Growth Arrest Specific 8 (Gas8) and G Protein-coupled Receptor Kinase 2 (GRK2) Cooperate in the Control of Smoothened Signaling

Received for publication, February 24, 2011, and in revised form, May 25, 2011 Published, JBC Papers in Press, June 8, 2011, DOI 10.1074/jbc.M111.234666

Tama Evron1, Melanie Philipp1,2,3, Jiuyi Lu1, Alison R. Meloni4, Martin Burkhalter4, Wei Chen4, and Marc G. Caron4

From the Departments of 1Cell Biology and 2Medicine, Duke University Medical Center, Durham, North Carolina 27710 and 3Department of Molecular Medicine and Max Planck Research Group on Stem Cell Aging, Albert Einstein Allee 11, University of Ulm, Ulm 89081, Germany

The G protein-coupled receptor (GPCR)-like molecule Smoothened (Smo) undergoes dynamic intracellular trafficking modulated by the microtubule associated kinase GRK2 and recruitment of β-arrestin. Of this trafficking, especially the translocation of Smo into primary cilia and back to the cytoplasm is essential for the activation of Hedgehog (Hh) signaling in vertebrates. The complete mechanism of this bidirectional transport, however, is not completely understood. Here we demonstrate that Growth Arrest Specific 8 (Gas8), a microtubule associated subunit of the Dynein Regulatory Complex (DRC), interacts with Smo to modulate this process. Gas8 knockdown in ciliated cells reduces Smo signaling activity and ciliary localization whereas overexpression stimulates Smo activity in a GRK2-dependent manner. The C terminus of Gas8 is important for both Gas8 interaction with Smo and facilitating Smo signaling. In zebrafish, knocking down Gas8 results in attenuated Hh transcriptional responses and impaired early muscle development. These effects can be reversed by the co-injection of Gas8 mRNA or by constitutive activation of the downstream Gli transcription factors. Furthermore, Gas8 and GRK2 display a synergistic effect on zebrafish early muscle development and some effects of GRK2 knockdown can be rescued by Gas8 mRNA. Interestingly, Gas8 does not interfere with cilia assembly, as the primary cilia architecture is unchanged upon Gas8 knockdown or heterologous expression. This is in contrast to cells stably expressing both GRK2 and Smo, in which cilia are significantly elongated. These results identify Gas8 as a positive regulator of Hh signaling that cooperates with GRK2 to control Smo signaling.

Smoothened (Smo) is a G protein-coupled receptor (GPCR)-like protein that serves as the main transducer of the Hedgehog (Hh) signaling pathway, regulating many aspects of embryonic development. Loss of function of components of this pathway leads to human developmental disorders, including holoprosencephaly, polydactyly, craniofacial, and skeletal malformation (1, 2), while inappropriate activation of the pathway is linked to a wide range of malignancies (3). Data from mammalian cell culture, mice, and zebrafish have implicated Smo trafficking into the primary cilia by intraflagellar transport (IFT) particles as an essential step in Hh downstream signaling (reviewed in Refs. 4, 5). Smo translocation results in the accumulation of transcriptionally active Gli (GliA) at the tip of the cilia, which is then transported out of the cilia to promote Hh target gene expression (4). Similar to classic GPCRs, phosphorylation of active Smo by the GPCR kinase 2 (GRK2) and recruitment of β-arrestin results in Smo internalization (6), and both GRK2 and β-arrestin play a positive role in Smo signaling as they do in selective forms of GPCR signaling (7, 8). Interestingly, GRK2 has been reported as a microtubule associated kinase that directly phosphorylates tubulin following GPCR stimulation (9). GRK2 has also been shown to promote signaling through phosphorylation of Smo (10) and enhancement of the direct interaction between Smo and β-arrestin (11). β-Arrestin 2 and GRK2/3 knockdown in zebrafish embryos provides further evidence for their association with the activation of Hh pathway in vertebrates (10, 12). Moreover, β-arrestin was shown to promote Smo translocation into the cilia by mediating its interaction with the kinesin motor protein Kif3A in mammalian cell culture (13).

Growth arrest specific 8 (Gas8, also called Gas11) is the vertebrate homolog of the Trypanosoma brucei protein trypanin (14, 15) and Chlamydomonas paralyzed flagellar 2 (PF2), in which mutations lead to defective flagella motility (16). This conserved family of microtubule-associated proteins encodes for the Dynein Regulatory Complex (DRC) subunit 4 (15, 16). Several different domains within Gas8 have been identified with different affinities for microtubules, including a microtu-

