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Smoothened Is a Fully Competent Activator of the Heterotrimeric G Protein G_i

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ABSTRACT

Smoothened (Smo) is a 7-transmembrane protein essential to the activation of Gli transcription factors (Gli) by hedgehog morphogens. The structure of Smo implies interactions with heterotrimeric G proteins, but the degree to which G proteins participate in the actions of hedgehogs remains controversial. We posit that the G_i family of G proteins provides to hedgehogs the ability to expand well beyond the bounds of Gli. In this regard, we evaluate here the efficacy of Smo as it relates to the activation of G_i, by comparing Smo with the 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor, a quintessential G_i-coupled receptor. We find that with use of [^{35}S]guanosine 5′-(3-O-thio) triphosphate, first, with forms of G_i endogenous to human embryonic kidney (HEK)-293 cells made to express epitope-tagged receptors and, second, with individual forms of $G\alpha_i$ fused to the *C* terminus of each receptor, Smo is equivalent to the 5-HT$_{1A}$ receptor in the assay as it relates to capacity to activate G_i. This finding is true regardless of subtype of G_i (e.g., G_{i2}, G_o, and G_z) tested. We also find that Smo endogenous to HEK-293 cells, ostensibly through inhibition of adenylyl cyclase, decreases intracellular levels of cAMP. The results indicate that Smo is a receptor that can engage not only Gli but also other more immediate effectors.

Introduction

Smoothened (Smo) is a 7-transmembrane protein essential to most of the actions of the hedgehog family of morphogens (Jiang and Hui, 2008; Wilson and Chuang, 2010). When derepressed through binding of hedgehogs to patched1 (Ptch1), Smo engages a series of proteins that lead to stabilization of the Gli2 and Gli3 zinc-finger transcription factors and to subsequent expression of Gli1. Consequent derepression and/or frank activation of diverse sets of genes leads to cell type–dependent programs of response.

Efforts to understand the means by which Smo stabilizes the Gli transcription factors (Gli) have focused mostly on transport of Smo to the primary cilium and subsequent forms of scaffolding (Wilson and Chuang, 2010). However, the structure of Smo is that of a G protein–coupled receptor, suggesting interactions with heterotrimeric G proteins and, thus, additional forms of transduction. We determined that Smo indeed activates members of the G_i family of G proteins (i.e., G_i, G_o, and G_z) (Riobo et al., 2006). We also determined that G_i is required in the activation of Gli in NIH 3T3 fibroblasts. The data are in accord with the effects of a *Bordetella pertussis*

toxin (Ptx) on selected aspects of eye, brain, and somite patterning in zebrafish (Hammerschmidt and McMahon, 1998) and the effects of dsRNA-mediated knockdown of $G\alpha_i$ in *Drosophila* on the activation of Cubitus interruptus, a Gli homolog (Ogden et al., 2008). The requirement for G_i in the activation of Gli is, however, not universal. Ptx has no impact on Gli-dependent patterning events in zebrafish beyond those noted above, nor does it have an impact on Gli-dependent chick neural tube patterning (Low et al., 2008).

The question of a role for G_i in hedgehog signaling placed in its most frequent context of Gli activation is, we believe, too narrowly framed. That Smo couples to G_i implies a potential for hedgehogs to regulate any number of pathways other than those that might bear on the activity of Gli. In this context, Smo may exhibit actions comparable to those of any other G_i-coupled receptor, whose importance need not relate to transcription much less Gli. We have referred to signaling achieved by Smo through G_i other than Gli as noncanonical (Brennan et al., 2012). In accordance with this notion, we found that migration of murine embryo fibroblasts in response to sonic hedgehog (Shh) through Smo requires G_i but does not involve Gli (Polizio et al., 2011). Migration requires, instead, sequential activation of phosphatidylinositol 3-kinase (PI 3-kinase) and the monomeric G proteins Rac and Rho. PI 3-kinase is a well-documented target for G_i, as is Rac for PI 3-kinase and, in some cells, Rho for Rac. Use of of

ABBREVIATIONS: 5-HT$_{1A}$, 5-hydroxytryptamine$_{1A}$; 8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin hydrobromide; BSA, bovine serum albumin; FITC, fluoresceine isothiocyanate; Gli, Gli transcription factors; GTPγS, guanosine 5′-(3-O-thio)triphosphate; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI 3-kinase, phosphatidylinositol 3-kinase; Ptch1, Patched1; Ptx, a *Bordetella pertussis* toxin; Shh, sonic hedgehog; Smo, smoothened.

G_i and PI 3-kinase by Shh is also noted for endothelial cells in tubulogenesis (Kanda et al., 2003; Chinchilla et al., 2010). G_i apart from Gli is additionally reported to be directly responsible for hedgehog-dependent calcium spike activity in embryonic spinal cells (Belgacem and Borodinsky, 2011).

