Identification of Glis1, a Novel Gli-related, Krüppel-like Zinc Finger Protein Containing Transactivation and Repressor Functions

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Yong-Sik Kim, Mark Lewandoski, Alan O. Perantoni, Shogo Kurebayashi, Gen Nakanishi, and Anton M. Jetten

From the Cell Biology Section, Division of Intramural Research, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the §Laboratory of Cancer and Developmental Biology and $Laboratory of Comparative Carcinogenesis, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702

In this study, we describe the identification and characterization of a novel Krüppel-like protein named Glis1. The Glis1 gene encodes a 84.3-kDa proline-rich protein. Its five tandem zinc finger motifs exhibit highest homology with those of members of the Gli and Zic subfamilies of Krüppel-like proteins. Glis1 was mapped to mouse chromosome 4C6. Northern blot analysis showed that expression of the 3.3-kb Glis1 mRNA is most abundant in placenta and adult kidney and expressed at lower levels in testis. Whole mount in situ hybridization on mouse embryos demonstrated that Glis1 is expressed in a temporal and spatial manner during development; expression was most prominent in several defined structures of mesodermal lineage, including craniofacial regions, branchial arches, somites, somites, vissel, and hair follicles, limb buds, and myotomes. Confocal microscopic analysis showed that Glis1 is localized to the nucleus. The zinc finger region plays an important role in the nuclear localization of Glis1. Electrophoretic mobility shift assays demonstrated that Glis1 is able to bind oligonucleotides containing the Gli-binding site consensus sequence GACCACCCAC. Although monohybrid analysis showed that in several cell types Glis1 was unable to induce transcription of a reporter, deletion mutant analysis revealed the presence of a strong activation function at the carboxyl terminus of Glis1. The activation through this activation function was totally suppressed by a repressor domain at its amino terminus. Constitutively active Ca++-dependent calmodulin kinase IV enhanced Glis1-mediated transcriptional activation about 4-fold and may be mediated by phosphorylation/activation of a co-activator. Our results suggest that Glis1 may play a critical role in the control of gene expression during specific stages of embryonic development.

Krüppel-like zinc finger proteins, named after the Drosophila segmentation gene Krüppel (1, 2) form one of the largest families of transcription factors. Typically, these proteins contain two or more Cys2-His2-type zinc fingers that are separated by the conserved consensus sequence, (T/S)\(^2\)GEKP/(Y/F)\(^2\). Krüppel-like zinc finger proteins can be divided into several subfamilies based on the number of zinc finger motifs, sequence homology between the zinc-fingers, and the presence of specific repressor and activation domains (3–6).

Gli and Zic form two closely related subfamilies of Krüppel-like zinc finger proteins that contain five Cys\(^2\)-His\(^2\)-type zinc finger motifs (7–11). Gli and Zic proteins can function as repressors and activators of transcription (12–14). They are closely related to the Drosophila proteins Cubitus interruptus and odd-paired, respectively (9, 15, 16), while odd-paired-like is the Zic homologue in Xenopus (17). Cubitus interruptus plays an important role in wing development, whereas odd-paired regulates segmentation and mid-gut development. Cubitus interruptus and Gli proteins function as downstream regulators of transcription in the Sonic hedgehog-Patched-Smoothened signal transduction pathway in Drosophila and vertebrates, respectively (8, 18–21). In addition, growing evidence supports a role for Wnt and bone morphogenic proteins upstream as well as downstream of Gli and Zic proteins (22–24). Gli and Zic proteins are essential for normal embryonic development (13, 25–31). Gli2 and Gli3 are required for skeletal development (12) and organogenesis of various tissues, including lung, trachea, and esophagus (25, 31), whereas Zic proteins play important roles in the development of the central nervous system and limb buds (7, 29, 32, 33). Gli proteins have been implicated in a number of human diseases. For example, Gli1 is amplified in human glioblastomas and rhabdomyosarcomas (34, 35) and both Gli1 and Gli2 are overexpressed in basal cell carcinomas of the skin (36, 37), whereas Gli3 has been implicated in Greig cephalopolysyndactyly and Pallister-Hall syndromes (11, 38, 39).