---

1 Recipient of a postdoctoral fellowship from The Machiah Foundation, a supporting foundation of the Jewish Community Federation of San Francisco, the Peninsula, Marin, & Sonoma Counties.
2 Present address: Dept. of Biochemistry and Molecular Biology, Albert Einstein Allee 11, University of Ulm, Ulm 89081, Germany.
3 Recipient of a Marie Curie Outgoing International Fellowship of the European Commission.
4 To whom correspondence should be addressed: Department of Cell Biology, Box 3287 or RM 487 Carl Bldg., Duke University Medical Center, Durham, NC 27710. Tel.: 919-684-5433; Fax: 919-681-8641; E-mail: caron002@mc.duke.edu.
5 The abbreviations used are: Smo, smoothened; Hh, hedgehog; Gas8, growth arrest specific 8; GRK2, G protein-coupled receptor kinase 2; DRC, dynein regulatory complex; IFT, intraflagellar transport; GMAD, Gas8 microtubule association domain; IMAD, inhibitor of microtubule association domain; Pol, polaris; shRNA, short hairpin RNA; MO, morpholino; hpf, hours post fertilization.
Gas8 and GRK2 Cooperate in Smoothened Signaling

bule association domain (GMAD) between residues 115–258 and the N-terminal inhibitor of microtubule association domain (IMAD) between residues 1–108 (17). In this study, an engineered protein consisting of the GMAD and C terminus domains of Gas8 (Gas8\textsubscript{GMAD-CT}) displayed the highest affinity to microtubules. In zebrafish embryos, Gas8 knock down results in abnormal ear development and defective cilia motility (14), providing the first evidence of Gas8 and DRC requirement for proper motile cilia functioning in vertebrates.

Here we show that Gas8 interacts with Smo and functions as a positive regulator of Hh signaling, a pathway dependent on non-motile cilia. In cells we find that Gas8 association with microtubules at the base of cilia and the presence of active GRK2 contribute to the positive effect of Gas8 on Smo signaling. In zebrafish embryos we show that Gas8 is important for proper slow muscle development and Hh-target gene expression, but not for cilia assembly. Altogether, these data are consistent with Gas8 and GRK2 influencing Hh pathway upstream of Gli.

**EXPERIMENTAL PROCEDURES**

*Plasmids*—The 16.2 Gli reporter plasmid and the Myc-Smo expression plasmid were obtained from M. Scott, Stanford University (18). The bovine GRK2, and bovine GRK2-K220R expression plasmids were described previously (19). A full-length mouse (m)Gas8 cDNA sequence (NM_018855.2) was cloned into pCDNA6 via NotI and XhoI following an upstream Flag sequence. Flag-Gas8 mutants were cloned in the same way for proper slow muscle development and Hh-target gene expression, but not for cilia assembly. Altogether, these data are consistent with Gas8 and GRK2 influencing Hh pathway upstream of Gli.

**Immunoblots and Immunofluorescence**—Immunoblots were performed according to standard protocols. A rabbit polyclonal and ~70% reduction in the reporter activity, but was eventually deleterious to survival of the cell. Thus, this shRNA was not used further. GFP-Smo cells (13) were infected with the shRNAs to generate double stable lines as above.

**Transfections and Luciferase Assay**—Luciferase reporter assay in NIH-3T3 cells was performed as described (13). Briefly, NIH-3T3 cells were transfected with 1.05 \( \mu \)g 9X Gli-binding site-luciferase plasmid and 0.15 \( \mu \)g of pRL-TK (Promega, Madison, WI) using Fugene 6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations.

**Immunoprecipitation Assay**—HEK 293 cells plated in 10-cm dish were transiently transfected with 3 \( \mu \)g of Myc-Smo, 2 \( \mu \)g of Flag-Gas8 or both constructs by the calcium phosphate method. DNA amount transfected was kept constant with the empty pCDNA3 plasmid. 48 h post-transfection, cells were harvested in 0.7 ml immunoprecipitation buffer containing 50 mM HEPES, 0.5% Triton X-100, 250 mM NaCl, 10% glycerol, 2 mM EDTA, and a 1 \( \times \) protease inhibitor mixture tablet (Complete; Roche Diagnostics). To avoid interference with the protein-protein interaction, the immunoprecipitation buffer contained non-ionic detergent that mainly solubilizes the cytoplasmic detergent-soluble fraction of Gas8 (20). This fraction may be enriched by the transient transfection of Flag-Gas8 and probably by microtubule de-polymerization in the absence of microtubule-stabilizer (17). An aliquot of the extract (2%) was used as the input, while the remaining extract was incubated with Flag antibody beads (Sigma) that had been pre-blocked with 0.5% bovine serum albumin for 1 h. Extracts and beads were allowed to mix at 4 °C overnight. Beads were then washed four times in the lysis buffer and four times with a high salt lysis buffer (750 mM NaCl). Sample loading buffer was then added directly to the washed beads and run on 10% Tris-glycine polyacrylamide gels (Invitrogen). The gels were transferred to a nitrocellulose membrane, which was then blotted with rabbit anti-Myc (1:2000, abcam, Cambridge, MA) and mouse anti-Flag M2 (1:1500, Sigma).
Gas8 and GRK2 Cooperate in Smoothened Signaling