Any assertion that Smo is similar to other G_i-coupled receptors in its capacity to control the range of effectors described for G_i remains, however, tenuous. One could argue that the connection between Smo and G_i is a more residual than robust feature, with Smo having evolved to a form more apt to serve as a scaffold than to activate a G protein. In this case, the activation of G_i by Smo would be perceived by only especially sensitive effectors and, perhaps, only under special circumstances. The primary cilium, moreover, remains a structure in which support of G protein coupling and consequent action is uncertain. The paucity of reports regarding the use of G_i in hedgehog signaling adds to the uncertainty of the value of the G protein to the morphogen's actions.

To understand better the notion of signaling through G_i as a meaningful facet of hedgehog action, we evaluated the efficacy of Smo specifically with regard to activation of the G protein, by comparing Smo with the 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor. The 5-HT$_{1A}$ receptor is a quintessential G_i-coupled receptor that, through G_i, links serotonin to a wide variety of effectors. We found, in the context of the assays that we used, that Smo is equivalent to the 5-HT$_{1A}$ receptor in activating G_i. We also extended the list of effectors targeted by hedgehogs through G_i from PI 3-kinase to adenylyl cyclase. Our data support Smo as a receptor with the potential to engage not only Gli but also many other, more immediate effectors.

Materials and Methods

Materials. Cyclopamine and purmorphamine were obtained from EMD Biosciences (San Diego, CA); 8-hydroxy-2-(dipropylamino) tetralin hydrobromide (8-OH-DPAT), fluorescein isothyocyanate (FITC)–conjugated goat antibody specific for rabbit IgG, and Ptx were purchased from Sigma-Aldrich (St. Louis, MO). Guanosine 5′-(3-O-thio) triphosphate ([^{35}S]GTPγS) was provided by PerkinElmer Life and Analytical Sciences (Boston, MA). Rabbit antibody specific for the hemagglutinin (HA) YPYDVPDYA sequence was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antisera specific for Gα-subunits were described previously (Riobo et al., 2006). Recombinant Shh (N-terminal domain) was synthesized and purified as described elsewhere (Polizio et al., 2011).

Plasmid Constructs. All expression constructs used the pcDNA3.1 vector. Those for human 5-HT$_{1A}$ wild-type and 3×HA-tagged (N-terminus) 5-HT$_{1A}$ receptor were obtained from the Missouri cDNA Resource Center (Rolla, MO). The construct for mouse Smo wild-type receptor was provided by Dr. Philip Beachy (Stanford University, Palo Alto, CA). cDNA for 3×HA-tagged Smo was constructed by polymerase chain reaction (PCR)–directed mutagenesis. The tag was inserted after the signal peptide: GGPGRGA$^{33(Smo)}$/YPYDVPDYA(x3)/A$^{34(Smo)}$LSGNVT. Constructs for Smo-Gα and 5HT$_{1A}$ receptor-Gα fusion proteins were achieved by PCR-directed mutagenesis. The primary sequences at splice sites were TGHSDDEPKR$^{566(Smo)}$/G$^{2(Gai2)}$CTVSAEDKA (Smo/Gα$_{i2}$) TGHSDDEPKR$^{566(Smo)}$/G$^{2(Gao)}$CTLSAEER (Smo/Gα$_o$), TGHSDDEPKR$^{566(Smo)}$/ G$^{2(Gaz)}$CRQSSEEK (Smo/Gα$_Z$), FQNAFKKIIK$^{416(5HT1A)}$/ G$^{2(Gai2)}$CTVSAEDK (5-HT$_{1A}$/Gα$_{i2}$), FQNAFK-KIIK$^{416(5HT1A)}$/ G$^{2(Gao)}$CTLSAEERA (5HT$_{1A}$/Gα$_o$), and FQNAFK-KIIK$^{416(5HT1A)}$/ G$^{2(Gaz)}$CRQSSEEKE (5HT$_{1A}$/Gα$_Z$). All DNA sequences after PCR-directed mutagenesis were verified by direct sequencing. The expression vector for mouse Gli2 and the 8×Gli luciferase reporter gene were obtained from Dr. Hiroshi Sasaki (RIKEN Center for Developmental Biology, Kobe, Japan).

Cell Culture and Transfection. Human embryonic kidney (HEK)-293 (CRL-1573) cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C with 5% carbon dioxide. Cells were transfected at 70–80% confluency with use of Lipofectamine (Gibco). Expression constructs were adjusted to provide equal expression levels (5 μg HA-tagged 5-HT$_{1A}$ receptor, 40 μg Smo, and 40 μg receptor-Gα fusion constructs as noted in Results). The cells were maintained after transfection under normal culture conditions up to 48 hours before experiments. C3H10T1/2 cells (CCL-266) were also obtained from American Type Culture Collection. They were maintained in Basal Medium Eagle with 10% fetal bovine serum at 37° with 5% carbon dioxide. They were transfected using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's suggested protocol.