In this study, we describe the cloning of a cDNA encoding a novel member of the Krüppel-like zinc finger family not previously described. We named this protein Gli-similar 1 (Glis1)\(^1\) based on its relationship to Gli proteins. Glis1 contains five tandem zinc finger motifs that show high homology with those of Gli and Zic proteins. The zinc finger region of Glis1 exhibits the highest identity (77%) with the Drosophila Gli-like protein Lame duck (Lmd, also named gleeful or glf) (40, 41). In adult mouse tissues, Glis1 mRNA is particularly abundant in placenta, kidney, and testis. Analysis of its expression during embryonic development demonstrated that Glis1 is expressed in both a temporal and spatial manner in the frontal nasal region, branchial arches, somites, vissel and hair follicles,
Identification of Glis1

and limb buds. Its nuclear localization and binding to oligonucleotides containing the Gli-binding site suggested that Glis1 might modulate gene transcription. This conclusion was supported by studies examining the transcriptional activity of Glis1 by monohybrid and deletion analysis. This analysis revealed that Glis1 contains both transactivation and repressor domains indicating that it may function as an activator and repressor of transcription. These observations suggest that Glis1 functions as a transcription factor that regulates specific stages of embryonic development.

**Experimental Procedures**

**Cell Culture**—Green monkey kidney fibroblast CV-1 and COS-7, Chinese hamster ovary, and human kidney 293 cells were obtained from ATCC. Human kidney epithelial PK-1 cells were obtained from Dr. Bonventre (Harvard Medical School). Cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum except for Chinese hamster ovary, which was cultured in Ham’s F-12.

**Yeast Two-hybrid Analysis**—Yeast two-hybrid analysis was performed using the ligand-binding domain of the nuclear orphan receptor RORγ (42) as bait and a PACT mouse lymphoma MATCHMAKER cDNA library following the manufacturer’s protocol (Clontech). The cDNA fragment encoding the ligand-binding domain of the nuclear receptor RORγ was cloned into pGADT (Clontech). One of the positive cDNA clones contained a 1.4-kb insert encoding the carboxy-terminal region of Glis1.

**cDNA Library Screening and 5’ SMART-RACE**—To obtain the remaining sequence of mGlis1, a mouse kidney cDNA library made in pDINAS (Invitrogen) was screened by PCR using two Glis1-specific primers. This screening was performed by IncyteGenomics (St. Louis, MO) and yielded one clone containing the full-length coding region of Glis1. To obtain the 5’-untranslated region, 5’ SMART-RACE was performed following the manufacturer’s protocol (Clontech). The obtained cDNA was PCR amplified with the universal primer (5’-CTAAATAGCTACATTAGGCGCAAGCTTGTAACACCGCAGCT) and Glis1-specific primer GSP5p (5’-CGCGTCTTTGACACCATATGCAGTTCGGAG). A second PCR reaction was performed using nested primers, a universal primer (5’-AAAGCATGTTACAAACCGCAGCT) and Glis1-specific primer GSP5’ (5’-GCGCAACCTGAGCGACGGCGGCGCCTGAC). The 5’-end fragments were then subcloned into pGemT vector (Promega) and sequenced.

**DNA Sequencing**—Plasmids were purified using Wizard miniprep or midiprep kits from Promega. Automatic sequencing was carried out using a Dynamic ET Terminator Cycle Sequencing Ready reaction kit (PerkinElmer Life Sciences) and an ABI Prism 377 automatic sequencer. DNA and deduced protein sequences were analyzed by the secedet software sequence analysis package.

**Northern Blot and RT-PCR Analysis**—A multitissue blot containing total RNA (25 μg) from 14 different mouse tissues was purchased from Seegene (Seoul, Korea). The expression of the mGlis1 gene was also examined by RT-PCR using total RNA (1 μg) from different mouse tissues as template and two Glis1-specific primers. The RT-PCR was carried out at the following conditions: 45 min at 54 °C and 2 min at 94 °C (1 cycle), followed by 0.5 min at 94 °C, 1 min at 60 °C, and 2 min at 68 °C (25 cycles). Finally, after a 7-min incubation at 68 °C, samples were analyzed on a TBE gel (0.8%) and transferred to a HyBond-N+ membrane. The blots were hybridized to a 32P-labeled probe for mGlis1. Hybridizations were performed at 68 °C for 1 h, the membranes were then washed twice with 2× SSC, 0.1% SDS at room temperature for 30 min and 0.1% SSC, 0.1% SDS at 55 °C for 30 min. Autoradiography was carried out with Hyperfilm-MP (Amersham Biosciences) at −70 °C.