Gas8/11 (Pep5) antibody corresponding to GMAD amino acids 111–127 (NNLTEMKAGTVMVMK) was a kind gift from R. H. Crosbie and K. Hill at UCLA, CA. Gas8/11 antibody was diluted 1:500 and yielded a 56 kDa band. Rabbit anti-GFP (FL, 1:1500, Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect Smo in the GFP-Smo cell line and mouse anti-ACTin (1:5000, Milipore/Chemicon, Billerica, MA) was used as loading control. The signal was detected by a SuperSignal West Femto maximum-sensitivity substrate (Pierce). For immunofluorescence, C3H10T1/2 cells were transfected using TransIT-LT1 reagent (Mirus, Madison, WI) at a density of 2.5 x 10⁶ cells/35 mm glass bottom culture dish (MatTek, Ashland, MA) with the indicated plasmids, incubated for 48 h and fixed with 4% paraformaldehyde. Samples were incubated in 0.1% Triton for 10 min and blocked in 10% normal goat serum (NGS) for 30 min and then incubated with the primary antibody for either 3 h at room temperature or at 4 °C overnight. Samples were washed in PBS and incubated with the appropriate secondary antibody for 2 h at room temperature. GFP-Smo cells were grown to confluence in 35 mm glass bottom culture dishes, starved for 2 h in 2% BCS, fixed, and stained according to the above protocol. All antibodies were diluted in 1% BSA, 2% NGS, 0.4% Triton X-100 and 0.05% Tween-20 in PBS. Primary antibodies used were: mouse anti-acetylated tubulin (1:1000, Sigma), mouse anti-γ-tubulin (1:500, Sigma), mouse anti-β-tubulin (1:1000, Sigma), rabbit anti-Myc (1:4000, Abcam), rabbit anti-GRK2 C-15 (1:500, Santa Cruz Biotechnology), and rabbit anti-GM130 (1:500, Sigma). Secondary antibodies included Alexa Fluor 568 goat anti-mouse, 568 goat anti-rabbit, and 488 goat anti-rabbit (Invitrogen/Molecular Probes, Eugene, OR). Lysotracker was used to label lysosomes (Invitrogen). DNA was labeled using either Hoechst stain (Invitrogen) or Draq5 (Alexis Biochemicals, San Diego, CA). Imaging was done using Zeiss LSM510 and LSM710 confocal microscopy systems. The public domain ImageJ software was used for the analysis of confocal Z-stacks.

Zebrafish Strains and Husbandry—A single outcross of ekwill and AB inbred lines (EK/AB) produced adult fish that were used for wild-type egg production. Smo−/− mutants (21) were kindly provided by M. Bagnat, Duke University, intercrossed, and screened for mutant embryos. All zebrafish were maintained according to standard procedures in accordance with Duke University approved animal use IACUC protocols.

Morpholino and mRNA Microinjections—The zGas8 ATG MO (5’-GCACGATTTTTCTTTTGCGTTGATT-3’) targeting the translation start site of the zGas8 mRNA was previously described (14). 5-bp mismatched ATG morpholino (5’-GCCACCTTTTTTCTTTTGCTTGGAT-3’) was used as control. zGRK2/3 ATG MO and its 5-bp mismatched morpholino were as in (10). β-Arrestin2 MO was previously described (12). Pol ATG MO was 5’-CTGGGACAAGATGCACATCTCGT-3’. All morpholinos were synthesized by Gene Tools (Philomath, OR). Two nanoliter of the morpholino stock solutions were injected into the yolk of 1–2-cell embryos using a Femtojet microinjector (Eppendorf, Fremont, CA). Morpholino Stock solutions were: 0.5 mm (final dose of 8 ng/embryo) Gas8 and its control MOs, 0.05 mm GRK2 and its control MOs, 0.25 mm Pol and β-arrestin2 MOs. Capped mRNAs for injections were generated using the T7 and SP6 message machine kit (Ambion) using linearized and purified cDNA as the template. Mutagenesis of the morpholino binding site was performed using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). For rescue experiments, 100 pg mRNA of a mutant Gas8 mRNA or the dnPKA mRNA was co-injected with the indicated morpholino. Injected embryos were kept at 28 °C. 0.003% 1-phenyl-2-thiourea (PTU, Sigma) was added to suppress pigmentation. Embryos were dechorionated prior to their analysis.

Whole Mount In Situ Hybridization and Immunofluorescence—In situ hybridization was performed following standard protocols, with one of the DIG-labeled probes for Nkx2.2, Pax2, and Shh. Embryos were fixed with 4% paraformaldehyde, digested with proteinase K, and hybridized with the probe at 70 °C. Alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) was used to detect the signals. After staining with BM purple substrate (Roche), embryos were re-fixed with 4% paraformaldehyde and supplemented with 10 mM EDTA and stored in PBS buffer. For imaging, embryos were photographed using a Leica microscope with a plan Apo 1× lens equipped with a Retiga Esi Fast CCD camera (QImaging). For Immunofluorescence, embryos were fixed 27 hpf (for muscle phenotypes, Figs. 6 and 7) or 30 hpf (for cilia analysis, Fig. 8), blocked and incubated overnight with the appropriate primary antibody in blocking solution at 4 °C. The blocking solution included 2% NGS, 1% BSA, 0.1% Fish skin gelatin (Sigma), 0.1% Triton X-100, 0.05% Tween-20, and 0.05% sodium azide in 1× PBS. The next day, the embryos were washed and incubated with a secondary antibody overnight at 4 °C. The 4d9 antibody (1:100), which recognizes the engrailed protein in the nuclei of muscle pioneer cells was a kind gift from N. Patel (University of California, Berkeley, CA; Ref. 22). The Prox1 antibody (1:500, Millipore, Temecula, CA) was used to label the nuclei of slow muscle fibers and the acetylated tubulin antibody (1:500, Sigma) was used to label cilia in the zebrafish neural tube. Fluorescent secondary antibodies were used for detection. Stained embryos were deyolked using forceps and flat mounted on slides with Vectashield Hard Set mounting medium (Vector Laboratories, Burlingame, CA). Imaging was performed using a Zeiss LSM510 confocal microscopy system.