Membrane Preparation. HEK-293 cells were rinsed with phosphate-buffered saline (PBS) and incubated with cell lysis buffer (20 mM Hepes [pH 8.0], 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM PMSF; 0.5 ml/100 mm dish) with rocking at 4°C for 20 minutes. The cells were collected into an eppendorf tube and passed 15 times through a 26G needle. Lysates were centrifuged at 2500g for 5 minutes at 4°C. Supernatants were transferred to new eppendorf tubes and centrifuged at 14,000g for 30 minutes at 4°C. Pellets (membranes) were resuspended in 100 μl cell lysis buffer by passing through a 26G needle and stored at −80°C.

[^{35}S]GTPγS Binding. The assay for receptor-promoted binding of [^{35}S]GTPγS to Gα subunits was performed as described previously (Windh and Manning, 2002). Membranes (20 μg protein) were incubated with vehicle, agonist, or antagonist for 10 minutes at 30°C, then with 1 nM [^{35}S]GTPγS (plus 10 μM GDP for G_i and G_o, and 0.1 mM GDP for G_z) for another 10 minutes before solubilization of the membranes and immunoprecipitation of endogenous or fused Gα subunits for analysis by scintillation spectrometry. Binding was a near linear function of time and membrane amount.

Flow Cytometry. HEK-293 cells transfected with HA-tagged Smo or HA-tagged 5-HT$_{1A}$ receptor expression constructs or with pcDNA 3.1 alone were trypsinized, washed, and incubated with PBS/1% bovine serum albumin (BSA) or PBS/1% BSA containing HA-directed rabbit IgG at a 1:100 dilution. The cells were then washed and incubated with PBS/1% BSA containing FITC-conjugated goat antirabbit antibody (1:50) for 20 minutes, washed, and analyzed by flow cytometry using FACSCalibur (BD Biosciences, NJ) and excitation/emission wavelengths of 488/530 nm.

cAMP Assay. HEK-293 cells were plated at a concentration of 1 × 10^5 cells/well in standard 96-well microplates and cultured overnight. Cells were stimulated the following day for 5 minutes with 10 μM isoproterenol or vehicle in triplicate. Some wells were treated with 5 μM purmorphamine or 1 μM 8-OH-DPAT for 5 minutes or with 2 μg/ml recombinant Shh for 16 hours at 37°C before stimulation with isoproterenol. Intracellular cAMP was extracted and assayed by enzyme immunoassay according to the manufacturer's instructions (Biotrak; GE Health Care Biosciences, Pittsburgh, PA). Absorbance values were transformed to cAMP concentration with use of a standard curve generated by plotting the percentage bound for each standard and sample as a function of the log cAMP concentration.

Gli Reporter Gene Assay. C3H10T1/2 cells were seeded at 1.5 × 10^4 cells/well in 24-well plates and transfected with 0.1 μg 8×Gli firefly reporter, 0.01 μg TK Renilla reporter, and, where noted, 0.13 μg Gli2 expression vector. At 48 hours, when the cells had reached confluence, the serum was lowered to 0.5% serum, at which point, Ptx (100 ng/ml) or vehicle was added. The cells were treated 20 hours thereafter with or without purmorphamine. Luciferase activities were evaluated 4 hours later with use of the Dual Luciferase Reporter Assay (Promega, Madison, WI).

Western Blotting. A total of 1 μg (HA-tagged receptors) or 10 μg (receptor-Gα fusion proteins) of membrane protein for each lane were dissolved in SDS-PAGE sample buffer and separated by SDS-PAGE.

The membranes were blotted with anti-HA (#3724S Cell Signaling, Danvers, MA) or with anti-Gα antisera at a 1:1000 dilution. Horseradish peroxidase–conjugated secondary antibodies with ECL immunoblot kits (GE Health Care Biosciences) were used for visualization.

Statistics. Comparison of two groups was made using a paired Student's t test with use of GraphPad Prism, version 5.0. N and P values are noted in figure legends.

Results

Smo couples directly to members of the G_i family of heterotrimeric G proteins (Riobo et al., 2006). Whether the activation of G_i by Smo is comparable in degree to that of other G_i-coupled receptors, however, is unknown, because expression in the previous study was neither controlled nor specifically evaluated. Toward this end, we constructed an epitope-tagged form of Smo and a set of Smo-Gα fusion proteins for comparison with similar forms of the 5-HT$_{1A}$ receptor.

HA-tagged forms of Smo and the 5-HT$_{1A}$ receptor were introduced into HEK-293 cells by transfection. Several bands for the receptors were evident in Western blots (Fig. 1A). In the case of Smo, the larger, more diffuse band(s) around 85 kDa corresponded to the size anticipated for the full-length tagged receptor (87.4 kDa). For the 5-HT$_{1A}$ receptor, the doublet around 46 kDa was near the size anticipated (49.4 kDa). Heterogeneity in banding may reflect glycosylation and/or proteolysis. Both receptors targeted to the cell surface, as noted by flow cytometry (Fig. 1B). The expression of Smo at the cell surface was nearly monodisperse, whereas that of the 5-HT$_{1A}$ receptor was divided into a subset of cells in which expression was equivalent to that of Smo and another subset in which expression was higher. Both receptors promoted binding of [^{35}S]GTPγS to subtypes of Gα_i present in the HEK-293 cells with or without agonist, as evaluated using an antiserum recognizing Gα_{i1}, Gα_{i2}, and Gα_{i3} (Fig. 1C). Activities without agonist are consistent with constitutive activity, a hallmark of Smo when unrepressed by Ptch1 (Riobo et al., 2006; Riobo and Manning, 2007; Jiang and Hui, 2008; Wilson and Chuang, 2010) and a characteristic of the 5-HT$_{1A}$ receptor (Barr and Manning, 1997; Martel et al., 2007). The activity of Smo toward G_i was not elevated further by the agonist purmorphamine. The activity of the 5-HT$_{1A}$ receptor toward G_i was doubled with 8-OH-DPAT. In the context of the assay, the activity of Smo with or without purmorphamine was equal to or greater than that of the 5-HT$_{1A}$ receptor under either circumstance.