**Fluorescence in Situ Hybridization**—The regional chromosomal localization was determined by fluorescence in situ hybridization using a Glis1 genomic fragment as a probe. Genomic clones containing the Glis1 gene were obtained by screening a library of BAC1 vectors containing 100–150-kb fragments of the mouse genome using a radiolabeled Glis1 cDNA as a probe. The identity of the clones was verified by restriction mapping using different cDNA fragments of Glis1 as probes. Genomic DNA derived from the Glis1 BAC clone was labeled with digoxigenin-DUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts in a solution of 50% formaldehyde, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein-conjugated anti-digoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole. The initial experiment resulted in specific labeling of chromosome 4 on the basis of 4,6-diamidino-2-phenylindole staining. The latter was confirmed when a probe specific for the centromeric region of chromosome 4 was co-hybridized with the Glis1 probe.

**Whole Mount in Situ Hybridization**—Whole mount in situ hybridizations were performed according to Haramis and Carrasco (43). Mouse embryos from 7.5 to 14.5 days postcoitus (E7.75–E14.5) were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline, washed twice for 10 min in phosphate-buffered saline containing 0.1% Tween 20 (PBST), and then dehydrated with a series of 10 min methanol/PBT washes (25, 50, and 75, and twice at 100%). Embryos were stored at −20 °C until probed. The Glis1 probe, encoding the region from nucleotide 502 to 1069, was generated by PCR using primers containing either EcoRI or an HindIII site. The PCR product was then cloned into pGEM3Zf (+). For labeling, the construct was linearized either with HindIII for T7-generated sense transcripts or with EcoRI for T7-generated antisense transcripts, and riboprobes were produced using digoxigenin-substituted UTP (Lofstrand Labs, Ltd.).

**Plasmids**—The reporter plasmid pFR-LUC, containing five copies of the Gal4 upstream activating sequence (UAS) upstream and referred to as (UAS)_5-LUC, was obtained from Stratagen. The pFR and pFR-(UAS)_5-LUC reporter plasmids were purchased from Clontech. pGEX-Glis1 deletion mutants were created by placing different Glis1 cDNA fragments at the 5’-end of Gal4DBD. These fragments were generated by PCR using Glis1-specific 5’- and 3’-primers that included either EcoRI or BamHI restriction sites, respectively, to allow the PCR fragments to be subcloned into the EcoRI or BamHI sites of the pFR vector. Details on the length of each deletion are described in the text and figure legends.

pEGFP-Glis1 constructs were generated by cloning full-length Glis1 into EcoRI and BamHI sites of the pEGFP-C1 vector (Clontech). The plasmids pEGFP-Glis1(1N266), pEGFP-Glis1(1N317), and pEGFP-Glis1(1N544) encoding Glis1 from Thr290, Lys317, and Ser444 to the carboxy-terminal end, respectively, were generated by PCR using Glis1-specific primers containing either a 5’-EcoRI or 3’-BamHI site. The plasmids of pEGFP-Glis1(1ZFD) encoding Glis1, in which the region between Arg392 and Ser444 was deleted, was constructed by PCR. To create pGE2-Glis1(1ZFD), encoding GST-Glis1(1ZFD), a region encoding the ZDF of Glis1 (from Lys317 to Pro375) was amplified by PCR and inserted into the EcoRI and SalI sites of pGEXX-3 (Amersham Biosciences). To generate pEGFP-GluI(1ZFD) encoding His6-Glis1(ZFD) Glis1(1N544), the GluI/ZFD region was amplified by PCR and inserted into the BamHI or HindIII sites of pQE32 (Qiagen). All constructs were verified by restriction analysis and DNA sequencing. The plasmids RSV-CaMKI(1–295), RSV-CaMII(1–290), and RSV-CaMKIV(1–313) encoding constitutively active Ca2+-calmodulin kinase I, II, and IV, respectively, were described previously (44) and kindly provided by Dr. R. Maurer (Oregon Health Sciences University, Portland, OR) and Dr. J. Amidon (University of California, San Francisco).

