Characterization of the Physical Interaction of Gli Proteins with SUFU Proteins*

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The Hedgehog signaling pathway is involved in both development and cancer induction in a wide range of organisms. The end point of the Hedgehog signal-transduction cascade is the Gli/Ci family of proteins, which is required for the interaction with SUFU. Mutational studies revealed that Gly122 and His123 are crucial for binding to SUFU, suggesting the importance of hydrophobicity for the correct binding conformation. Functional analysis revealed that the activity of GLI transcription factors with mutations in this motif is no longer suppressed by co-expression of SUFU. Moreover, we have found that a C-terminal 19-amino acid deletion in SUFU (Δ465) is sufficient to abrogate interaction with GLI1. Interestingly, this SUFU mutant localizes in the nucleus, most probably because it is not efficiently sequestered in the cytoplasm. Taken together, we identified a novel motif in the Gli/Ci family of proteins that is essential both for protein-protein interaction with SUFU and for functional repression of GLI1 by SUFU.

GLI1 was originally isolated as a highly amplified gene in a malignant glioma and subsequently implicated in the development of other tumor types, including liposarcoma, rhabdomyosarcoma, osteosarcoma, and astrocytoma (1–3). Later it was shown that GLI1 encodes a transcription factor that is a downstream nuclear component of the Sonic hedgehog-patched signaling pathway. This pathway is evolutionary conserved and plays important roles in tissue patterning during early embryogenesis in vertebrates and invertebrates (4, 5). In addition to GLI1, two other isoforms have been identified in vertebrates, Gli2 and Gli3, each encoded by separate genes (6). All Gli proteins bind to DNA through five Zn-finger domains that recognize the consensus sequence 5'-TGGGTGTC-3' (7). The role of each Gli isoform in mediating the hedgehog signal is not clear yet but recent studies have shown overlapping roles and indicated some functional redundancy (8, 9). There are indications that Gli1 is mainly a transcriptional activator, whereas Gli2 and Gli3 can act both as activators and repressors depending on specific post-translational modifications (10). Gli proteins include both a nuclear export signal and a nuclear localization signal, suggesting that they shuttle between the nucleus and the cytoplasm.

Gli and Ci (Drosophila homolog of Gli proteins) are known to interact with a number of proteins such as the Ser/Thr kinase Fused (11), Suppressor of Fused (SUFU)1 (12, 13), and a kinase-like protein Costal-2 in Drosophila (14). This complex is assumed to receive and transmit a signal induced by hedgehog ligands to activate or repress certain genes. Moreover, Gli2 and Gli3 have potential binding sites for interaction with cAMP-response element-binding protein/p300 (a coactivator-acetyltransferase), whereas Gli1 lacks such a site (15).

SUFU encodes a 484-amino acid cytoplasmic protein. Genetic screens in Drosophila first identified Su(fu) as a suppressor of mutants in the Fused serine/threonine kinase Fused (11), Suppressor of Fused (SUFU)1 (12, 13), and a kinase-like protein Costal-2 in Drosophila (14). This complex is assumed to receive and transmit a signal induced by hedgehog ligands to activate or repress certain genes. Moreover, Gli2 and Gli3 have potential binding sites for interaction with cAMP-response element-binding protein/p300 (a coactivator-acetyltransferase), whereas Gli1 lacks such a site (15).

Three alternatively spliced transcripts of SUFU with tissue specific expression have been identified. One SUFU variant has a trinucleotide insertion resulting in an extra glutamine and a protein of 485 amino acids. A second variant, SUFU-Lk, lacks exon 10 encoding a protein of 388 amino acids. The third variant, SUFU-Tt, has an extra exon after exon 8 (exon 8a) resulting in a protein containing 359 amino acids. The latter two of these variants have lost the ability to interact with GLI1 (21). In addition, an alternative splice variant of 433 amino acids lacking the C terminus was identified and reported to retain ability to suppress Gli-mediated activation of transcription (16). These observations underscore the importance of the C-terminal domain of SUFU for biological function.