We evaluated coupling additionally with receptor-Gα fusion proteins. Fusion proteins have proven to be useful in studies of efficacy (Seifert et al., 1999; Milligan, 2000; Zhang et al., 2006) and, because of a common Gα subunit, permit quantitation of relative expression with Gα-directed antibodies. Western blotting confirmed expression of Smo-Gα_{i2} and 5-HT$_{1A}$-Gα_{i2} (Fig. 2A). Smo-Gα_{i2} appeared primarily as two bands, one at ~115 kDa, near the predicted molecular weight of 99.7 kDa, and the other at the top of the separating gel, likely representing aggregated protein. 5-HT$_{1A}$-Gα_{i2} appeared mostly as doublet around 85.8 kDa, close to the predicted molecular weight of 86 kDa. A substantive constitutive activity was noted for Smo-Gα_{i2}, as it was noted above for Smo alone with endogenous G_i (Fig. 2B). The activity was
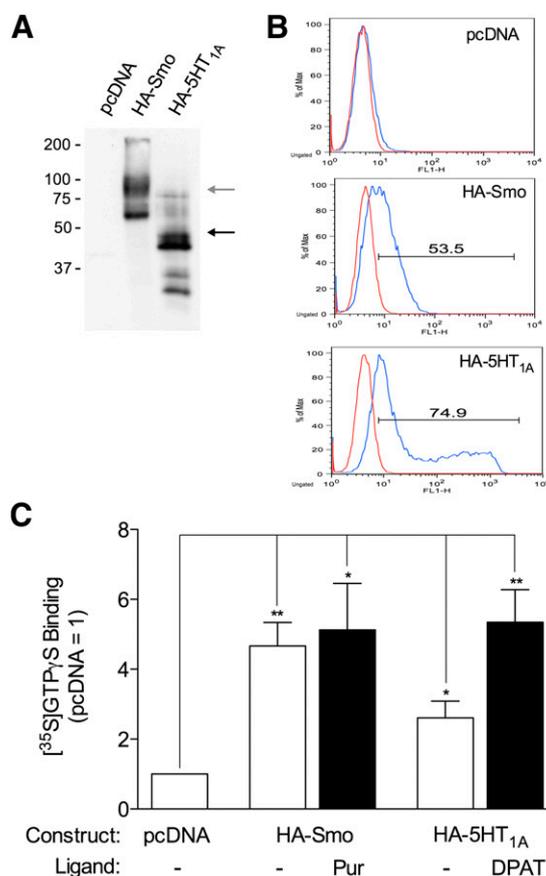

Fig. 1. Activation of G_i endogenous to HEK-293 cells by Smo and 5-HT$_{1A}$ receptor. HEK-293 cells near confluence (10-cm plates) were transfected with pcDNA 3.1 alone or the vector encoding HA-tagged Smo or HA-tagged 5-HT$_{1A}$ receptor. Cells or subsequently prepared membranes were evaluated 48 hours after transfection. (A) Expression of the receptors was evaluated for membranes (1 μg membrane protein) by a Western blot using HA-directed antibody. Positions of molecular weight standards are noted. Arrows refer to mobilities predicted for full-length receptors (gray arrow, HA-tagged Smo; black arrow, HA-tagged 5-HT$_{1A}$ receptor) based on molecular weight standards. The depiction is representative of four experiments total. (B) Expression of receptors was evaluated for intact cells by flow cytometry using either no primary antibody (red) or HA-directed antibody (blue) with FITC-conjugated secondary antibody. Horizontal bars represent cutoffs for expression and equate to transfection efficiency. The depiction is representative of three experiments total. (C) Activity of the receptors toward one or more forms of G_i endogenous to HEK-293 cells was evaluated in membranes by [^{35}S]GTPγS binding. Binding of the radioligand was examined with or without 10 μM purmorphamine (HA-Smo) or 1 μM 8-OH-DPAT (HA-5-HT$_{1A}$) and normalized to binding obtained with pcDNA alone. The data represent means $\pm$ S.E. for six independent experiments. Differences from pcDNA alone are noted (*$P \leq 0.05$; **$P \leq 0.01$).

not the result of the appended Gα subunit in some way operating apart from the receptor, because it was largely suppressed by the inverse agonist cyclopamine. Purmorphamine was found to increase the activity of Smo-Gα_{i2}. Both constitutive and agonist-promoted activities exhibited by Smo-Gα_{i2}, as measured by the assay, were greater than those exhibited by 5-HT$_{1A}$-Gα_{i2}.