Statistical analysis was performed using both Excel and GraphPad Prism software (San Diego, CA). p values were calculated by either two tailed Student’s t-test or one way Anova with Bonferroni’s post hoc test for multiple group comparison as indicated in the text. Prior to each t-test, an F-test was applied to determine whether the variances are significantly different.

RESULTS

Gas8 Is a Smo-binding Protein—To identify novel Smo-binding proteins we have applied a yeast two-hybrid screen using the last 211 residues of human Smo C terminus as bait. Several potential partner proteins from a human cDNA library enabled the survival of yeast clones expressing human Smo C terminus. Of these, Gas8 appeared in four cDNA fragments; one containing the last 93 residues of Gas8 while the other 3 clones included the last 122 residues. To confirm this potential interaction we first repeated the two-hybrid assay in yeast using the same bait, but with 2 versions of Gas8 cDNA as a prey, either encoding...
Gas8 residues 357–478 (Gas8, Fig. 1A) or a shorter C terminus, harboring the last 93 residues (Gas8CT, Fig. 1B) under screening conditions lacking histidine and adenine (-HIS, -ADE). C, Smo and Gas8 specifically interact in HEK cells. HEK cells were transiently transfected with pcDNA3.1 control vector, Myc-tagged Smo, Flag-tagged mouse Gas8, or both Myc-Smo and Gas8. Lysates were immunoprecipitated (IP) with an antibody directed to the Flag tag and immunoblotted for the presence of Myc-Smo or the Flag-tag, WCL, whole cell lysate.

**FIGURE 1.** Gas8 is important for Smo signaling and ciliary localization. A and B, association between Smo and Gas8 was assayed in vitro by yeast two-hybrid technique using the last 211 residues of human Smo as a bait (SmoCT) and Gas8 cDNA as prey, either encoding Gas8 residues 357–478 (Gas8, A) or its 93 C-terminal residues (Gas8CT, B) under screening conditions lacking histidine and adenine (-HIS, -ADE). C, Smo and Gas8 specifically interact in HEK cells. HEK cells were transiently transfected with pcDNA3.1 control vector, Myc-tagged Smo, Flag-tagged mouse Gas8, or both Myc-Smo and Gas8. Lysates were immunoprecipitated (IP) with an antibody directed to the Flag tag and immunoblotted for the presence of Myc-Smo or the Flag-tag, WCL, whole cell lysate.

D–K, lentiviral shRNA-mediated Gas8 knockdown. D, Gas8 protein levels were detected in lysates from NIH-3T3 cells stable for either Gas8 shRNA or a scramble control (scr) shRNA. Anti-actin antibody was used for loading control. Averages ± S.E. were calculated from 4 experiments and presented as percent of scr control. E, shRNA stable cell lines were transiently transfected with a Gli luciferase reporter and Renilla luciferase internal control. Cells were treated with either SAG or DMSO for 24 h and then starved overnight in the induction media and lysed. Averaged fold induction in luciferase activity values ± S.E. (SAG/DMSO) were calculated. *, p < 0.05. F, GFP-Smo cell line was infected with the lentiviral shRNAs to generate double stable lines, plated, and stained for acetylated tubulin (red). G–I, percent of cells with cilia (G), the averaged length of the cilia (H), and the percent of GFP-Smo containing cilia (I) ± S.E. were calculated from four experiments in GFP-Smo cells stable for either Gas8 or scr shRNAs. **, p < 0.01. J, GFP-Smo protein levels were detected in the double stable cell lines using an antibody against GFP. K, Gas8 protein levels were detected upon scr shRNA following stimulation with SAG or DMSO. In both J and K, anti-actin antibody was used for loading control. All p values were calculated by two-tailed Student’s t test.

Gas8 Is Important for Shh Signaling and Smo Ciliary Localization—To assess whether the interaction between Gas8 and Smo is functional, we analyzed Gas8 loss of function in NIH-3T3 cells by lentiviral shRNA-mediated knockdown which yielded 50% reduction in Gas8 protein levels (Fig. 1D). We examined the Gli-dependent transcriptional activity upon Gas8 knockdown using Gli-dependent luciferase reporter assay (13). We found that SAG stimulation leads to a 15-fold induction in the reporter activity levels in the control scramble (scr) shRNA, while Gas8 shRNA significantly reduced the reporter activities by ~50% (7.7-fold induction, Fig. 1E). There was no significant change in Gas8 protein levels in the control cells under SAG treatment, indicating that it is not controlled by Shh pathway (Fig. 1K). Gas8 has been previously shown to co-localize with the microtubule organizing center (MTOC), centrosomes and the Golgi apparatus. In ciliated cells, Gas8 is localized to the basal body of the cilia (20). To assess whether Gas8 knockdown interferes with Smo accumulation in the cilia, we
followed Smo subcellular localization in GFP-Smo cells (13) upon stable infection of Gas8 shRNA. Gas8 shRNA does not affect cilia formation or elongation as the percent of ciliated cells and the averaged length of the cilia remain the same (Fig. 1, F–H). Consistent with a previous report (13), 56% of the cilia in the control (scr) cells contain GFP-Smo, even without ligand stimulation (Fig. 1, F and I). However, upon Gas8 shRNA, only 24% of the cilia contain GFP-Smo, suggesting a role for Gas8 in Smo transport to the cilia. This loss of Smo in the cilia is not due to reduced expression levels of GFP-Smo because similar protein levels were detected upon Gas8 shRNA (Fig. 1J).

