Identification of a Suppressible Mechanism for Hedgehog Signaling through a Novel Interaction of Gli with 14-3-3*

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Gli transcription factors are central effectors of Hedgehog signaling in development and tumorigenesis. Using a tandem affinity purification (TAP) strategy and mass spectrometry, we have found that Gli1 interacts with 14-3-3, and that Gli2 and Gli3 also bind to 14-3-3 through homologous sites. This interaction depends on their phosphorylation, and cAMP-dependent protein kinase (PKA), a known negative regulator of Hedgehog signaling serves as a responsible kinase. A Gli2 mutant engineered to eliminate this interaction exhibited increased transcriptional activity (2 – 3×). Transcriptional repression by 14-3-3 binding was also observed with Gli3, when its N-terminal repressor domain was deleted. The phosphorylation sites responsible for the binding to 14-3-3 are distinct from those required for proteolysis, the known mechanism for PKA-induced repression of Hh signaling. Our data propose a novel mechanism in which PKA down-regulates Hedgehog signaling by promoting the interaction between Gli and 14-3-3 as well as proteolysis. Given the certain neuronal or malignant disorders in human caused by the abnormality of 17p13 encompassing 14-3-3 interaction, we used a tandem affinity purification (TAP) strategy in conjunction with mass spectrometry, to isolate and characterize human protein complexes containing Gli.

The signaling proteins of the Hedgehog (Hh) family play central roles in many developmental processes in organisms from flies to humans, particularly processes involving patterning of the neural tube, notochord, and prechordal mesoderm (1, 2). Hedgehog protein, the ligand that initiates Hh signal transduction pathway, is a developmental morphogen that elicits a graded cellular response depending on the distance between the recipient cell and the ligand-secreting cell. This gradient-dependent signaling is thought to be important in the determination of cell fate. Deregulation of Hh signaling results in a variety of congenital malformations and cancers (3–5). Given the importance of the Hh signaling pathway, there has been considerable interest in understanding the mechanism by which a target cell interprets Hh signaling to yield an appropriate response.

In Drosophila, activation and repression of Hh target genes is mediated by a single Gli zinc finger transcription factor known as Cubitus interruptus (Ci). In the absence of Hh signaling, Ci is phosphorylated by cAMP-dependent protein kinase (PKA) (6), glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CKI). After proteolytic processing into an N-terminal repressor form, Ci translocates to the nucleus, where it silences expression of its target genes. Hh signaling triggers a series of events that prevents this phosphorylation and proteolysis of Ci, to block formation of the repressor form, and allow the full-length, activating form of Ci to enter the nucleus and promote induction of specific target genes (1, 7).

In vertebrates, three Hedgehog proteins (sonic, Indian, and desert), and three Gli proteins, Gli1, Gli2, and Gli3, are involved in transcriptional control of Hh target genes (2, 8). Each Gli protein has a unique role: Gli3 functions chiefly as a transcriptional repressor, Gli2 is chiefly a transcriptional activator, and Gli1 functions only as a transcriptional activator (1). Like Ci, Gli3 is phosphorylated and processed in the absence of Hh; it responds to Hh by relocating to the nucleus without cleavage (9). Though Gli1 knockout mice develop normally, Gli2 and Gli3 knockouts have serious developmental abnormalities (4), suggesting that Gli2 and Gli3 signaling is likely to be intensely regulated in response to Hh ligands. To better understand the molecular mechanisms underlying Gli activation and regulation, we used a tandem affinity purification (TAP) strategy involving a novel mammalian TAP tag, called MEF (10, 11), in conjunction with mass spectrometry, to isolate and characterize human protein complexes containing Gli.

**EXPERIMENTAL PROCEDURES**

Cell Cultures and Transient Transfection—The human embryonic kidney cell line 293T, the human hepatoma cell line PLC/PRF/5, the human cervical carcinoma cell line HeLa, and the mouse fibroblast cell line NIH3T3 (Riken Cell Bank, Tsukuba Science City, Japan) were maintained in Dulbecco’s
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modified Eagle’s medium (Sigma) containing 10% heat-inactivated fetal bovine serum (293T, PLC/PRF/5, and HeLa) or bovine serum (NIH3T3). All cells were incubated at 37 °C and 5% CO$_2$. Cells were seeded on culture plates, and transfections were carried out using Effectene transfection reagent (Qiagen, Hilden, Germany) for PLC/PRF/5 and NIH3T3 cells or FuGENE 6 (Roche Applied Science, Indianapolis, IN) for 293T and Hela cells, as described in the manufacturers’ protocol.

