1 were obtained from Sigma-Aldrich. Transforming growth factor-β and a goat antibody specific for Gli1 were obtained from R&D Systems (Minneapolis, MN). SB202190 and LFM-A13 were obtained from Tocris Bioscience (Ellisville, MO). 5-Fluro-2-indolyl des-chlorohalopemide, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid (U46619), and halopemide were obtained from Cayman Chemicals (Ann Arbor, MI). Chloroethyltinin-8 (CCK-8) was obtained from Peninsula Laboratories (Belmont, CA). [35S]GTPγS was purchased from PerkinElmer Life Sciences. Rabbit antisera specific for Ga subunits were described previously (24).

**Plasmid Constructs**—Expression constructs for constitutively active Ga subunits (in which leucine is substituted for glutamine in the DVGQQ motif) were obtained from the Missouri cDNA Resource Center (Rolla, MO) and Dr. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). Vectors for SmoWT, SmoM2, and Shh were provided by Dr. Philip Beachy (Stanford University, Palo Alto, CA). Constructs for mouse Gli2 and both normal and mutated 8xGli luciferase reporters were obtained from Dr. Hiroshi Sasaki (RIKEN Center for Developmental Biology, Kobe, Japan) and, for experiments with pancreatic cells, Dr. Chi-chung Hui (Research Institute, Toronto, Ontario, Canada). The Bcl-2 promoter luciferase reporter was provided by Dr. Linda Boxer (Stanford University). TPα-Gα_{13} was constructed as described previously (25). Smo-Gα_{13} was constructed in a similar fashion, i.e., by PCR-directed mutagenesis, using mouse Smo cDNA; the sequence was confirmed by automated sequencing. The sequence of the fusion immediately distal to the 7-TM domain of Smo was 5′-RRTWCRLT-GHSDDPEKR^{566}−2ADFLPSR5V11 (Smo/G_{13}). shRNA was designed as described previously (26). The following shGli1-targeted sequences were used: CCGTCCCTGTCCAGCTATGGTcaagagaTCTAGCTGGACGGAGCAGG (shGli1, sense), CCTGCCATTCTTGCA-CCATcttcagagaATGGTCAGAATGGCGAG (scrambled, sense), CCTGCCATTCTTGCACTcttcagagaATGGTCGACGAATGGCGAG (scrambled, antisense); capital letters represent the target sequences of the shRNA.

**Cell Culture and Transfection**—C3H10T1/2 cells (CCL-266), MC3T3 cells (CRL-2593), and HEK293 cells (CRL-1573) were obtained from the American Type Culture Collection (Manassas, VA). C3H10T1/2 cells were maintained in basal medium Eagle (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. MC3T3 were maintained in minimal essential medium α supplemented with l-glutamine, ribonucleosides, and deoxyribonucleosides, without ascorbic acid (Invitrogen). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum added. All cell lines were grown at 37 °C with 5% CO₂. For transfection, C3H10T1/2 and MC3T3 cells were seeded at 1.5 × 10⁴ cells/well in 24-well plates and transfected with 0.1 µg firefly reporter, 0.01 µg TK Renilla reporter, and 0.13 µg construct DNA per well using FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s suggested protocol. HEK293 cells were seeded at 3.5 × 10⁴ cells/well and transfected with 0.17 µg 8xGli reporter, 0.017 µg TK Renilla reporter, and 0.2 µg of construct DNA per well using Lipofectamine 2000 (Invitrogen). Serum was usually decreased to 0.5% upon attainment of confluence, and production of luciferases was assayed 24–36 h thereafter. Where noted, pertussis toxin (100 ng/ml) was added at the time serum was decreased; enzyme and channel inhibitors were added as noted 24 h prior to assay. Luciferase activities were determined using the Dual-Luciferase Reporter Assay (Promega, Madison, WI). Firefly luciferase activity was normalized to Renilla luciferase activity.