Gas8 and GRK2 Cooperate in Smoothened Signaling

To assess which domain of Gas8 is important for its effect on Smo and to tests whether altering the ability of Gas8 to interact with microtubules results in differential effect on Smo signaling we generated four different Flag- or GFP-tagged Gas8 fusion proteins consisting of the different mouse (m)Gas8 domains initially described in Bekker et al. (17) and illustrated in Fig. 2A: mGas8FL (full-length), mGas8GMAD-CT, mGas8IMAD-GMAD, and mGas8CT. The last construct consists of the 93 amino acids that bind Smo C terminus and is shorter than the C-terminal construct designed by Bekker et al. (17). We followed the subcellular localization of GFP-tagged mGas8 fusion proteins in C3H10T1/2 cells, which we have previously used to monitor Smo-dependent signaling (11). The majority of GFP-mGas8FL-expressing cells display a punctated cytoplasmic distribution of Gas8, probably in vesicles

FIGURE 2. Gas8 polypeptides display differential subcellular localization and exert various effects on Smo ciliary accumulation and activity. A, schematic representation of the recombinant Gas8 polypeptides that were fused to GFP or Flag tag. B, C3H10T1/2 cells were transiently transfected with one of the four Gas8-GFP fusion constructs, fixed 72 h post transfection and immunostained using anti-γ-tubulin (red), which marks the cilia basal body. Scale in insets is 5 μm. C, C3H10T1/2 cells stably expressing Myc-Smo were transfected with one of the four Gas8-GFP fusion constructs, fixed 48 h post-transfection and immunostained using an antibody against the Myc tag (red). Bar graphs in D indicate the averaged percent of GFP-positive cells in C that displayed GFP-positive cilia basal body. **, p = 0.0001, One-way ANOVA with Bonferroni’s post hoc test indicating that Gas8GMAD-CT is different from all other groups. Bar graphs in E indicate the averaged percent of GFP-positive cells in C that contained Myc-Smo within the cilia shaft. *, p < 0.05, two tailed Student’s t-test as compared with the empty GFP vector group. Averages ± S.E. in D and E were calculated from three independent experiments. F, effect of different Gas8 polypeptides on Gli luciferase reporter activity. C3H10T1/2 cells were transiently transfected with a Gli luciferase reporter, a β-Gal transfection control and the indicated recombinant Flag-tagged Gas8 expression plasmids along with Smo, SmoGRK2 or SmoK220R plasmids. Fold increase refers to the increase in luciferase activity relative to a sample containing the reporter, Smo and the empty vector pcDNA3.1 (n = 6–9 independent experiments for Smo and Smo+GRK2 and 3 for Smo+K220R; *, p < 0.05, two tailed Student’s t-test).
along cellular microtubules (Fig. 2B and supplemental Fig. S1, left panels). A smaller fraction (15–20%) of Gas8FL is localized to the basal body of the cilia in addition to its localization in vesicles. Gas8\textsubscript{GMAD-CT} that lacks the N-terminal IMAD sequence is co-localized with γ-tubulin in the cilia basal body of the majority of the transfected cells (Fig. 2B and supplemental Fig. S1). The observation that only a small fraction of Gas8\textsubscript{FL} is distributed to the cilia basal body could reflect a weak and transient binding to microtubules (17). Interestingly, Gas8\textsubscript{CT} and the truncated Gas8\textsubscript{IMAD-GMAD} constructs are not associated with microtubules, but accumulate in the cytoplasm or in aggregates, respectively. Using markers for the Golgi apparatus (GM130) and lysosomes (LysoTracker), we found that Gas8\textsubscript{IMAD-GMAD} aggregates are not co-localized to either of these organelles, although they are found in proximity to the Golgi (supplemental Fig. S2). To test which of Gas8 domains supports changes in Smo subcellular localization, we transfected C3H10T1/2 cells, which stably express Myc-Smo with the different GFP-Gas8 constructs. Smo in those cells is constitutively active (11) and accumulates in the cilia (Fig. 2, C and E). The accumulation of Gas8\textsubscript{GMAD-CT} in the cilia basal body does not affect the percent of Smo positive cilia (Fig. 2, C–E). However, all other forms of Gas8 that do not accumulate in proximity to the cilia basal body prevent Smo ciliary localization (Gas8\textsubscript{FL}, Gas8\textsubscript{IMAD-GMAD}, and Gas8\textsubscript{CT} in Fig. 2, C–E), suggesting a role for the GMAD-CT domain in Smo targeting to the cilia.