Antibodies and Materials—Mouse monoclonal anti-c-Myc Ab (9E10) and its agaroase-conjugated form, rabbit polyclonal anti-14-3-3 Ab (K-19), and normal rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-FLAG M2 Ab and its agaroase-conjugated form and mouse monoclonal anti-α-tubulin Ab (DM1A) were purchased from Sigma. Mouse monoclonal anti-Xpress Ab was obtained from Invitrogen (Carlsbad, CA), and mouse monoclonal Ab (4E2) specific for the phospho-(Ser) 14-3-3 binding motif, rabbit monoclonal Ab (100G7E) specific for the phospho-PKA substrate, and rabbit polyclonal anti-Gli1 (nos. 2553 and 2534) and anti-14-3-3ε Ab were from Cell Signaling Technology (Beverly, MA). Alexa Fluor 488 goat anti-mouse IgG secondary Ab was obtained from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit Abs were purchased from GE Healthcare (Piscataway, NJ). Forskolin (FSK) and Smo agonist (SAG) were obtained from Sigma and Alexis Biochemicals, respectively.

Plasmids—Human Gli1 and Gli3 cDNAs (from K. W. Kinzler and B. Vogelstein (2, 8)) and mouse Gli2 cDNA (from H. Sasaki (12)) with deletions of their 5’-untranslated sequences were cloned into the vector pcDNA3-MEF, a mammalian expression plasmid for the MEF method described previously (10, 11). PCR-based mutagenesis was used, and genes encoding wt and mutant ΔN-Gli3 were prepared utilizing the unique BamHI site. ΔPKA-Gli2 was generated by a combination of restriction digestion and PCR strategies to delete the region from Ser-781 to Leu-861. All PCR-generated constructs were verified by sequencing. The reporter plasmid GliBS-Luc, the Myc-tagged SUFU expression vector, the vector expressing the constitutively active catalytic subunit of PKA (PKA-CA), and the 14-3-3 inhibitor peptide expression vector EYFP-difopein were kindly provided by H. Sasaki (13), R. Toftgard (14), G.S. McKnight (15), and H. Fu (16), respectively.

MEF Purification and Tandem Mass Spectrometry—MEF purification was carried out as described previously (10, 11). Briefly, 40 10-cm dishes containing ~8 × 10^7 293T cells in total were transfected with 80 μg of pcDNA3-MEF-Gli1 or pcDNA3-MEF plasmid. After 48 h, the cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (w/v) glycerol, 100 mM NaF, 10 mM EGTA, 1 mM Na$_2$VO$_4$, 5 μM ZnCl$_2$, 0.05% SDS, 1% (w/v) Nonidet P-40, and a protease inhibitor mixture (Complete Mini; Roche Applied Science)), and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was removed and incubated with anti-Myc Ab-conjugated agaroase beads for 90 min at 4 °C. The beads were then washed five times with wash buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (w/v) glycerol, 0.05% SDS, 0.1% (w/v) Nonidet P-40) and twice with buffer B (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% SDS, 0.1% (w/v) Nonidet P-40). The beads were then mixed with 15 units of tobacco etch virus (TEV) protease (Invitrogen) in buffer B at room temperature for 1 h to release the protein complex from the beads.

The protein complex was incubated with anti-FLAG Ab-conjugated agarose beads for a second immunoprecipitation at room temperature for 1 h. After three washes with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), the beads were eluted with FLAG peptide (80 μg/ml) in buffer A. The eluted proteins were concentrated, separated by 10/20% SDS-PAGE, and visualized by silver staining.