The human pancreatic cancer cell lines Panc1 (CRL-1469) and AsPC1 (CRL-1682) and the rat pancreatic acinar cell line AR42J (CRL-1492) were obtained from the American Type Culture Collection. The human cancer cell line L3.6 was kindly provided by Dr. Isaac Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). L3.6 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 100 units/ml penicillin/100 units/ml streptomycin. L3.6 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum sodium pyruvate, nonessential amino acids, l-glutamine, and a 2-fold vitamin solution (LifeTechnologies, Rockville, MD). All pancreatic cells but Panc1 cells were transfected by electroporation at 350 V using a 10-µs pulse; Panc1 cells were electroporated at 260 V using two 10-µs pulses (BTX, Harvard Apparatus, Holliston, MA); 4 × 10⁵ cells were placed in each cuvette together with 2 µg of firefly luciferase reporter. Where noted, 5 µg hCKK_{α} or Gα_{13}QL were additionally included. After electroporation, 2.5 × 10⁵ cells were seeded into each well of a six-well plate for luciferase assays, whereas
shown in Fig. 1 contrast to the data for Gi, no activation of G13 by Smo was evident with or without agonist (Fig. 1B). Membranes (20 μg protein) were incubated with vehicle or agonist for 10 min at 30 °C then additionally with 5 nM [35S]GTPγS for another 10 min prior to solubilization and immunoprecipitation with Ga α subunit-directed antibodies and scintillation spectrometry.

RESULTS

We and others (15, 18, 19, 21) demonstrated the capacity of Smo to couple to the Gi family of G proteins. Our data regarding specificity, wherein Smo was deemed unable to couple to Gαs, Gαq, Gα12, or Gα13, were based on a reconstitution paradigm using membranes from SF9 cells (15). Kasai et al. (22), however, have argued the involvement of Gα13 and/or Gα11 using HEK293 cells, noting inhibition of an Shh-activated Gli reporter by the RGS domain of p115 RhoGEF. Because processing and/or targeting of Smo might differ between SF9 and HEK293 cells, and because activation of Gα12 and/or Gα13 in the latter report was not measured directly, we evaluated the activation of the two G proteins in HEK293 cells. Smo was introduced into the cells by transfection, and activation was evaluated in subsequently isolated membranes by [35S]GTPγS binding to selected Ga α subunits. As shown in Fig. 1A, Smo promoted the binding of [35S]GTPγS to the one or more forms of Gαi endogenous to HEK293 cells, as anticipated. The activity of Smo without agonist was equivalent to that with agonist (purmorphamine). The lack of agonist-promoted activity is expected for cells not expressing Ptch1 or in which levels of Smo otherwise exceed the repressive actions of Ptch1. The 5-HT1A receptor was used for comparison. In C3H10T1/2 cells are responsive to Gαq, and Gα12 and Gα13 in these cells.

In extending experiments with the GaQL mutants to another commonly employed model of Hedgehog signaling, C3H10T1/2 cells, we found that the subunits are not always without effect on Gli activity. Substantial activation of the Gli reporter was noted for Gα13QL in these cells (Fig. 2); activation was equivalent to that of SmoM2. Activation was achieved to some degree as well by GαqQL and Gα12QL, whereas reporter activity was suppressed by GαsQL. The activation by Gα13QL was specific for the Gli recognition sequence, as no activation was achieved for a reporter in which the octameric recognition sequences were mutated (5-GAAAGTGGGA; Fig. 2, inset). Thus, in contrast to HEK293 and NIH3T3 cells, one or more forms of Gli in C3H10T1/2 cells are responsive to GαQL subunits, with activation by Gα13QL the most substantive.

To address the possibility that Gα13QL in C3H10T1/2 cells operates through production of Shh or some other Hedgehog, i.e. that it proceeds through autocrine signaling, we employed KAAD-cyclopamine, which as an inverse agonist for Smo not only inhibits the actions of Hedgehogs but decreases Smo constitutive activity (15). We found in C3H10T1/2 cells that KAAD-cyclopamine inhibits the actions of Shh but fails to block those of Gα13QL (Fig. 3). This result demonstrates that Gα13QL acts downstream or entirely apart from pathways engaged by Smo.

The previous experiments employed constitutively active Ga subunits. Wild type Gα12 and Gα13 were also found to promote activity of the Gli reporter (Fig. 4A), albeit the actions of these (and again Gα12QL) are considerably less than that of Gα13QL. We compared the activities of these subunits in relation to the SRF reporter gene as well (Fig. 4B). Whereas activities among the subunits toward Gli differed considerably, those toward SRF did not. These data suggest that Gli(s) is more sensitive to the nature of the subunit than SRF. Also depicted in Fig. 4 are data that show a GTPase-deficient form of RhoA (RhoA(G14V)), though activating SRF, is unable to activate Gli.

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The remaining cells were plated on a 10-cm plate for expression controls. Cells were harvested and prepared for luciferase assays. To control for intersample variations in transfection efficiency, total protein for samples on each plate was quantitated using Bio-Rad protein assay (Bio-Rad), and luciferase readouts were normalized to protein content. For knockdown of expression, 18 μg of shGli1 or scrambled shRNA were used.