**Electrophoretic Mobility Shift Assay (EMSA)**—Escherichia coli BL21(DE3) transformed with pGEX-Gli1(ZFD) or pQE32-Gli1(ZFD) were grown at 37 °C to mid-log phase and then treated with isopropyl-β-D-thiogalactopyranoside (0.5 mM final concentration) for 3 h. GST-Glis1(ZFD) was purified over glutathione-Sepharose 4B beads. His6-GluI(ZFD) was purified using nickel-nitrilotriacetic acid resin (Qiagen). Double-stranded oligonucleotides containing the consensus Gli-binding site TCTAACAGCAGCCACACATGATGGTTA were end-labeled with [-32P]ATP by T4 polynucleotide kinase (Promega). GST-Glis1(ZFD) or His6-GluI(ZFD) recombinant proteins (2 μg) were incubated in binding buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl2, 10 μM ZnSO4, 1 mM dithiothreitol, 0.1% Nonidet P-40, 12% glycerol) with c32P-end-labeled, double-stranded oligonucleotides for 1 h at room temperature. The protein-DNA complexes were then separated on a 6% native polyacrylamide gel and visualized by autoradiography.

**Nuclear Localization**—pEGFP-mGlis1, pEGFP-mGlis1(1N266), pEGFP-mGlis1(1N317), pEGFP-mGlis1(1N544), pEGFP-mGlis1(-ΔZFD), or pEGFP-C1 plasmid DNA were transfected into CV-1 cells using Lipofectamine 6. After 36 h, the cells were fixed with 4% paraformaldehyde and 0.1% Triton X-100 and local mouse LSM 510 NLO (Zeiss, Thornwood, NY). The excitation and emission frequencies were 488 and 505 nm, respectively. Differential interference contrast images were obtained simultaneously with fluorescence images.
with 0.25 \mu g of (UAS)\textsubscript{5}-LUC, 0–1.0 \mu g of pBSK, and 0.25 \mu g of pCMV\textsubscript{6}, which served as an internal control to monitor transfection efficiency. Cells were transfected in Opti-MEM (Invitrogen) and 3–6 \mu l of FuGENE 6 transfection reagent (Roche Molecular Biochemicals). Cells were incubated for 48 h and then assayed for \beta-galactosidase and luciferase activity. Luciferase activity was assayed with a luciferase kit (Promega). The level of \beta-galactosidase activity was determined using a luminescent \beta-galactosidase detection kit (CLONTECH) according to the manufacturer's instructions. Transfections were performed in triplicate and each experiment was repeated at least twice.

RESULTS

Cloning of Full-length Glis1 cDNA—Yeast two-hybrid analysis using the LBD of the nuclear orphan receptor ROR\textsubscript{y} (42) as bait pulled out several cDNAs encoding fragments of proteins interacting with ROR\textsubscript{y}. One of these cDNAs encoded part of a novel protein, referred to as Gli-similar 1 (Glis1) because of its relationship to Gli proteins as will be discussed further below. Although Glis1 interacted with ROR\textsubscript{y} in the yeast two-hybrid assay, we have not yet been able to observe any interaction between these two proteins in mammalian two-hybrid or pull-down analysis. The proline-rich region is underlined. The zinc finger domain is shaded. The Cys and His residues involved in the tetrahedral configuration in the zinc finger motifs are underlined and in bold. Sequences were submitted to GenBank\textsuperscript{TM} under the accession number AF486579.

Fig. 1. The nucleotide and amino acid sequences of mouse Glis1. The nucleotide and deduced amino acid sequences of mouse Glis1 are shown in the first and second lines, respectively. The start and stop codons are indicated in bold. Putative nuclear localization motifs are indicated by a dashed line. The proline-rich region is underlined. The zinc finger domain is shaded. The Cys and His residues involved in the tetrahedral configuration in the zinc finger motifs are underlined and in bold. Sequences were submitted to GenBank\textsuperscript{TM} under the accession number AF486579.
composed of five tandem Cys-His-type zinc finger motifs with the consensus Cys-X$_{7}$-Cys-X$_{2,10}$-His-X$_{5}$-His that are separated by sequences showing homology to the consensus sequence (T/S)GEKP(Y/F)X typically found in Krüppel-like zinc finger proteins (47).

Comparison of the amino acid sequence of Glis1 with those in the GenBank$^\text{TM}$ data base revealed that its ZFD exhibited high homology with those of the Krüppel-like zinc finger proteins of the Gli and Zic subfamily (7, 9, 18, 48, 49), the recently described Glis2 (50), and with the ZFD of the Drosophila Gli-like zinc finger protein glf/Lmd (40, 41) (Fig. 2). Although these proteins share a highly conserved five zinc finger repeat, they exhibit little homology in regions outside their ZFD. The ZFD of Glis1 exhibits highest identity (77%) with the ZFD of glf/Lmd.