Protein bands were excised from the gel and digested with trypsin, and the resultant peptide fragments were analyzed using a direct nano-flow LC-MS/MS system equipped with an electrospray interface reversed-phase column, a nano-flow gradient device, and a high-resolution quadrupole time-of-flight hybrid mass spectrometer (Q-TOF2; Micromass, Manchester, UK). To identify the proteins, all of the MS/MS spectra were searched against the non-redundant protein sequence database maintained at the National Center for Biotechnology Information using the Mascot program (Matrixscience, London, UK). The MS/MS signal assignments were confirmed manually.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as previously described (11, 17). After 293T cells were lysed, the cellular proteins were incubated with anti-FLAG agarose-conjugated beads for 1 h at 4 °C. To detect endogenous interaction, cell lysates from PLC/PRF/5 cells were incubated for 1 h at 4 °C with anti-Gli1 or anti-14-3-3ε Ab or normal IgG control, followed by an additional 4 h of incubation with protein G. The beads were then washed, and the immunoprecipitated proteins were eluted by boiling in SDS sample buffer. The proteins were separated by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was probed with primary Ab (anti-14-3-3, anti-14-3-3ε, anti-Gli1, anti-phospho-14-3-3 binding site, anti-phospho-PKA substrate, anti-Myc, or anti-FLAG at 1:1,000 dilution, and anti-α-tubulin at 1:5000) and with secondary Ab (HRP-conjugated anti-mouse or anti-rabbit Ab at 1:1000 to 1:5000 dilution). The HRP signal was detected using an enhanced chemiluminescence system (ECL Plus, GE Healthcare).

Expression and Purification of Recombinant Gli1, Gli2, and Gli3 Proteins and in Vitro Kinase Assay—The regions surrounding the 14-3-3 binding sites on Gli1 (aa 617–688), Gli2 (aa 924–1057), and Gli3 (aa 981–1101) were amplified by PCR and inserted in-frame into the BamHI and Xhol (Gli1) or NotI (Gli2 and Gli3) sites of vector pGEX-6P-1 (GE Healthcare). The resulting expression vectors were transformed into Escherichia coli BL21(DE3) cells (Strategene). Expression was induced with 1 mM isopropyl-1-thio-β-d-galactopyranoside at 25 °C for 3 h. The cells were then pelleted and lysed in a buffer consisting of 50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1 mM dithiothreitol, 1 mg/ml lysozyme, and 1% Triton X-100. The lysates were sonicated and clarified by centrifugation (10,000 × g for 10 min at 4 °C). The fusion proteins were purified from the cleared bacterial lysates using glutathione-Sepharose 4B (GE Healthcare).

Purified GST fusion proteins were incubated with 25 ng of recombinant PKA catalytic subunit (Upstate, Lake Placid, NY),
20 μl of Assay Dilution Buffer I (Upstate) containing 10 μg of bovine serum albumin, and Magnesium/ATP Mixture (Upstate) containing 10 μCi of [γ-32P]ATP (GE Healthcare). After 30 min at 30 °C, the reactions were terminated by addition of 13 μl of 4X SDS-PAGE sample buffer, and phosphorylated proteins were visualized by SDS-PAGE and autoradiography.

**Knockdown of 14-3-3ζ using Lentiviral-based shRNA Vector**—pLKO.1-based lentiviral 14-3-3ζ shRNA vectors, TRCN0000062233 and 000062236 for PLC/PRF/5 and TRCN0000012385 and 000012386 for NIH3T3 were obtained from Open Biosystems. The non-target pLKO.1-scrambled shRNA (designated non-target-RNAi) was from Sigma. To prepare viral particles, 293T cells were co-transfected with 750 ng of psPAX2 packaging plasmid, 250 ng of pMD2.G envelope plasmid, and 2 μg of viral vector using Fugene6. Supernatants containing lentivirus particles were collected 36–60 h of post-transfection and filtered.

For lentivirus infection, PLC/PRF/5 or NIH3T3 cells in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum or BS and 8 μg/ml polybrene were incubated with an equal volume of lentivirus for 24 h. Stably transfected cells were selected in puromycin (2 μg/ml for PLC/PRF/5, 3 μg/ml for NIH3T3) and tested for 14-3-3ζ expression by Western blot.