**Gas8 Stimulation of Smo Activity in Cells Is Dependent of Gas8 C Terminus and GRK2**—To assess which domain of Gas8 is important for its effect on Smo-dependent signaling we have transiently transfected C3H10T1/2 with the different Flag-tagged Gas8 fusion proteins and measured Gli-dependent luciferase reporter activities. Because GRK2 enhances Smo-mediated transcriptional activity in cells (10, 11), we measured the reporter activity following co-transfection of Myc-Smo, Gas8, and GRK2. As expected, GRK2 and Myc-Smo induced a 2-fold increase in the reporter activity as compared with Myc-Smo alone (Fig. 2F). In the presence of GRK2, Gas8\textsubscript{GMAD-CT} induced a statistically significant 3-fold increase in activity (Fig. 2F, *, p < 0.05, two tailed Student’s t-test). However, the effect of Gas8\textsubscript{CT} on the reporter activity was highly variable and thus did not reach statistical significance. No significant induction was observed either in the absence of GRK2 or with the kinase dead GRK2 mutant (GRK2 K220R, Ref. 23) as well as following co-transfection of GRK2 and either Gas8\textsubscript{IMAD-GMAD} or Gas8\textsubscript{CT}. These findings suggest that GRK2 and Gas8\textsubscript{GMAD-CT} potentially cooperate to activate Gli-dependent transcription in the presence of Smo.

**Catalytically Active GRK2 Facilitates Cilia Elongation and Smo Accumulation in Cilia**—Earlier reports showed that GRK2 can phosphorylate both tubulin and Smo (9, 10). Given the GRK2-dependent effects of Gas8 on Smo signaling, we sought to examine whether GRK2 is involved in targeting Smo to ciliary microtubules. We studied cilia from 4 lines of C3H10T1/2 cells stably transfected with GRK2, Myc-Smo, Myc-Smo with GRK2 (Smo\_GRK2) or Myc-Smo with GRK2 K220R (Smo\_K220R, (11)). Surprisingly, Smo\_GRK2 stable cells assemble 2.2-fold longer cilia as compared with GRK2 or Smo alone and visualized by acetylated tubulin staining (Fig. 3, A and C). This structural change is accompanied by an induction of Gli activity, which can be blocked by the Smo antagonist cyclopamine (Fig. 3E and Ref. 11). Cilia from GRK2 or Smo cells are indistinguishable from cilia of cells expressing empty vectors only (Fig. 3, A and C). Moreover, cilia from Smo\_K220R cells were similar to those of Smo cells, suggesting that the kinase activity of GRK2 is important for its effect on cilia elongation. Population distribution analysis further confirms the shift in cilia length in our Smo\_GRK2 cell line (supplemental Fig. S3). We hypothesized that in the presence of GRK2 and Smo, longer cilia may allow more Smo to accumulate within the cilia and that this accumulation may contribute to the observed higher transcriptional activity levels. Indeed, we found that cilia which are positive for Myc-Smo were significantly longer in the Smo\_GRK2 line as compared with Smo and Smo\_K220R lines (Fig. 3, A and C). In those positive cilia, Smo was found along the cilium shaft and at the distal tip (Fig. 3A, insets). However, GRK2 showed cytoplasmic localization (Fig. 3B). The ratio between the length of cilia that are positive for Myc-Smo and the length of cilia labeled with acetylated tubulin was similar among the different cell lines (80–90% of the cilium shaft is filled with Myc-Smo, Fig. 3). Moreover, the staining intensity of Myc-Smo signal was also unchanged (Fig. 3D), suggesting that Smo accumulation within cilia is proportional to the increased length of the cilium, an effect that is GRK2-dependent.

**Gas8 Regulates Zebrafish Early Development and Hh Target Gene Expression**—Loss of zebrafish (z)Gas8 leads to various developmental abnormalities, including hydrocephaly, disorganized somites, pericardial edema, left-right asymmetry, and abnormal ear development (14). Given that regulated Hh signaling is essential for normal inner ear development in zebrafish (24) and based on the interaction between Smo and Gas8, we hypothesized that at least some of the reported abnormalities may be associated with disrupted Hh signaling pathway. Thus, we injected fertilized zebrafish eggs with a translation blocking morpholino against zGas8 (Gas8 MO) or its control morpholino which lacks the ability to bind Gas8 mRNA due to 5 point mutations (Gas8 CT MO). As is shown in Fig. 4A and E, 189/269 Gas8 morphant embryos displayed phenotypes including pericardial edema, misshaped somites and hydrocephaly, while 128/171 embryos injected with the control morpholino which lacks the ability to bind Gas8 mRNA in 67/93 and 51/72 injected embryos, respectively. Co-injection of a dominant negative ver-
sion of PKA (dnPKA) mRNA, which has been previously shown to induce constitutive activation of Hh at the level of Gli (26), reversed both the phenotype (Fig. 4, A and E, 33/56 embryos developed normally) and the attenuated expression of Hh target genes that were caused by zGas8 inhibition (Fig. 4, B, C, F, and G, 34/56 and 38/53 embryos displayed unaffected Nkx2.2 and Pax2 mRNA levels, respectively).