Chromosomal Localization of the Glis1 Gene—The chromosomal localization was determined by fluorescence in situ hybridization using a genomic fragment of the mouse Glis1 gene as a probe. DNA was labeled with digoxigenin-dUTP by nick-translation and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblasts. This resulted in the specific labeling of the middle region of chromosome 4. Labeling with a specific marker for the centromere of chromosome 4 confirmed its localization (not shown). Measurements of 10 specifically labeled chromosomes indicated that Glis1 was located at a position that was 62% of the distance from the heterochromatic-euchromatic boundary to the telomere of mouse chromosome 4, an area that corresponds to band 4C6 (Fig. 3).

Tissue-specific Expression—To examine in which tissues Glis1 was expressed, we performed Northern blot analysis using RNA from mouse placenta and 13 adult tissues. Fig. 4A shows that the radiolabeled Glis1 probe hybridized to a single 3.3-kb transcript that was most highly expressed in placenta and kidney. A lower level of Glis1 expression was observed in testis. Northern blot analysis showed little Glis1 expression in the other tissues analyzed. Glis1 expression was also examined by RT-PCR (Fig. 4B). These results confirmed the high expression of Glis1 in kidney and indicated low levels of mRNA expression in brain, colon, brown fat tissue, testis, and thymus. Glis1 mRNA was undetectable in lung, spleen, liver, pancreas, and muscle.

Whole Mount in Situ Hybridization—To determine the expression pattern of Glis1 during mouse development we performed whole mount in situ hybridization on embryos from stages E7.75 through E14.5. At early headfold stages Glis1 transcripts were detected in extraembryonic mesoderm at E7.75 (Fig. 5A) and in lateral mesoderm at E8.0 (Fig. 5B). No Glis1 transcripts were detected at E8.5 (data not shown). At E10.5, Glis1 expression was detected in the branchial arches, somites, proximal genital tubercle, and ventral mesenchyme of the tail (Fig. 5, C and D). Sectioning revealed that transcripts in branchial arches were localized distally to the mesenchyme and were most prominent in the posterior aspect of the arch (data not shown). Transverse sections of E10.5 embryos revealed that Glis1 transcripts extended throughout the presumptive dermomyotome in the interlimb region (Fig. 5E) but were restricted to lateral mesoderm (apparent hypaxial myo-
Identification of Transactivation and Repressor Functions—Mammalian monohybrid analysis was performed to assess the transcriptional activity of Glis1. For this purpose CV-1 cells were co-transfected with (UAS)5-CAT reporter and pM-Glis1 expression plasmid DNA encoding the Gal4(DBD)-Glis1 fusion protein. Fig. 8 shows that full-length Glis1 was unable to induce transcription of the reporter gene effectively.

Next, we examined the effect of a series of amino- and car-
boxyl-terminal deletions on the transcriptional activity of Glis1. As shown in Fig. 8, carboxyl-terminal deletions had little effect on the transactivation activity of Glis1. In contrast, deletions at the amino terminus had a major impact on Glis1 activity. Amino-terminal deletions up to Leu98 had little effect on Glis1 activity. However, deletion of the amino terminus up to Lys317 (Glis1ΔH9004N317) caused a 30–40-fold induction of Glis1-dependent transactivation, whereas an additional 4–5-fold increase in transactivation was observed when regions up to Ser544 were deleted (Glis1ΔH9004N544). Although the level of transactivation decreased 70% upon further deletion (as for Glis1ΔN697 and Glis1ΔN757), these mutant Glis1 proteins were still able to substantially activate transcription of the reporter. These results suggest that Glis1 contains a strong activation domain at its carboxyl-terminal region and that the activity of this activation function was suppressed by a repressor domain at its amino-terminal half.

To further map the amino-terminal repressor domain(s), the effect of several additional amino-terminal deletions was examined. As shown in Fig. 9A, analysis of several Glis1ΔN mutants demonstrated that deletion of the amino terminus up to Phe150 has little effect, whereas deletion up to Arg200 induced transcriptional activation about 6-fold. An additional 12- and 3-fold increase in transactivation was observed when regions up to Lys317 and Ser544 were deleted. These results appear to indicate the presence of a major repressor function within the region between Phe150 and Lys317, whereas an additional repressor function was associated with the region between Gly459 and Ser544.