Interaction domains for each individual protein involved in the intracellular signaling complex have not yet been precisely mapped, and no known protein-protein interaction motifs have been found by sequence analysis. Therefore, we decided to

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‡ The abbreviations used are: SUFU, Suppressor of Fused; Hh, hedgehog; HA, hemagglutinin; PBS, phosphate-buffered saline; LMB, leptomycin B.
pinpoint domains of Gli proteins and SUFU, which specifically contribute to their interaction. We have used a combination of immunoprecipitation in vivo and in vitro and Far Western blot analysis to define the required fragment and specific amino acids of GLI1 that are essential for full SUFU-binding activity.

We show that this fragment comprises residues 116–125, which are necessary for SUFU association. Designed mutant proteins corresponding to alterations in the SYG8 amino acid motif within the interaction region selectively disrupted this protein-protein interaction. Functional analysis revealed that the activity of GLI1 transcription factors with mutations in this motif is no longer suppressed by co-expression of SUFU. We have also demonstrated the requirement of the C-terminal domain (19 amino acids) of SUFU for the interaction with GLI1. Deletion of these amino acids led to relocation of SUFU from the cytoplasm to the nucleus. We assume that this effect can be explained by loss of sequestering of SUFU in the cytoplasm.

MATERIALS AND METHODS

Plasmids—To express MYC-tagged and hemagglutinin (HA)-tagged proteins, relevant sequences were amplified by PCR and subcloned into pCMV- and pcDNA3 vectors. Sequences included full-length human GLI1 and SUFU. The deletion series of GLI1 such as GLI1-(55–407), -(78–407), -(111–407), -(125–407), -(424–1111) were also cloned in the same vectors. The following regions GLI1-(55–116), -(55–125), -(111–407), GLI2-(203–300); GLI3-(250–350) were used to generate thioredoxin fusion proteins. The fragments were PCR-amplified using specific primers and cloned into the pBAD/TOPO vector (Invitrogen). All constructs were sequence verified.

Immuno precipitation Studies and Western Blot Analysis—Human 293T cells (embryonic kidney epithelial cell line) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were transiently transfected with expression constructs encoding HA-tagged GLI1 or Myc-tagged SUFU using the Superfect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Before harvesting, cells were washed twice with 1× PBS and lysed in 1 ml of immunoprecipitation buffer (1× PBS, 1% Nonidet P-40, complete protease inhibitor mixture (Roche)). Cell suspensions were centrifuged at 14,000 rpm for 20 min to remove nuclei and unbroken cells. Immunoprecipitations were performed using antibodies to the HA- or c-Myc epitopes conjugated with agarose. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with antibodies to the HA or Myc epitopes, using the chemiluminescence detection system (PerkinElmer Life Sciences).

Immunoprecipitation in vitro was performed as follows; GLI1 fragments and SUFU were synthesized by in vitro translation in the rabbit reticulocyte lysate system (TNT, Promega). The in vitro translation mixture was diluted with 1× PBS buffer and immunoprecipitated with anti-Myc antibodies. Pellets were washed four times with 1× PBS or other washing conditions as indicated, resuspended in sample buffer, and resolved by SDS-PAGE.

Expression of Thioredoxin Fusion Proteins—The Escherichia coli Top10 strain was transformed with different deletion constructs of GLI1. Fusion proteins were generated with the N-terminal-attached thioredoxin according to the manufacturer's protocol (pBAD/Topo™ ThioFusion™ Expression System, Invitrogen). After induction with different concentrations of arabinose for 4 h, the cells were harvested by centrifugation and resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 1 mM imidazole). The cells were disrupted by sonication, and the lysate was subjected to centrifugation for 30 min at 14,000 rpm.

Far Western Blot—E. coli cells expressing GLI1 fragments were collected by centrifugation and lysed by boiling in SDS sample buffer. Lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). Proteins were visualized by staining with Ponceau-S (0.5% in 1% acetic acid). Membranes were blocked in buffer A containing 10% glycerol, 100 mM NaCl, 20 mM Tris-HCl at pH 7.5, 0.5 mM EDTA, 0.1% Tween 20, 5% skim milk powder for 1 h at room temperature.