To assess the severity of Gas8 MO effects we compared Gas8 morphants to Smos294 mutant fish, where the Smo gene is mutated (21) and to fish injected with 2 other MOs targeting either /H9252-arrestin2 or Polaris (Pol/Tg737/IFT88), an anterograde IFT protein important for cilia formation (27). Already at 24 hpf, Gas8 morphants showed similar, although less severe phenotypes like those observed in /H9252-arrestin2 morphants and Smos294 mutant fish (Fig. 5, C as compared with D and E). Interestingly, those phenotypes seem to be stronger than in Pol morphants at 24 hpf, but milder at 72 hpf (Fig. 5 F). The observed down-regulation of Nkx2.2 and Pax2 mRNAs following Gas8 knockdown is similar to their attenuated expression in /H9252-arrestin2 morphants (Fig. 5, I and O as compared with Fig. 5, J and P) but only partially similar to Pol morphants, which had normal Nkx2.2 levels (Fig. 5L). Smo^{294} mutant displayed more dramatic reduction in both mRNAs, consistent with severe impairment of the Hh pathway (Fig. 5, K and Q). These findings suggest a selective but moderate attenuation of Hh signaling in embryos with reduced zGas8 expression.

**FIGURE 3.** Stable transfection of GRK2 modifies ciliary structure and Smo accumulation. A, cilia of C3H10T1/2 cells stably expressing GRK2, Myc-tagged Smo (Smo), Myc-tagged Smo, and GRK2 (Smo-GRK2), Myc-tagged Smo and the kinase dead GRK2 (Smo-K220R) or two empty vectors were labeled using antibodies against acetylated tubulin (A, upper panel) and the Myc tag (A, middle panel, merged images in the bottom panel). Insets in A show co-staining of γ-tubulin (red) and anti Myc (green). The inset’s scale is 2 μm. In B, GRK2 and acetylated tubulin co-staining show that GRK2 is not enriched within the cilia. C averages ± S.E. of general cilia length and Myc-Smo positive cilia length, calculated from three samples per indicated cell line and 50–100 cilia analyzed in each sample. **, p < 0.001, Student’s t-test. D, averages ± S.E. of Myc-Smo staining intensities in the cilia analyzed in C and displayed as a ratio of Smo. All measurements were done on confocal Z stacks. E, C3H10T1/2 cells stably expressing the indicated proteins were transfected with the Gli reporter and a β-gal transfection control, treated as indicated and assayed for luciferase activity 72 h post-transfection.
than in Pol morphant embryos (Fig. 6, E, G, and H). The numbers of both slow muscle and muscle pioneer cells were rescued by the co-injection of Gas8 MO and dnPKA mRNA (see also Fig. 5). 72 hpf embryos are shown laterally. B–D, whole mount in situ hybridization of Nkx2.2 (B), Pax2 (C), and Shh (D), showing attenuated Nkx2.2 expression in the floor plate (fp) and hind brain (hb), and Pax2 in the otic vesicle (ov) of 24 hpf embryos injected with Gas8 MO, but not with its CT MO. Normal expression levels achieved by co-injection of zGas8 or dnPKA mRNAs. Shh expression levels remained similar under all conditions. E–H, stacked bar graph analysis of the percentage of embryos with normal or abnormal phenotype (E), and the percentage of embryos that displayed attenuation or no change in the indicated transcript levels (F–H) following the different injections. Stacked bar graphs summarize at least 4 separate injections of Gas8 MO and their controls. Two of these experiments included zGas8 mRNA and the other two included dnPKA mRNA for rescue. Numbers of injected embryos are indicated in the text.

zGas8 and zGRK2/3 Regulate Hh Signaling but Are Not Essential for Cilia Assembly in Zebrafish—To determine whether Gas8 and GRK2/3 function in the same pathway, we co-injected GRK2/3 MO and zGas8 mRNA and then examined Nkx2.2 expression levels. As previously reported (10), GRK2/3 MO caused a moderate reduction of Nkx2.2 mRNA in the developing brain of 46/60 zebrafish embryos 24 h post injection (Fig. 8B) as compared with 5/35 embryos injected with its control MO (Fig. 8A). Nkx2.2 expression levels were rescued in...
36/53 GRK2/3 morphants by the co-injection of zGas8 mRNA (Fig. 8D), suggesting a genetic interaction between Gas8 and GRK2/3. zGas8 mRNA did not affect Nkx2.2 levels in 33/38 control embryos (Fig. 8C). Given the effect of GRK2/3 on cilia elongation, we explored whether Gas8 or GRK2/3 MOs can affect cilia formation in vertebrates. Acetylated tubulin staining 30 hpf revealed that Pol MO reduces the numbers of cilia in the caudal neural tube, while neither Gas8 nor GRK2/3 MOs affect cilia numbers or architecture, suggesting that both proteins are not required for ciliogenesis in zebrafish (Fig. 8F).

**DISCUSSION**

In this study, using gain and loss of function approaches, we identified Gas8 as a novel interacting partner of Smo that cooperates with GRK2 and thus promotes Smo signaling in vitro and in vivo.