**Quantitative Real-time RT-PCR**—Total RNA was collected with the RNeasy Mini Kit (Qiagen) and subjected to the RT reaction using oligo-dT primer and ImProm-II Reverse Transcriptase (Promega) according to the manufacturer’s instructions. Quantitative real-time PCR for murine Gli1 and Pch1 genes was performed using the cDNA of NIH3T3 cells treated with DMSO or 100 nM SAG/ml Polybrene or overnight at 4 °C and harvested 24 h after transfection and assayed for luciferase activity using the PicaGene Dual SeaPansy System (Toyo Ink, Tokyo, Japan) and a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). Independent experiments were performed at least three times.

**Electrophoretic Mobility Shift Assays**—Expression vectors for constitutively active PKA-CA and for FLAG-tagged wt and S956A Gli2 were co-transfected into 293T cells in 10-cm tissue culture dishes. The cells were lysed in 1 ml of lysis buffer, and cellular proteins were immunoprecipitated with anti-FLAG Ab-conjugated agarose beads for 2 h at 4 °C. After the beads were washed with lysis buffer, the bound proteins were eluted with buffer containing 100 μg/ml of 3X FLAG peptide (Sigma).

This eluted protein lysate was used in electrophoretic mobility-shift assays performed using a LightShift EMSA Kit ( Pierce Biotechnology), according to the manufacturer’s instructions. Briefly, eluted protein lysate was incubated for 30 min with biotinylated DNA probe (Gli-RE) (18) and non-biotinylated competitor DNA oligonucleotide (100-fold molar excess) in binding buffer supplemented with 2.5% glycerol, 5 mM MgCl2, 0.05% Nonidet P-40, and 50 ng/μl poly(dI-dC). The resulting complexes were resolved in a nondenaturing 6% polyacrylamide gel, transferred onto a membrane, and detected using HRP-conjugated streptavidin and a chemiluminescent substrate.

**RESULTS**

**Identification of 14-3-3ζ as a Novel Interacting Protein with Gli1**—The MEF (Myc-TEV-FLAG) technique uses two different affinity modules (Myc and FLAG) separated by a cleavage affinity module (Myc and FLAG) separated by a cleavage

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was carried out as previously described (11). HeLa cells were transfected with expression vectors 24 h after seeding. After another 36 h, including 12 h exposure to 40 μM FSK in the growth medium, the cells were fixed with 2% paraformaldehyde for 15 min at room temperature, rinsed with phosphate-buffered saline (PBS), and permeabilized with 0.25% Triton X-100/PBS for 15 min. After blocking, the cells were incubated with anti-MyC Ab (1 μg/ml) overnight at 4°C and then with Alexa Fluor 488 goat anti-mouse IgG secondary Ab (1:100 dilution) for 1 h at room temperature. The nuclei of the cells were stained with propidium iodide (Sigma). Fluorescence images were obtained using a Leica TCS SL confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) and photographed using Leica confocal software (Leica Microsystems).

**Luciferase Assays**—Luciferase assays were performed as described previously (11). Approximately 1 × 104 293T cells were plated into each well of a 12-well tissue culture plate (Iwaki Glass, Chiba, Japan) and transiently transfected after 24 h. The transfection mixtures contained 250 ng of GliBS-Luc, 125 ng of PKA-CA, 2.5 ng of pRL SV40 (internal control), and 125 ng of effector construct or empty vector. The cells were harvested 24 h after transfection and assayed for luciferase activity using the PicaGene Dual SeaPansy System (Toyo Ink, Tokyo, Japan) and a luminometer (Lumat LB 9507; EG & G Berthold, Bad Wildbad, Germany). Independent experiments were performed at least three times.

**Negative Regulation of Gli by PKA and 14-3-3ζ**

Gli1 Interacts with 14-3-3ζ through Ser-640—14-3-3ζ is a member of evolutionarily conserved regulatory proteins that can bind to many molecules, including transcription factors (20, 21). Intriguingly 14-3-3ζ is known to contribute to the neu-
ronal development and axial patterning where Hh signaling plays important roles (22–24). 14-3-3 proteins bind to phosphoserine/threonine (pSer/pThr)-containing motifs on most of their ligands through Lys-49, a critical residue in the phospho-substrate-binding pocket (25, 17, 26). To investigate whether the interaction between Gli1 and 14-3-3 proteins is pSer/pThr-dependent, we examined the affinity of Gli1 for a K49E mutant form of 14-3-3, in which Lys-49 was replaced by Glu. We co-expressed Xpress-tagged wt or K49E 14-3-3/H9280 with FLAG-tagged Gli1 in 293T cells and immunoprecipitated Gli1 using anti-FLAG Ab (M2) beads. As shown in Fig. 2A, only the wild-type 14-3-3/H9280, but not the K49E mutant, could be efficiently co-precipitated with Gli1. This result suggests that Gli1 interacts with 14-3-3 proteins in a phosphorylation-dependent manner.