SUFU proteins were synthesized by coupled in vitro translation/translation (Promega TNT with T7 RNA polymerase) in the presence of [35S]methionine. Reactions were diluted with buffer and passed over Millipore spin columns to remove unincorporated methionine. Subsequently, the blocked membranes were incubated for 1 h in the same buffer containing [35S]-labeled SUFU. Filters were washed three times with buffer A, allowed to dry and exposed to x-ray film (Eastman Kodak).

Site-directed Mutagenesis—Point mutations were introduced by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. A fragment of GLI1 (55–407) in pBAD or full-length GLI1 in pEFGP-C2 (Clontech) were used as the template for mutagenesis. For all constructs, mutagenesis was confirmed by sequence analysis.

Luciferase Assay—The expression vectors for GLI1 and SUFU used in this study have been described previously (13). All constructs were verified by DNA sequencing. The NIH-3T3 cell clone Shh-LIGHT having a stably incorporated GLI-luc reporter (22) was used for the luciferase reporter assay. One day before transfection the cells were plated onto 24-well plates so that on the next day the cell density was ~80%. Cells were transfected for 3 h using 0.4 μg of GLI1 wild-type or mutant DNA and 0.2 μg of SUFU DNA or empty vector using Biofect (InBio) according to the manufacturer's instructions at a ratio of DNA to Biofect of 1:3 (w/w). After 24 h the medium was replaced with low serum media and cells were incubated for an additional 24 h. Subsequently cells were lysed and luciferase activity was measured with a luciferase kit from Tropix (Bedford) according to the manufacturer's instructions using an Ascent Fluoroscan combined fluorim- and luminometer (Thermo Lab Systems). All experiments were repeated three times and normalized for alkaline phosphatase activity.

Cell Fractionation Experiments—293T cells were transfected with full-length SUFU or SUFU-(445) deletion constructs. To isolate nuclear and cytoplasmic fractions, cells were washed twice with cold PBS and lysed with hypotonic buffer (HEPES pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA). After incubation for 10 min at 4 °C, cells were treated with 0.5% Nonidet P-40 and centrifuged at 1,000 rpm for 10 min. After recovery of supernatants representing cytoplasmic components, the pellets were washed twice with hypotonic buffer, centrifuged at 1,500 rpm for 5 min, and lysed with 2× Laemmli sample buffer. Samples were analyzed by Western blot analysis using an anti-SUFU polyclonal antibody (Santa Cruz).

RESULTS

Mapping the GLI1 Domain That Is Responsible for SUFU Interaction in Vivo—As a first step we determined the affinity of the GLI1-SUFU interaction by examination of its sensitivity to elevated salt concentration. An N-terminal deletion construct (1–407) of GLI1 and Myc-tagged SUFU were produced by in vitro translation in the presence of [35S]methionine, and the translation mixture was subjected to immunoprecipitation using an anti-Myc antibody. GLI1 and SUFU associate with high affinity, as these associations occur in a 600 mM NaCl buffer (Fig. 1A).

It was previously shown that the GLI1 (1–407) amino acid region is capable of binding SUFU (13). To further analyze this interaction, we mapped the region of GLI1 required for interaction with SUFU. Expression constructs encoding different lengths of HA-tagged GLI1 were cotransfected into 293T cells with Myc-tagged SUFU. Fig. 1 shows a schematic diagram of the amino acid regions of GLI1 used for the experiments. GLI1 fragments and SUFU were coimmunoprecipitated from lysates of transfected cells using monoclonal antibodies specific for the Myc and HA epitope tags. The presence of the proteins in the immunoprecipitates was monitored by Western blot analysis (Fig. 1, A and C). Proteins containing GLI1 fragments 111–407 and 78–407 interacted with SUFU, whereas GLI1 fragments containing amino acids 125–407 or 143–407 failed to maintain binding to SUFU. Thus, our results indicate that the N-terminal region of GLI1 including residues 111–125 defines a SUFU-binding domain.