Previously, Gas8 has been suggested to serve as a linker between the DRC and microtubules through its GMAD domain, regulating dynein and microtubule sliding in motile cilia and flagella (16, 17, 20, 30). We show here that 50% reduction in Gas8 protein levels is sufficient to attenuate Smo-dependent signaling and leads to the loss of Smo within the cilia without affecting ciliogenesis. We also find that altering the ability of Gas8 to interact with microtubules results in differential effect on Smo signaling. mGas8GMAD-CT accumulates in the cilium basal body and induces Smo signaling in cells in a GRK2 dependent manner. Truncated forms of Gas8 that do not localize to the cilium decrease Smo ciliary accumulation and are unable to increase signaling. However, the full-length Gas8 could be detected both at the base of the cilium and in vesicles in the cytoplasm, which may explain the high variability in the levels of Smo stimulation as compared with the induction achieved by the removal of IMAD from the expression vector. This may also indicate that the IMAD has to be sequestered to allow maximal interaction with microtubules as was previously suggested (17).

Whereas Gas8 knockdown in ciliated cells and zebrafish embryos is sufficient for moderately attenuating Hh signaling, its stimulatory effect on activity depends on GRK2 being catalytically active. The mechanism of this dependence is not clear.
Gas8 and GRK2 Cooperate in Smoothened Signaling

AUGUST 5, 2011•VOLUME 286•NUMBER 31
JOURNAL OF BIOLOGICAL CHEMISTRY 27685

but it may be partially explained by the structural modification in ciliary length caused by simultaneous expression of GRK2 and Smo in stable cell lines, where higher levels of Smo can be targeted to the cilia. Cilia length is regulated by cAMP levels (31, 32). However, the observation that stable expression of Smo and GRK2 can affect cilia length is consistent with a previous suggestion that the Hh pathway by itself may influence the length of primary cilia (33). Surprisingly, GRK2 does not have to traffic into the cilia or to the basal body in order to exert its effect. Possibly, GRK2 phosphorylates cytoplasmic tubulin or other proteins that would enter the cilium and modulate microtubule organization. Alternatively, recent studies have shown that temporary down-regulation of GRK2 is required for normal cell cycle progression (34) and that depletion of β-arrestin results in uncontrolled cell proliferation and reduced cilia numbers (35), suggesting an indirect mechanism involving cell cycle control. Yet, other mechanisms can certainly not be ruled out.

Our findings suggest a role for Gas8 in vertebrate Hh signaling. Gas8 knockdown in zebrafish results in various developmental abnormalities, including impaired development of slow muscles, a process that depends on Hh signaling (28, 29). Those abnormalities are accompanied by attenuated Hh transcriptional responses, further confirming the involvement of Gas8 in...
Gas8 and GRK2 Cooperate in Smoothened Signaling

this pathway. The mild loss of function phenotypes of Gas8 compared with those of Smo294 point to Gas8 as a moderate or transient effector of the Hh pathway that exerts its activity upstream of Gli. The aberrant slow muscle development as well as the attenuation in Hh target gene expression are similar to those of GRK2/3 and as the attenuation in Hh target gene expression are similar to upstream of Gli. The aberrant slow muscle development as well

REFERENCES

1. Huangfu, D., and Anderson, K. V. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 1118–1123
2. Blesschotnova, T. Y., Kolpakova-Hart, E., Guan, Y., Zhou, J., Olsen, B. R., and Shah, J. V. (2010) Curr. Biol. 20, 182–187
3. Ou, Y., Ruan, Y., Cheng, M., Moser, J. J., Rattner, J. B., and van der Hoorn, F. A. (2009) Exp. Cell Res. 315, 287–298
4. Barski, M. J., Stiekny, H. L., and Devoto, S. H. (2000) Development 127, 2189–2199
5. Lewis, K. E., Currie, P. D., Roy, S., Schauerte, H., Haffter, P., and Ingham, P. W. (1999) Dev. Biol. 216, 469–480
6. Heuser, T., Raytchev, M., Krell, J., Porter, M. E., and Nicastro, D. (2009) J. Cell Biol. 187, 921–933
7. Besschnettova, T. Y., Kolpakova-Hart, E., Guan, Y., Zhou, J., Olsen, B. R., and Shah, J. V. (2010) Curr. Biol. 20, 182–187
8. Ou, Y., Ruan, Y., Cheng, M., Moser, J. J., Rattner, J. B., and van der Hoorn, F. A. (2009) Exp. Cell Res. 315, 2802–2817
9. Kim, H. R., Richardson, J., van Eeden, F., and Ingham, P. W. (2010) BMC Biol. 8, 65
10. Penela, P., Rivas, V., Salcedo, A., and Mayor, F., Jr. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 1118–1123
11. Molla-Herman, A., Boularan, C., Ghossoub, R., Scott, M. G., Burtey, A., Zarka, M., Saunter, S., Concordet, J. P., Marullo, S., and Benmerah, A. (2008) PLoS ONE 3, e3728
12. Haycraft, C. J., Banizs, B., Ayydin-Son, Y., Zhang, Q., Michaud, E. J., and Yoder, B. K. (2005) PLoS Genet. 1, e53
13. Huangfu, D., and Anderson, K. V. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 11325–11330
14. Huangfu, D., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L., and Anderson, K. V. (2003) Nature 426, 83–87
15. Qin, J., Lin, Y., Norman, R. X., Ko, H. W., and Eggenschwiler, J. T. (2011) Proc. Natl. Acad. Sci. U.S.A. 108, 1456–1461
16. Huang, P., and Schier, A. F. (2009) Development 136, 3089–3098
17. Lunt, S. C., Haynes, T., and Perkins, B. D. (2009) Dev. Dyn. 238, 1744–1759