Phospho-dependent interactions between 14-3-3 and their targets are typically mediated through the consensus 14-3-3 binding sequences, RSXpSXR or RX/YFXpSXR, where pS is phosphoserine (or phosphothreonine) (25). To identify the site(s) on Gli1 responsible for binding to 14-3-3, we used Scan-site, a Web-based peptide library-based searching algorithm that identifies sequence motifs likely to bind to specific protein domains (27). With medium stringency parameters, Scan-site identified four putative serine-containing sequences (Ser-545, -640, -659, and -963) as 14-3-3-binding motifs (Fig. 2B). We made Ser → Ala mutations in each of these candidate sequences and performed immunoprecipitation studies using each of the mutant proteins (Gli1 S545A, S640A, S659A, and S963A). The affinity of only Gli1 S640A for 14-3-3 was significantly reduced compared with that of the wt Gli1 (Fig. 2C), suggesting that the phosphorylation of Ser-640 in Gli1 is critical for its interaction with 14-3-3.

14-3-3 Binding Site Is Phosphorylated by PKA in Vitro—The sequence surrounding Ser-640, RRXpS, is identical to the consensus phosphorylation sequence for PKA. This motif is conserved in other Gli proteins, including Ci (Fig. 3A) and has already been reported as a putative site for PKA phosphorylation (6, 9, 28). To confirm whether PKA directly phosphorylates Gli1 Ser-640, in vitro kinase assays were performed. Wild-type and S640A mutant sequences flanking Gli1 Ser-640 were fused to GST and the recombinant fusion proteins examined for incorporation of $^{32}$P from $[^{32}P]$-γATP by the recombinant PKA catalytic subunit. As seen in Fig. 3B, the wt fusion protein was efficiently phosphorylated by PKA whereas S640A mutation abolished this phosphorylation. Taken together, these data suggest that Gli1 associates with 14-3-3 proteins through Ser-640, and that PKA, a negative regulator of Hh signaling, can serve as a responsible kinase for Ser-640.

Gli2 and Gli3 Also Bind to 14-3-3 in a Phosphoserine-dependent Manner—We next examined whether Gli2 and Gli3, the other Hh-regulated transcription factors with unique roles distinct from Gli1 (1), are also phosphorylated by PKA and bind to
14-3-3 proteins. The amino acid sequence surrounding Ser-640 of Gli1 is highly conserved among Gli transcription factors; the corresponding serine residues in mouse Gli2 and human Gli3 are Ser-956 and -1006, respectively (Fig. 3A). We expressed the Gli2 and Gli3 peptides flanking these serine residues as GST fusion proteins and performed in vitro kinase assays with recombinant PKA. Both of the Gli2 and Gli3 fusion proteins were efficiently phosphorylated by PKA, whereas the Gli2 S956A and Gli3 S1006A mutant fusion proteins were not (Fig. 3B).

Next, to examine the interaction of Gli2 and Gli3 with 14-3-3, we expressed FLAG-tagged Gli2 and Gli3 in 293T cells under PKA-activated conditions, either by co-expressing the constitutively active PKA-CA (15) or by the treatment with FSK, a PKA activator. Gli2 and Gli3 were then immunoprecipitated using anti-FLAG Ab beads and blotted for co-associated 14-3-3, as well as the Gli3 double mutant S1006A/S1026A (Fig. 3, C and D), confirming the importance of the Gli1 Ser-640 homologs Ser-956 and Ser-1006 in Gli2 and Gli3, respectively, for conferring phosphorylation-dependent 14-3-3 binding. Finally, we demonstrated that PKA induces the in vitro phosphorylation of the 14-3-3 consensus phospho-binding sequence RXpSXP in Gli2, using the phosphorylation-specific Ab against this motif. In addition, we showed that the interaction of Gli2 and 14-3-3 increased in proportion to the level of Gli2 phosphorylation (Fig. 3E).