[^3S]GTPγS Binding—The assay for receptor-promoted binding of [35S]GTPγS to Ga subunits was performed as described previously (27). Membranes (20 μg protein) were incubated with vehicle or agonist for 10 min at 30 °C then additionally with 5 nM [35S]GTPγS for another 10 min prior to solubilization and immunoprecipitation with Ga α subunit-di-
We evaluated the activation of the Gli reporter by G\textsubscript{13}QL in several other cells as well. C3H10T1/2 cells are equivalent to mesenchymal stem cells (28), therefore we examined MC3T3 and C2C12 cells, which reside in osteoblastic and myogenic lineages, respectively. Activation of the reporter by G\textsubscript{13}QL was noted in MC3T3 (Fig. 5) but not C2C12 cells (data not shown). We also examined human pancreatic cancer cell lines, for which Smo-independent forms of Gli activation have been reported (29–31). Activation of the reporter was noted in all the cells examined, i.e. L3.6, PANC1, and AsPC1 cells. These results do not constitute an...
extensive survey of course; however, they support the notion that the activity of G_{13}QL toward Gli, although not universal, is not uncommon.

We also evaluated the actions of G_{13}QL in the context of the bcl-2 promoter, which contains three Gli-binding sites and mediates up-regulation of bcl-2 by Gli in settings of cell survival (32, 33). We used L3.6 cells in these experiments, as the electroporation protocol devised for them represents an efficient means of introducing DNA and monitoring sequellae for a large population of cells. G_{13}QL activates the reporter containing the bcl-2 promoter (Fig. 6A, filled bars), as it does that bearing the concatameric Gli recognition sequences (see above). The activation is inhibited by Gli1-targeted shRNA (open bars). Panel B shows that Gli1 is expressed in L3.6 cells, does not increase in response to G_{13}QL, and is reduced by ~70% by the targeted shRNA. These data demonstrate that the bcl-2 promoter is activated by G_{13}QL and that the activation requires Gli1 but is not attributable to an increase in this transcription factor.

CCK_{A} is a G_{a} and G_{12/13}-coupled receptor for cholecystokinin (34, 35). To evaluate activation of Gli in response to the activity of such a receptor, we turned to PANC1 cells in which CCK_{A} was introduced through transfection. Activation of the Gli reporter was found to occur in this setting (Fig. 7A), ostensibly through receptor-constitutive activity. We turned as well to the pancreatic AR42J acinar cell line, in which CCK_{A} is expressed normally. We found that treatment of AR42J cells with the agonist CCK-8 effects activation of the Gli reporter. Thus, the activation of Gli(s) occurs not only in response to introduced wild type and constitutively active G_{12/13} subunits, but to a 7-TM receptor (other than Smo) as well.

The mechanism by which G_{13} activates Gli remains to be determined. Studies with C3 exotoxin and Rho-targeted siRNA in C3H10T1/2 cells offer no remarkable insight, nor do inhibitors of Rho kinase (Y27632), c-Jun N-terminal kinases (SP600125), phospholipase D2 (halopemide, 5-fluoro-2-indolyl des-chlorohalopemide), and the protein tyrosine kinase PYK2 (dantrolene). We find that G_{a}catenin, which is sometimes an effector for G_{13} (36, 37) and is cited to be a mediator of TGF-β activation of Gli2 (38), is not activated by G_{13} nor able to activate Gli in these cells. Inhibitors of p38(s) (SB202190) and Tec tyrosine kinases (LFA-A13) inhibit G_{a}catenin-stimulated Gli activity by 40–60% (Fig. 8); however, additional work will be required to evaluate the exact identity of the
targeted proteins. KN-93, an inhibitor of Ca\(^{2+}\)/calmodulin kinase II (39), another target for G\(_{\text{q}}\) (39, 13), has a substantial effect on Gli reporter activity, but so does KN-92, an analog of KN-93 having no activity toward the kinase. Both KN92 and KN93 inhibit L-type Ca\(^{2+}\) channels (40), among other targets (41–43); however, additional inhibitors of these channels, i.e. nifedipine, verapamil, and diltiazam, are without effect on G\(_{\text{q}}\)QL-stimulated Gli reporter activity.