Far Western Binding Assay with N-terminal Fragments of GLI1—To confirm the importance of the 111–125 amino acid domain of GLI1 for the binding of SUFU, we constructed and expressed in E. coli a series of deletion derivatives of GLI1 fused with thioredoxin. Cleared bacterial lysates of each fusion protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. The interaction of GLI1 fragments...
with SUFU was determined by Far-Western blot analysis using in vitro translated 35S-labeled SUFU as a probe (Fig. 2). The GLI1 fragment including amino acids 55–407 was used as a positive control. Thioredoxin itself was used as a negative control. The GLI1 fragment including amino acids 55–116 failed to interact with SUFU. Inclusion of the 116–125 amino acid domain recovered SUFU binding. Thus, the results show that the minimal required region for GLI1-SUFU binding is amino acid residues 116–125 of GLI1.

**Interaction of SUFU with Other Members of the Gli Family**—We aligned the pinpointed region to reveal the homology with other Gli/Ci proteins, as shown in Fig. 3A. In addition, we used the PSI-BLAST program to detect weak but biologically relevant sequence similarities (23). All identified proteins belong to the Gli/Ci transcription factor family. Four amino acids, SYGH, were highly conserved in all analyzed proteins including Ci. To confirm that similar regions of Gli2/Gli3 would be responsible for SUFU interaction, we designed deletion constructs of Gli2 and GLI3. The derivatives including amino acids 205–300 of Gli2 and amino acids 250–350 of GLI3, respectively, were positive in the Far Western assay and bound SUFU with similar efficiency (Fig. 3B). Both fragments encompass the SYGH motif. Thus, SYGH is implicated as a required motif in all members of the Gli/Ci family to allow SUFU binding.

**Mutation Analysis of the SYGH Motif in GLI1**—To determine which amino acids in the SYGH motif of GLI1 are essential for the interaction with SUFU, we used site-directed mutagenesis.
Characterization of Gli-SUFU Interaction

...to change conserved amino acids to alanine. Mutants were expressed in E. coli and tested for interaction by the Far Western assay. The results of the mutagenesis study are summarized in Fig. 4A. Substitution of Ser\(^{120}\) to alanine resulted in SUFU binding with higher affinity. Mutation of the tyrosine at position 121 had no effect on the interaction. Mutations of the glycine residue at position 122 and the histidine residue at position 123 to alanine almost eliminated GLI1-SUFU interaction. In addition, we introduced double alanine substitutions of Ser120 and Y121, which also led to reduced binding. These results indicate that amino acids Gly\(^{122}\) and His\(^{123}\) are critical for GLI1-SUFU interaction.

The NetPhos 2.0 prediction program showed that the serine at position 120 is a good candidate for phosphorylation by serine kinases (score 0.814). Therefore, we assumed that phosphorylation of GLI1 might be involved in regulation of its regulation and localization. To test this hypothesis, we substituted Ser\(^{120}\) to glutamic acid, which would mimic a phosphorylated state of GLI1. Surprisingly, this replacement led to an increased affinity in comparison to wild-type, as did an alanine substitution (Fig. 4B). Therefore, we conclude that most likely this substitution led to the alteration of protein folding.

To further strengthen the functional relevance of the GLI1 mutations crucial for SUFU interaction, we used a reporter gene assay. SUFU, mutated GI22A, and H123A GLI1 full-length proteins were co-expressed in NIH3T3 cells, and the effect of these mutations on GLI1-activated transcription was examined. This reporter was activated by wild-type GLI1, whereas SUFU itself had no effect (Fig. 4C). Co-transfection of SUFU led to suppression of the reporter gene activation by GLI1. However, SUFU had no effect on activation of reporter gene activity by mutated GI22A or H123A GLI1. Thus, these results confirm the importance of these amino acid residues of GLI1 for SUFU interaction. In addition, we observed that the activity of mutated forms of GLI1 was higher in comparison with wild-type. We assume that wild-type GLI1 translocated into NIH3T3 cells can form a complex with endogenous Sufu and can be sequestered in the cytoplasm, because NIH3T3 cells do express Sufu. Mutated GLI1 does not bind Sufu and may escape cytoplasmic retention.