Binding of Gli Proteins to 14-3-3 Decreases the Hh Signaling Activity—The effect of 14-3-3 binding on the transcriptional activity of each Gli protein was examined by luciferase assay using a Gli-regulated luciferase reporter under the control of eight tandem copies of the HNF3-β Gli-binding site (8×GBS-luciferase) (13). Gli expression vectors and the 8×GBS luciferase reporter plasmid were co-transfected into 293T cells together with PKA-CA to potentiate the interaction between Gli and 14-3-3. As shown in Fig. 4, A and B, co-transfection of wt Gli1 with 8×GBS-luciferase resulted in a 30-fold increase in reporter activity compared with co-transfection of 8×GBS-luciferase with vector control. The Gli1 S640A mutation up-regulated the reporter activity to the comparable level to that wt Gli1 did. Gli2 also increased reporter activity nearly 3–8-fold, and importantly, the analogous 14-3-3-binding mutant Gli2 did not. In a while, neither wt nor the 14-3-3 binding mutant of Gli3 activated this reporter, consistent with its proposed function as a transcriptional repressor (12). Because deletion of the N-terminal repressor domain from Gli3 has been reported to restore its transcriptional activity (12), we constructed an N-terminal deletion mutant of Gli3 (ΔN-Gli3). We found that ΔN-Gli3 functions as a transcriptional activator like Gli2 (Fig. 4B) and that ΔN-Gli3 defective in 14-3-3 binding demonstrated the higher activity compared with that of ΔN-Gli3 (Fig. 4, A and B). These findings support that the phosphorylated sequence. FLAG-tagged Gli1 was co-expressed with Xpress-tagged wt or K49E 14-3-3 in 293T cells. Cell lysates were subjected to immunoprecipitation using monoclonal anti-FLAG Ab to detect associated 14-3-3 or using anti-FLAG Ab to monitor the efficiency of Gli1 protein expression (middle blot). Lysate samples were also subjected to immunoblot analysis with anti-Xpress Ab (lower blot). B, Scansite prediction of the 14-3-3 binding site on Gli1. Mode1 and mode2 are consensus sequences for the 14-3-3 binding site. The amino acid sequences containing Ser-545, -640, -659, and -963 were predicted to bind to 14-3-3. As shown in Fig. 3, A and B, Scansite prediction of the 14-3-3 binding site on Gli1. Mode1 and mode2 are consensus sequences for the 14-3-3 binding site. The amino acid sequences containing Ser-545, -640, -659, and -963 were predicted to bind to 14-3-3. The 14-3-3 consensus phospho-binding sequence RXpSXP in Gli2, using the phosphorylation-specific Ab against this motif. In addition, we showed that the interaction of Gli2 and 14-3-3 increased in proportion to the level of Gli2 phosphorylation (Fig. 3E).
phosphorylation-dependent binding to 14-3-3 of Gli2 or Gli3 affects their transcriptional activity.

To confirm that the observed transcriptional repression requires the binding to 14-3-3 as well as the phosphorylation of Gli proteins, we constructed a new Gli2 mutant in which Pro-958 was mutated to Ala (P958A). Pro-958, which is at the position relative to the pSer-956, is an important residue for 14-3-3 binding (25) but is not involved in PKA phosphorylation. As seen in Fig. 4C, the P958A mutation abolished 14-3-3 binding to Gli2. In luciferase assays performed under PKA-CA expression, the transcriptional activities of P958A and S956A mutants of Gli2 were comparable to each other, but 2-fold higher compared with those of wt or S975A Gli2 (Fig. 4D). These results suggest that 14-3-3 binding is essential for the phosphorylation-dependent repression of Gli transcriptional activities.

A recent report suggested that the phosphorylation of Gli2 by PKA, CKI, or GSK-3β, influences the transcriptional activity through its ubiquitination and degradation (28). In addition, phosphorylation-dependent 14-3-3 binding of MDMX has been reported to influence its ubiquitination and degradation (29). To address whether the proteasome-dependent degradation affects the 14-3-3-dependent repressive effect, we generated the Gli2 mutant lacking the regions essential for its degradation by the E3 ubiquitin ligase (PKA). The deleted region containing the four responsible PKA phosphorylation sites, Ser-789, -805, -817, and -848 for degradation does not include Ser-956. As shown in Fig. 4E, the additional mutation in
Ser-956 clearly enhanced the transcriptional activity of ΔPKA Gli2, similar to the case of wt Gli2. These findings indicate that the 14-3-3-dependent repressive mechanism against Gli2 activity is independent on the degradation by the ubiquitin-proteasome system.