The location of the transactivation function was further mapped by examining the effect of a series of carboxyl-terminal deletions on the transactivation activity of Glis1ΔN544 that lacks the repressor function. As demonstrated in Fig. 9B, Glis1ΔN544 induced transcriptional activation of the LUC reporter about 100-fold. Deletion of the first 15 amino acids at the carboxyl terminus reduced reporter activity by more than 90%, indicating that this region was essential for Glis1-mediated transactivation (Fig. 9A). The results in Figs. 8 and 9B suggest that the region between Ala618 and Thr789 of Glis1 was required for optimal transactivation activity.

Glis1-mediated Transactivation Is Cell-type Dependent

To determine whether the transcriptional activity by Glis1 was limited to CV-1, we compared the transactivating activity of Glis1ΔN317 and Glis1ΔN544 in several different cell lines using monohybrid analysis. As shown in Fig. 10, Glis1ΔN317 and Glis1ΔN544 induced transcriptional activation of the LUC reporter in all cell lines tested, however, the magnitude of the induction differed greatly. Glis1ΔN544 induced a 270-fold increase in reporter activity in 293 cells and a 120- and 65-fold increase in CV-1 and COS-7, respectively, compared with a 6- and 15-fold induction in Chinese hamster ovary and PK-1 cells. These differences in the degree of transactivation among cell lines may be related to different levels of expression or activation of co-activators able to interact with Glis1.
Stimulation of Glis1-dependent Transcriptional Activation by CaMKIV

The activity of transcription factors was often regulated by specific protein kinase signaling pathways. Fig. 11A demonstrates that Glis1(H9004N317)-dependent transcrip-
tional activation can be modulated by Ca2+/calmodulin kinases. Co-transfection with a plasmid encoding a constitutively active CaMKIV (CaMKIV*) caused a 4–7-fold enhancement in Glis1(H9004N317)-dependent transactivation. CaMKI* increased reporter activity about 1.5–2-fold, whereas CaMKII* had little effect. Addition of the CaMK inhibitor KN93 reduced the CaMKIV*-induced stimulation in a concentration-dependent manner (Fig. 11B). As shown in Fig. 11C, CaMKIV* increased the transcriptional activity of Glis1 (ΔN317), Glis1(ΔN544), and Glis1(ΔN757) but had little effect on the transactivation activity of full-length Glis1, Glis1(ΔC594), or Glis1(Δ462). These results indicate that the transactivation enhancing effect of CaMKIV does not involve the repressor domain of Glis1 but only depends on the carboxyl-terminal region containing the transactivation function. Sequence analysis of the carboxyl terminus did not reveal any potential CaMKIV phosphorylation sites suggesting that the stimulation by CaMKIV may not be because of phosphorylation of Glis1 itself.

DISCUSSION

In this study, we describe the cloning and sequence of a cDNA encoding a novel Krüppel-like zinc finger protein that was named Glis1 based on its relationship to Gli proteins. As
Gli proteins, Glis1 contains a ZFD that comprises five tandem zinc finger motifs with the consensus Cys-X$_{2,4}$-Cys-X$_{12,15}$-His-X$_{3,4}$-His. The motifs are separated by sequences homologous to the consensus sequence (T/S)GEKP(Y/F)X, a typical feature in Krüppel-like zinc finger proteins. The ZFD of Glis1 exhibits highest homology with members of the Gli and Zic subfamily of Krüppel-like zinc finger proteins, the Gli-related proteins Glis2, and Drosophila glf/Lmd. These proteins exhibit little homology outside their ZFDs.