We evaluated interactions of the receptors with other members of the G_i family, again using receptor-Gα fusion proteins. Although Smo-Gα_z was more prone to aggregation after electrophoresis than was Smo-Gα_{i2} (Fig. 3A), the [^{35}S]GTPγS binding data for Smo-Gα_z compared with 5-HT$_{1A}$-Gα_z were nearly identical to those for Smo-Gα_{i2} compared with

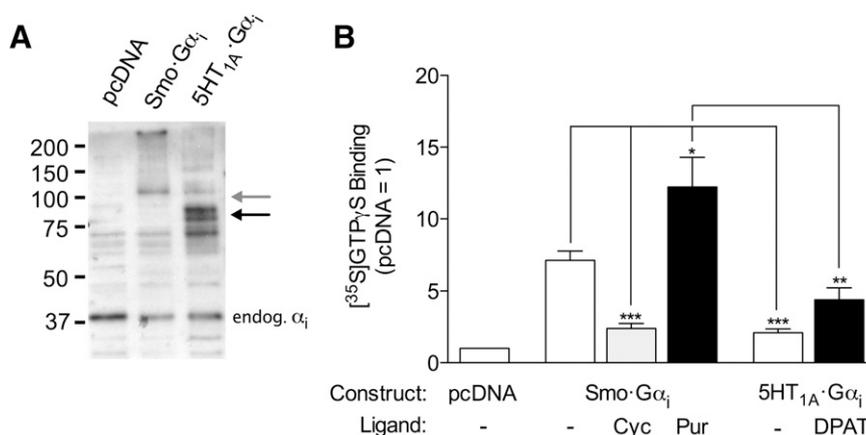**Fig. 2.** Activation of Gα$_i$ within Smo-Gα$_i$ and 5HT$_{1A}$-Gα$_i$ fusion proteins. HEK-293 cells were transfected with pcDNA 3.1 alone or the vector encoding Smo-Gα$_{i2}$ or 5-HT$_{1A}$-Gα$_{i2}$ fusion protein. Membranes were evaluated 48 hours later. (A) Western blot using a Gα$_{i2}$-directed antibody (1 μg membrane protein per lane). Arrows refer to mobilities predicted for full-length Smo-Gα$_{i2}$ (gray arrow) and full-length 5-HT$_{1A}$-Gα$_{i2}$ (black arrow); Gα$_i$ endogenous to the cells is also noted. The depiction is representative of three experiments total. (B) [^{35}S]GTPγS binding for the fusion proteins. Binding was evaluated with or without 10 μM purmorphamine (Smo-Gα$_{i2}$), 10 μM cyclopamine (Smo-Gα$_{i2}$), or 1 μM 8-OH-DPAT (5-HT$_{1A}$-Gα$_{i2}$) and normalized to binding obtained with pcDNA alone. The data represent means ± S.E. for 5–7 independent experiments. Differences from Smo-Gα$_{i2}$ without ligands and between Smo-Gα$_{i2}$ and 5HT$_{1A}$-Gα$_{i2}$ with agonists are noted (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

5-HT$_{1A}$-Gα$_{i2}$ above. Smo-Gα$_o$ resolved as a doublet with an apparent molecular weight higher than anticipated (Fig. 3B). The reason for the shift from the anticipated molecular weight is unknown. The data for this construct compared with 5-HT$_{1A}$-Gα$_o$ were nonetheless similar to those of Smo-Gα$_{i2}$ compared with 5-HT$_{1A}$-Gα$_{i2}$, with the exception that binding of [^{35}S]GTPγS relative to control, pcDNA, was higher. Notwithstanding the noted electrophoretic anomalies and

consequent difficulties in evaluating precise expression levels, the activities of Smo-Gα in each case appeared in the assay to be at least comparable to those of 5-HT$_{1A}$-Gα.