**14-3-3 Interaction Does Not Affect the Intracellular Localization and the Relationship with SUFU of Gli2**—14-3-3 binding affects the function of various proteins by sequestering from nucleus to cytoplasm (17, 20, 21). The change in the subcellular localization of Gli transcription factors, principally Ci, by their processing following PKA phosphorylation has also been investigated (7). To investigate whether 14-3-3 binding influenced the subcellular localization of Gli2, Myc-tagged wt and S956A Gli2 were expressed in HeLa cells in the presence of 40 μM FSK. Immunocytochemistry using anti-Myc antibody revealed that both wt and S956A Gli2 localized in the nucleus (Fig. 5A). In addition, PKA-CA and GFP-tagged Gli2 were co-expressed in 293T cells showed similar results (data not shown). Because it is reported that Gli proteins are sequestered to the cytoplasm by SUFU (14), we also examined whether 14-3-3 binding affects the interaction with SUFU. SUFU repressed the transcriptional activity of Gli2 independently of its interaction with 14-3-3 (Fig. 5B). The co-expression of Myc-tagged SUFU, Gli2, and PKA-CA showed that the interaction between Gli2 and 14-3-3 had no effect on the affinity of Gli2 for SUFU (Fig. 5C). Thus, 14-3-3 binding to Gli2 does neither affect cellular localization nor interaction with SUFU.

S956A Gli2 Protein Maintains the Affinity for Target Oligonucleotide in Vitro—To assess the direct effect of 14-3-3 on DNA binding by Gli2, we performed EMSA using immunopurified FLAG-tagged wt or S956A Gli2 from cell lysates co-expressing PKA-CA. Both wt and S956A Gli2 specifically decreased the electrophoretic mobility of the Gli-RE oligonucleotide (Fig. 5D). Addition of excess unlabeled Gli-RE dramatically reduced the intensity of retarded oligonucleotide bands in both the wt and S956A samples. These findings suggest that, at least in an in vitro condition, 14-3-3 does not affect the binding capacity of Gli2 to the synthetic naked oligonucleotides.
PKA Stimulation Down-regulates the Expression of Gli1, Which Is a Representative Target of Hh Signaling in a 14-3-3-dependent Manner—PKA is reported as a suppressor of Hh signaling by promoting degradation of Gli proteins. Given that PKA phosphorylates Gli proteins in the sites significant for 14-3-3 binding, PKA should be one of the putative kinases responsible for the 14-3-3 dependent regulation of the transcriptional activities of Gli proteins. Then we addressed whether PKA affects the transcriptional activities of Hh signaling in a 14-3-3-dependent manner. As Gli1 is a representative target protein of Hh signaling (30), the Gli1 protein level was compared between FSK-treated PLC/PRF/5 cells and DMSO-treated cells. Overnight treatment of 40 μM FSK reduced the Gli1 expression, which was consistent with the notion that PKA is a negative regulator of this signaling (6). However, in 14-3-3α knockdown PLC/PRF/5 cells, FSK treatment reduced Gli1 protein level to a lesser extent, which suggested that 14-3-3α played a role in PKA-dependent repression of Hh signaling (Fig. 6A).

To certify the effect of PKA-14-3-3 axis on Hh signaling, we also used mouse fibroblast NIH3T3, where Hh signaling is inactive. To activate Hh signaling in the cells, they were treated with SAG, which is a potent activator of Hh signaling (31). Quantitative real-time PCR showed that incubation with 100 nM SAG for 30 h induced NIH3T3 cells to express Gli1 and Ptch1 in mRNA level, and the addition of 1 μM FSK suppressed it (Fig. 6B). Given that Gli1 and Ptch1 are representative targets of Hh signaling, it means that SAG is able to activate Gli transcriptional activities in NIH3T3 cells. We also found that Gli1 protein as well as its mRNA was induced by the treatment of SAG (Fig. 6C). To elucidate the role of 14-3-3α in this assay condition, we prepared an expression vector for the fusion protein EYFP-difopein, a specific peptide that can compete away 14-3-3α.
PKA-dependent Gli binding to 14-3-3 is not involved in its ubiquitination and proteolysis. Thus, our results suggest the distinct PKA-dependent mechanisms involved in the repression of Hh signaling, through 14-3-3 binding or degradation, respectively.