Mapping the SUFU Domain That Is Responsible for Gli Interaction—To map the region of SUFU required for interaction with GLI1, SUFU deletion constructs were tested using immunoprecipitation (Fig. 5, A and B). Myc-tagged SUFU or its deletion constructs were cotransfected into 293T cells with HA-tagged GLI1 and immunoprecipitated with HA or c-Myc antibodies. The immunoprecipitates were separated by SDS-PAGE, blotted, and detected with c-Myc or HA antibodies. The expression of all proteins was confirmed by Western blotting of whole-cell extracts (data not shown). C-terminal deletion constructs of SUFU (Δ465) and (Δ425) failed to interact with GLI1. These results indicate that the C terminus of SUFU is absolutely required for the formation of a complex with GLI1.

To confirm the importance of the C-terminal domain of SUFU for the binding of GLI proteins, we constructed two N-terminal deletion derivatives of SUFU referred to as 100–484 and 200–484, respectively. Their interaction with GLI proteins was determined by Far Western blot analysis using in vitro translation for labeling (Fig. 5B). We tested the following constructs of GLI proteins, GLI1 55–125, GLI2 205–300, and GLI3 250–350, expressed in E. coli. We detected an interaction of both SUFU-deletion derivatives with all GLI proteins. However, deletion of the SUFU N terminus led to reduced binding compared with wild-type. A possible explanation for this result could be that any truncations of SUFU affect the overall conformation of the protein and that tertiary structure is critical for interaction with GLI proteins.

Subcellular Distribution of SUFU and LMB Treatment—SUFU is assumed to be a cytoplasmic-nuclear shuttling protein, because it is detected in both cytoplasm and nucleus (13, 16). Its localization might be an important factor in regulation of the Hh signaling pathway. To examine subcellular distribution of SUFU, we transfected 293T cells with either wild-type or C-terminally truncated SUFU (Δ465), and protein levels in cytosolic and nuclear fractions were detected by Western blot analysis (Fig. 6A). The purity of the fractions was determined with lamin B antibodies as a marker for nuclear fractions (Fig. 6A, lower panel). Whereas wild-type SUFU showed a predominant cytosolic localization, the C-terminally truncated form was mainly observed in the nuclear fraction. We also analyzed NIH3T3 cells, which express endogenous Sufu. The distribution of endogenous Sufu in NIH3T3 cells was similar to that observed in 293T cells transfected with wild-type SUFU (Fig. 6B).
The surprising pattern of nuclear localization of the SUFU (H9004465) mutant can be explained either by disruption of one or more SUFU interactions with other proteins, which might sequester SUFU in the cytoplasm, or a loss of ability to be exported from the nucleus. To determine whether the C-terminal deletion interferes with export of SUFU from the nucleus, we treated NIH3T3 cells with leptomycin B (LMB), a fungal metabolite that inhibits nuclear protein export by binding to the CRM1 receptor for nuclear export signals (24). LMB treatment for either 2 or 24 h had no effect on the subcellular distribution of endogenous Sufu. Most of the Sufu protein was present in the cytosolic fraction (Fig. 6C). These results suggest that Sufu is not exported from the nucleus through the CRM1/exportin pathway in NIH3T3 cells. Thus, it is likely that a motif within the twenty C-terminal amino acids has a role in mediating subcellular localization.

DISCUSSION
In this study, using deletion analysis coupled with in vivo coexpression and Far Western assays, we have identified the required region, the 116–125 amino acid residues, within the GLI1 N-terminal region responsible for interaction with SUFU. Deletion of this domain resulted in a complete loss of its association with SUFU. Although N-terminal residue conservation in the Gli/Ci protein family is quite modest, a motif containing the SYGH amino acids is specifically conserved from fly to man (25; Fig. 3). Analysis of alanine-substitution mutants of the GLI1 SYGH motif strengthened its important role for SUFU interaction. We demonstrated that substitutions of the glycine residue at position 122 and the histidine residue at position 123 as well as the double mutant of Ser120 and Tyr121 led to a dramatic reduction in SUFU binding, indicating that they are essential for the GLI1-SUFU interaction. Thus, we have identified a novel motif, which is involved in protein-protein interactions within the biologically very important Hh signaling pathway.