HEK-293 cells express endogenous Ptch1 and Smo (Jiang et al., 2009; Atwood et al., 2011), neither of which had an appreciable impact on [^{35}S]GTPγS binding in the context of receptor overexpression above. We found, in experiments to evaluate effectors downstream of G$_i$, however, that
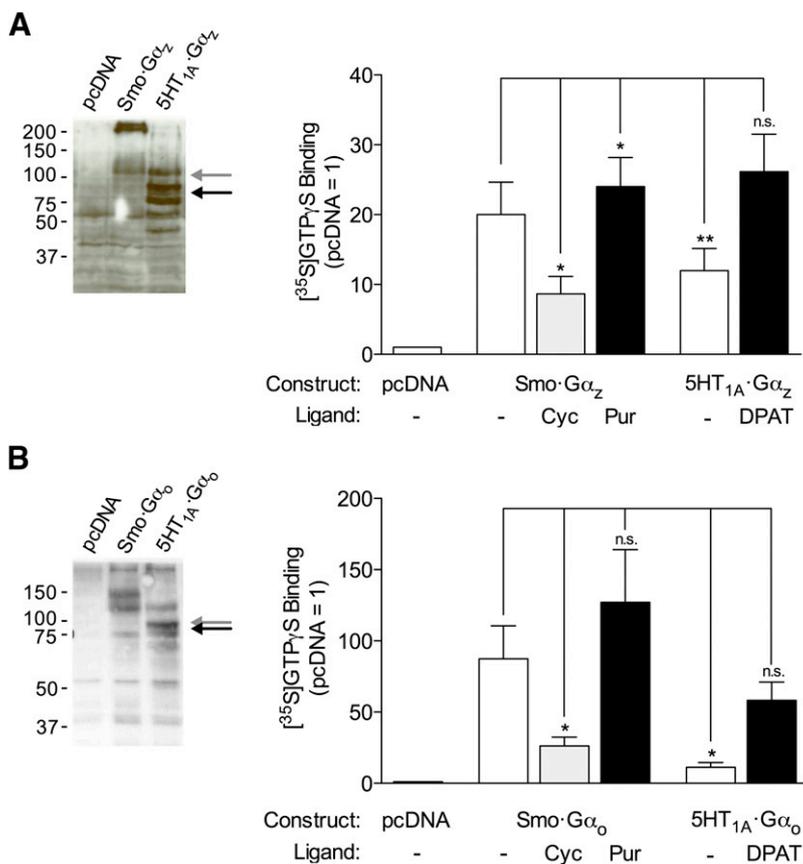

Fig. 3. Activation of Gα$_o$ and Gα$_z$ in receptor-Gα fusion proteins. HEK293 cells were transfected with pcDNA 3.1 alone or the vector encoding (A) Smo-Gα$_z$ or 5-HT$_{1A}$-Gα$_z$, or (B) Smo-Gα$_o$ or 5-HT$_{1A}$-Gα$_o$. Arrows refer to mobilities predicted for full-length Smo-Gα (gray arrows) and full-length 5-HT$_{1A}$-Gα (black arrows). [^{35}S]GTPγS binding assays were performed as described in Fig. 2 but using Gα$_o$- and Gα$_z$-directed antibodies and represent means ± S.E. for 4–5 and 6–8 independent experiments, respectively. Differences from Smo-Gα without ligand are noted (*$P \leq 0.05$; **$P \leq 0.01$; n.s., not significant).

endogenous Smo is fully capable of mediating inhibition of adenylyl cyclase. As shown in Fig. 4, addition of purmorphamine or Shh to HEK-293 cells achieved significant inhibition of isoproterenol-stimulated increases in cAMP. The inhibition was no greater with overexpressed Smo (as evaluated for purmorphamine; data not shown). HEK-293 cells also express endogenous 5-HT_{1D} receptor (Atwood et al., 2011), which similar to the 5-HT_{1A} receptor recognizes 8-OH-DPAT as an agonist and couples to G_i (Andrade et al., 2012). Similar to endogenous Smo, it had little impact on the [^{35}S]GTPγS binding above. 8-OH-DPAT, similar to purmorphamine and Shh, inhibited isoproterenol-stimulated increases in cAMP. Smo endogenous to HEK-293 cells reduces cAMP to the same extent as does the presumptive 5-HT_{1D} receptor.

We also evaluated the requirement of Gli for G_i, because unequivocal data for the requirement in mammalian cells are limited to a single type of cell, the NIH 3T3 fibroblast (Riobo et al., 2006). We had found little if any effect of purmorphamine on Gli activity in HEK-293 cells (Douglas et al., 2011). We turned therefore to C3H10T1/2 cells, a mesenchymal cell line used often as a model for Smo action on Gli. As noted (Wu et al., 2004), purmorphamine activates the Gli reporter gene in C3H10T1/2 cells, as does Gli1 (Fig. 5). The activation of the reporter gene by purmorphamine in these cells was sensitive to Ptx, with the toxin inhibiting activation by 50%. The activation caused by Gli1 was, as anticipated, not sensitive to the toxin.