Previously, 14-3-3 binding has been shown to directly affect the DNA binding activity of several transcription factors including Miz1, DAF16, and FKHRL1. Miz1 binds to 14-3-3 following AKT phosphorylation. The phosphorylation/14-3-3 binding site on Miz1 is located directly in the DNA binding zinc finger region, explaining how 14-3-3 binding might reduce its affinity for DNA (33). In contrast, the DNA binding Forkhead domain in DAF-16 and FKHRL1 is distinct from the AKT phosphorylation/14-3-3 binding sites flanking both its N- and C-terminal ends (34). Similar to DAF-16 and FKHRL1, the DNA-binding regions (zinc fingers) of Gli transcription factors, are distinct from its target proteins (16). The plasmid expressing a mutated version of difopein (R18Lys) that does not inhibit 14-3-3 was used as a negative control. Interestingly, the suppressive effects of FSK treatment for Gli transcriptional activities were recognized under the co-transfection of the EYFP-difopein plasmids as well as that of EYFP-R18Lys (Fig. 6E). These findings indicate that the molecular systems for PKA-induced suppression of Gli transcriptional activities include both 14-3-3-dependent and -independent mechanisms.

**DISCUSSION**

We used a proteomic approach to identify 14-3-3ε as a novel Gli-interacting protein and found that this interaction negatively regulates Hh signaling through Gli2, and an N-terminally truncated form of Gli3. This interaction depends on Gli phosphorylation, and PKA, the negative regulator of Hh signaling (32) can serve as a responsible kinase. One reported mechanism by which PKA influences Gli-dependent transcription involves the ubiquitin-dependent proteolytic processing of Gli into its repressor form (6, 9, 28); however, the PKA phosphorylated residues responsible for this processing event are different from the one for 14-3-3 binding. In addition, loss of binding to 14-3-3ε in undegraded Gli2 (APKα) enhanced its transcriptional activity (Fig. 4E), indicating that
demonstrated; however the overexpression of the binding mutant Gli1 did not increase the transcriptional activity (Fig. 4A). The binding of Gli1 to 14-3-3 might have an alternative effect for Hh signaling, different from those of Gli2 or Gli3.

The 14-3-3e isoform, which we have identified here as a Gli-interacting partner, appears to play a particularly important role in neuronal development and tumorigenesis. The 14-3-3e gene is located on chromosome 17p13, and heterozygous deletions in this chromosome result in human neuronal disorder, Miller-Dieker syndrome (MDS). Although mutations in the LIS1 gene PAFAH1B1, which is located in 17p13, contributes to MDS, the severity of MDS is suggested to be caused by the co-deletion of 14-3-3e in addition to LIS1 (22). Toyo-oka et al. (23) showed that mice deficient in 14-3-3e had defects in brain development and neuronal migration, whereas Muslin and co-workers (24) found that Xenopus embryos lacking 14-3-3e had prominent axial patterning defects.

Inappropriate activation of Hh signaling occurs in ~60% of medulloblastomas and the most common medulloblastoma-associated genetic lesion is loss of the chromosome 17p13 region (35). This evidence supports the importance of chromosome 17p13 in neuronal development and tumorigenesis. Determining whether the novel interaction between Gli and 14-3-3, especially the 14-3-3e isoform, is involved in these human neurological diseases will require further investigation.

Acknowledgments—We thank K. W. Kinzler and B. Vogelstein for human Gli1 and Gli3 cDNA; H. Sasaki for mouse Gli2 cDNA and GliBS-Luc; R. Toftgard for Myc-tagged SUFU plasmid; G. S. McKnight for the constitutively active PKA catalytic subunit expression vector; and H. Fu for EF1α-diopein and R18K expression vector. We also thank Mitsuko Tsubouchi for technical assistance.

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