Next, our results demonstrated that substitution of Ser120 to alanine enhanced binding of GLI1 to SUFU in comparison with wild-type GLI1. Because phosphorylation is a common mechanism for regulation of protein function, we assumed that phosphorylation of Ser120 would be functionally important for SUFU interaction. It has been demonstrated in Drosophila that Hh regulates the phosphorylation of downstream signal-
Characterization of Gli-SUFU Interaction

1 SUFU 484

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(13 16)
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FIG. 7. A summary of the results reported in the literature from analysis of Sufu domains involved in the interaction with Gli proteins.

1) Using the yeast two-hybrid system, the central domain of mouse Sufu between residues 109–325 was shown to be required for the interaction. The results obtained with glutathione S-transferase pull-down assays showed that the N-terminal domain of Sufu (amino acids 13–173) could interact only with Gli3, whereas the C-terminal region (amino acids 174–484) could bind Gli1 and Gli3, but not Gli2 (35). It has been speculated that the interaction domains of the Sufu protein with three Gli proteins are different.

2) An alternatively spliced isoform of Sufu, lacking 52 amino acids at the C terminus (referred to as Sufu-433), coimmunoprecipitated with Gli1 in transiently transfected 293 cells (16). Hence, it was concluded that the C terminus of Sufu (amino acids 433–484) is not involved in its interaction with Gli proteins.

3) Deletion of the C-terminal domain (amino acids 386–484) led to disruption of Gli1-Sufu interaction in the same cell type (13). Similar results were found for two additional Sufu natural isoforms referred to as Sufu-Lk (amino acids 1–388) and Sufu-Tt (amino acids 1–359) lacking 96 and 125 amino acids at the C termini (21). These data suggest that the C-terminal domain (amino acids 386–484) is required for Sufu-Gli interaction.

4) Sufu lacking the C-terminal half (designated Sufu-Δex8) was not able to bind Gli proteins. In addition, the Sufu (212–484) mutant also lost this ability (20).

A summary of these results is presented in Fig. 7. Based on available data, it is difficult to draw any firm conclusion about which domains of Sufu is crucial for the interaction with Gli proteins. The best way to resolve the discrepancies would be high resolution crystallographic studies of Sufu and the Sufu-Gli protein complex.

As we show here, deletion of 19 amino acids at the C terminus of Sufu caused its relocation from the cytoplasm to the nucleus. Similar results were found in SW-480 cells, where Sufu mutants with C-terminal deletions showed significant nuclear accumulation (36). Moreover, Sufu-Δex8 was localized in the nucleus as well (20). The Sufu C-terminal domain shares no apparent sequence homology to any other proteins. Altered subcellular localization could be explained either by disruption of one or several interactions with other proteins, which sequester Sufu in the cytoplasm, or by a loss of ability to be exported from the nucleus. Meng et al. (36) have presented data suggesting that Sufu is actively exported from the nucleus, because it could be readily detected in the nuclear compartment of LMB-treated SW-480 cells. In contrast, no substantial nuclear accumulation of Myc-Sufu could be observed in untreated or LMB-treated wing discs in the Drosophila (17). Moreover, Sufu inhibited nuclear accumulation of Gli1 in the presence of LMB (13). Our results have further demonstrated that LMB treatment of NIH3T3 cells did not alter subcellular localization of endogenous Sufu. In addition, no concensus leucine-rich nuclear export signal is present in Sufu. Taken together, these data are most consistent with a model in which Sufu Δ485 relocates to the nucleus because it cannot be efficiently sequestered in the cytoplasm. However, additional work is necessary to identify the mechanism and protein factors involved in Sufu subcellular distribution.

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