Discussion

Our most important finding is that Smo is comparable to the 5-HT_{1A} receptor in its capacity to activate G_i as measured in the [^{35}S]GTPγS binding assay. Equivalence was demonstrated in two ways: first, with forms of G_i endogenous to HEK-293 cells made to express epitope-tagged receptors and, second, with individual forms of $G\alpha_i$ fused to the C terminus of each receptor. We also found that Smo endogenous to HEK-293 cells inhibits adenylyl cyclase, a finding that corroborates the efficacy of Smo through G_i and extends the list of effectors
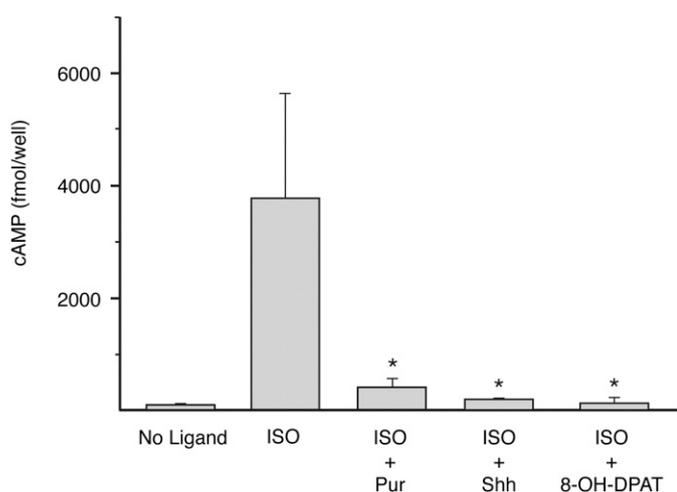
controlled by Smo through G_i beyond PI 3-kinase. Finally, the requirement for G_i in the activation of Gli was documented for C3H10T1/2 cells, demonstrating that the requirement is not unique to a single type of mammalian cell.

Both Smo and the 5-HT_{1A} receptor exhibited agonist-independent (constitutive) receptor activity. The activity was particularly prominent for Smo, with little if any additional activity achieved in response to purmorphamine. Although HEK-293 cells express Ptch1, it is likely that levels of Smo introduced into the cells exceed those that endogenous Ptch1 can suppress or that Ptch1-mediated repression of Smo is disrupted with preparation of membranes. Thus, we believe that the activity noted for the introduced Smo represents a propensity of the receptor to enter into a nearly fully active conformation. It is possible that the Smo-$G\alpha$ constructs (Smo-$G\alpha_{i2}$ and Smo-$G\alpha_z$) exhibit some degree of response to purmorphamine because of an inhibitory constraint of the appended subunit on constitutive activity. The 5-HT_{1A} receptor, while exhibiting constitutive activity, can, in all cases, be activated further by agonist. The activity of constitutively active Smo toward G_i in the context of the [^{35}S]GTPγS assay is nonetheless equal to or greater than that of either constitutively active or agonist-activated 5-HT_{1A} receptor.

It was important in these studies to be assured of comparable receptor expression. HA tagging permitted analysis of expression at the cell surface that showed differences in expression to be modest or in favor of the 5-HT_{1A} receptor. However, although the HA-tagged 5-HT_{1A} receptor has been extensively documented in terms of functionality, the mammalian HA-tagged Smo has not. For this reason, we adopted the additional strategy of using as tags $G\alpha$ subunits appended to truncated receptor tails. As noted, receptor-$G\alpha$ fusion proteins have been effectively used to evaluate efficacy as it relates to all classes of $G\alpha$ subunit. The results regarding activities for the Smo-$G\alpha$-tagged compares with 5-HT_{1A}-$G\alpha$–tagged receptors were the same as those for the HA-tagged receptors (i.e., Smo was at least the equivalent of the 5-HT_{1A} receptor, as measured in the assay).

Previous work with membranes of Sf9 cells made to express Smo and individual G proteins suggested that G_z was more readily activated than was G_o (Riobo et al., 2006). We found, in

Fig. 4. Decreases in isoproterenol-elevated cAMP with purmorphamine, Shh, and 8-OH-DPAT. HEK-293 cells were treated with vehicle, 10 μM isoproterenol alone, or 10 μM isoproterenol after pretreatment with 5 μM purmorphamine, 2 μg/ml Shh, or 1 μM 8-OH-DPAT. cAMP was measured by enzyme immunoassay. The three ligands were each found to decrease isoproterenol-elevated cAMP (*$P \leq 0.05$). The data represent the mean ± S.E. of three independent experiments.
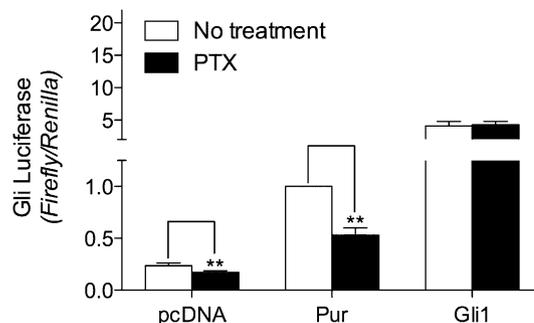

Fig. 5. Ptx-sensitivity of Gli activation in C3H10T1/2 cells. C3H10T1/2 cells were transfected with vectors for 8×Gli firefly and TK Renilla luciferase reporters and, where noted, Gli1. At 48 hours, after attainment of confluence by the cells, serum was lowered to 0.5% and (black bars) Ptx was added to a concentration of 100 ng/ml. Purmorphamine (10 μM) was added 20 hours thereafter. Luciferase activities were assayed after an additional 4 hour. The data represent means ± S.E. of ratios of firefly to Renilla activities normalized to those obtained for purmorphamine without Ptx for six independent experiments performed in triplicate. ***$P < 0.001$.

the present study using receptor-Gα fusion proteins, little difference between Smo and the 5-HT$_{1A}$ receptor in activation of individual subtypes of Gα$_i$ (i.e., Gα$_{i2}$, Gα$_z$, and Gα$_o$). This finding does not imply that one or another Gα subunit is not selectively activated by Smo, only that Smo does not differ from the 5-HT$_{1A}$ receptor in this regard. This would imply that any selectivity is intrinsic to the G protein and not to receptors. Transactivation of endogenous Gα$_i$ can possibly occur for any of the fusion proteins (Burt et al., 1998; Molinari et al., 2003); however, the subtype selectivity of the antibodies helps to ensure that the subunits assayed in the case of Gα$_z$ and Gα$_o$ are those appended to the receptor.