**DISCUSSION**

We demonstrate here the capacity of the G\(_{\text{13}}\) subunit of the heterotrimeric G protein G\(_{\text{13}}\), and of G\(_{\text{q}}\) and G\(_{\text{12}}\) as well, to activate one or more members of the Gli family of transcription factors. The mechanism employed by G\(_{\text{13}}\) is distinct from that engaged through canonical Hedgehog signaling: the activation does not require Smo, nor does it necessarily involve an increase in Gli1. We demonstrate as well that while activation of Gli(s) can be achieved through a receptor (CCK\(_{\alpha}\)) coupled to G\(_{\text{13}}\) Smo is not one such receptor. We posit that G\(_{\text{13}}\), and to some extent G\(_{\text{q}}\) and G\(_{\text{12}}\), serve as a means of increasing Gli transcriptional activity in a fashion altogether independent of Hedgehogs.

We were unable to corroborate the arguments of Kasai et al. (22) that the \(\alpha\) subunit of G\(_{12}\) or G\(_{13}\) plays a significant role in signaling through Smo in HEK293 cells. Smo does not activate G\(_{12}\) or G\(_{13}\) directly in these cells, as it is unable to promote exchange of GDP for \(^{35}\)S-GTP\(_{\gamma}\)S on any Gs subunit endogenous to them except those of the G\(_{i}\) family. Smo is also unable to activate G\(_{\alpha_{13}}\) to which it is fused for the purposes of amplification. Our data regarding specificity for the G\(_{i}\) family are in agreement with those obtained by us previously for SF9 cells.
made to express Smo and individual G proteins (15). It is conceivable that G13 can be activated indirectly by Smoothened, however the specific events by which this might occur are unclear. We were also unable to demonstrate an effect of Gαq, or of Gα12, on Gli activity in these cells. Kasai et al. (22) used a reporter under control of the genomic sequence of the gli1 promoter region (−397 to +216), with an intended selectivity for Gli3 activity. Our reporter was a concatenated set of GAACACC-CCA sequences, which is responsive to Gli1, Gli2, and conceivably Gli3. The difference in results may therefore relate to Gli-binding elements. Our studies using mutation of the concatenated reporter and Gli1-targeted shRNA preclude this from Gli-binding elements. Our studies using mutation of the concatenated reporter and Gli1-targeted shRNA preclude this from Gli-binding elements. Our studies using mutation of the concatenated reporter and Gli1-targeted shRNA preclude this from Gli-binding elements.

Distinctions among Gli1, Gli2, and Gli3 as the transcription factors targeted by Ga subunits, and the mechanism by which the subunits elicit activation of one or more of them, remain to be investigated. We suspect from our work with Ga13 in PANC1 cells that the activation is selective for Gli1, as activation of the bcl-2 promoter requires Gli1, and expression of Gli1 does not increase with Ga13, consistent with absence of input from Gli2 and Gli3. The latter observation is also a departure from what is observed with Hedgehog signaling (16).

Defining the path by which G13 activates Gli1 will, of course, be key to understanding the selectivity of activation among cells. RhoA is often utilized by G13; however, we find no evidence that it is employed in the context of Gli activation. β-catenin and TGF-β, too, are without effect, implying that any Ga13-initiated TGF-β autocrine loop is irrelevant. Other targets for G13 have been evaluated, with p38 and Tec tyrosine kinase offering promise; however, the list of targets to be evaluated is far from complete.

Activation of Gli transcription factors has long been thought to be the domain of Hedgehogs alone, but additional reports have challenged this notion. TGF-β controls the expression of
Gli1 and Gli2 in a variety of cells independently of Hedgehogs through Smad-dependent processes (31, 38, 44). The transcriptional nature of regulation of Gli2 in the case of TGF-β, owing to SMAD and lymphoid enhancer factor/T cell factor binding elements in the promoter region of gli2 (38), departs from the mechanism by which Hedgehogs activate Gli2, which involves inhibition of Gli2 degradation (45). Oncogenic K-Ras activates Gli1 in a variety of cells independent of Hedgehogs as well. Mechanisms involve an increase in Gli1 expression (29, 46) and facilitated translocation of Gli1 to the nucleus (46). The absence of an increase in Gli1 expression with Gα13 is a clear distinction from these reports.

Heterotrimeric G proteins can confer to agonists beyond Hedgehogs the potential to increase Gli activity. We believe this notion is exemplified by CCK. The nature and scope of signaling by such agonists will certainly differ from that by Hedgehogs. Beyond probable differences in selectivity among Gli transcription factors, agonists will certainly engage transcription with Gα13 and is germane as well, we believe, to many of the actions exerted through Smo that are presumed or known to be non-genomic in nature (17, 47–49). Gα13 does not serve in the same capacity, as Gα13 does not directly couple to Smo. Yet, Gα13 has an impact on Gli-mediated transcription and hence at least a subset of the events controlled through Smo.

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