Whether the potential of a receptor to communicate with a G protein as deduced through [^{35}S]GTPγS binding is fully realized in an endogenous setting depends on several factors. One of the most important is levels of expression of the receptor. Work with adenylyl cyclase here and activation of Rac and Rho through PI 3-kinase in murine embryo fibroblasts (Polizio et al., 2011) demonstrates that, in at least some cells, for at least two effectors, sufficient levels of expression are achieved. The subcellular distribution of a receptor is another factor that can impact on a receptor's capacity to interact with a G protein. In the case of Smo, whether the primary cilium to which it is recruited after activation contains G$_i$ or any one or more of G$_i$'s targets is unclear. Gα$_{i2}$ and Gα$_{i3}$, when overexpressed in cerebellar granular neuronal precursors, target the cilium (Barzi et al., 2011); however, targeting of endogenous subunits in these cells or elsewhere may be different. Our studies with HEK-293 cells were performed at confluence, a condition that supports formation of primary cilia. Our previous studies with murine embryo fibroblasts regarding Rac and Rho were performed at subconfluence. We suspect, therefore, that Smo can communicate with G$_i$ both in and beyond primary cilia.

Our study, to our knowledge, is the first to show that the activation of mammalian Smo decreases intracellular cAMP in an endogenous setting. The impact of vertebrate Smo on cAMP was indicated previously only after overexpression (DeCamp et al., 2000). The data for cAMP in HEK-293 cells are in agreement with findings for *Drosophila* (Ogden et al., 2008). A decrease in the activity of the most common target for cAMP, the cAMP-dependent protein kinase, is widely considered to be a requirement in the activation of Gli by hedgehog (Epstein et al., 1996; Barzi et al., 2010; Milenkovic and Scott, 2010). Our finding and that for *Drosophila* (Ogden et al., 2008) are consistent with the decrease being caused by activated Smo directly through the effects of G$_i$ on adenylyl cyclase before or after entry of Smo into the primary cilium. The decrease in cAMP was noted at 16 hours for Shh, suggesting that the interaction of Smo with G$_i$ is less susceptible to desensitization than are those of most other receptors, for which the effects are generally transient. One possibility is that the interaction of Smo with arrestin, after initiated, lasts only until Smo is translocated to the primary cilium, allowing interactions of Smo with G$_i$ to occur not only before but after the process of translocation. It is also possible that the affinity of arrestin for Smo is less strong than that for other receptors, allowing G$_i$ to more effectively compete for access, or that a population of G$_i$ targeting adenylyl cyclase is, for some reason, unavailable to arrestin. Smo-effected decreases in cAMP may not always be easily discerned. Barzi et al. (Barzi

et al., 2010) suggest that a decrease in cAMP may be localized to the primary cilium, such that overall cellular levels of cAMP may not seem to change. If localization of Smo to the primary cilium holds true in HEK-293 cells, the proportion of adenylyl cyclase that is inhibited or the degree to which it is inhibited must be large. A pressing question, given the consensus regarding the cAMP-dependent protein kinase, is why G$_i$ is not always required in the activation of Gli. A possible requirement for G$_i$ may be related to cell-intrinsic levels of cAMP. If levels are sufficiently high to block activation of Gli, G$_i$ would be required. If not, G$_i$ would not be required. This possibility remains to be tested.

The equivalence of Smo to the 5-HT$_{1A}$ receptor in activation of G$_i$ and decrease through endogenous Smo of cAMP provides evidence of Smo as a receptor that can engage not only Gli but other more immediate effectors. Effectors thus far identified are PI 3-kinase and adenylyl cyclase; however, the list for G$_i$ extends to other enzymes and ion channels. The regulation of these, in turn, may explain the increasing number of hedgehog-induced events that occur too quickly for any action of Gli or that can otherwise be mimicked by agonists working through G$_i$-coupled receptors other than Smo. Such events include axonal guidance (Yam et al., 2009), proliferation of neuronal stem cells (Lai et al., 2003; Banasr et al., 2004; Jin et al., 2004; Tran et al., 2007), β-selection of thymocytes (Shah et al., 2004), and potentiation of CD4$^+$ T cell activation (Stewart et al., 2002; Chan et al., 2006). Our documentation of noncanonical signaling (Polizio et al., 2011) and, here, efficacy of Smo acting on G$_i$ provides a rationale for evaluating these events and the prediction of others.

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