ZFDs have been implicated in many functions, including DNA recognition, protein/protein interactions, transcriptional repression, and nuclear localization (52–56). Examination of the secondary structure of the Glis1 zinc finger domain indicated that the second half of each zinc finger consists of an a-helix. Crystal structure analysis of DNA-bound GLI1 revealed that the $\alpha$-helices in the 4th and 5th zinc finger motifs in particular are involved in making DNA contacts and therefore in the recognition of specific DNA elements (57, 58). Because these $\alpha$-helices exhibit a 90% or more identity among Gli, Zic, and Glis1 proteins, one may predict that these proteins interact with very similar DNA response elements. Both Gli and Zic proteins have been demonstrated to bind DNA elements with the consensus sequence GACCACCCA (52, 56). EMSA (Fig. 7) showed that Glis1 was also able to interact with oligonucleotides containing this consensus sequence in a specific manner. Confocal microscopic analysis demonstrated that Glis1 was localized primarily to the nucleus where it was distributed in a speckled-like pattern. The latter suggests that Glis1 was associated with a larger protein complex. Deletion mutant analysis showed that absence of the amino terminus containing the first two putative NLS motifs had little effect on nuclear localization and demonstrated that the region containing the ZFD and the bipartite NLS was essential for nuclear localization of Glis1. In transcription factors, NLS motifs have been demonstrated to often overlap with the DNA-binding domain (59), and zinc finger motifs themselves can be involved in nuclear translocation as has been demonstrated for Gli and Zic proteins (55). The nuclear localization of Gli proteins was regulated at multiple levels that involves Sonic hedgehog signaling, nuclear import and export signals, and interactions with Fused (FU) and Suppressor of Fused (SUFU) (60–62). In addition, the nuclear localization of Gli proteins can be facilitated through heterodimerization with Zic proteins (56). Future studies have to ascertain the precise mechanisms that determine the nuclear transport of Glis1.

The demonstration that Glis1 is a nuclear protein and can bind the consensus GBS suggested that Glis1 functions as a transcription factor. Monohybrid analysis in several cell lines using the full-length Glis1 showed that Glis1 was not a very effective inducer of transactivation. However, deletion of the amino terminus converted Glis1 into a strong transcriptional activator suggesting the presence of a repressor domain at the amino terminus. The amino-terminal region does not exhibit any resemblance with the Krüppel-associated box, an evolutionarily conserved repressor domain found in approximately one-third of the Krüppel-like zinc finger proteins (63, 64), and does not contain a SCAN box, a repressor domain identified in several Krüppel-like zinc finger proteins (6). The repressor
domain in Glis1 appears to be novel and does not exhibit homology with the repressor domains recently identified in Glis2, a protein related to Glis1 (50). Repressor functions are often mediated through an interaction of the repressor domain with nuclear co-repressors. For example, the repression by the Kruppel-associated box is mediated through interaction with the co-repressor TIF1 (4, 5, 65). As mentioned above, the speckled-like distribution of Glis1 in the nucleus suggests association with a larger protein complex and possibly a co-repressor complex. Two-hybrid analysis using the Glis1 repressor domain as bait may help to identify (novel) co-repressors interacting with Glis1.

Deletion analysis demonstrated that the region from Ala 618 and Thr 789 at the carboxyl terminus of Glis1 was important for its transactivation function. The region has only a weak resemblance to the TAFII31 sequence identified in Gli proteins (66). Because full-length Glis1 is not a very effective inducer of transcription, its function as a transcriptional activator will likely require activation through a specific mechanism that results in the release of a co-repressor, and/or association with a co-activator. Although little is known about the signaling pathways that regulate the activity of Zic proteins, the Sonic hedgehog-Patched-Smoothened signaling pathway has been linked to activation of Gli proteins (8, 11), which in the case of Gli2 and Gli3 involves removal of an amino-terminal repressor domain by proteolytic cleavage (14). This might be a potential mechanism of Glis1 activation because deletion of the amino-terminal region converts Glis1 into an effective inducer of transcription. As reported for Gli/Cubitus interruptus proteins (11, 62, 67), activation of Glis1 could involve many other mechanisms, including (de)phosphorylation of Glis1. Alternatively, phosphorylation of a co-repressor or co-activator might cause, respectively, its release from or its association with Glis1. The observed increase in Glis1-mediated transcriptional activation by CaMKIV appears to be one such mechanism. CaMKIV has been reported to preferentially phosphorylate substrates with the consensus motif ΦRX(S/T), in which Φ and X are, respectively, a hydrophobic and any amino acid (68). Based on this consensus motif, Glis1 contains three potential CaMKIV phosphorylation sites at Ser172, Ser187, and Thr458. Because CaMKIV is also able to enhance transcription by the deletion mutant Glis1(ΔN544) that does not contain any potential CaMKIV phosphorylation sites, it appears unlikely that the increase in transactivation by CaMKIV involves phosphorylation of Glis1 itself. Relief of repression of the transcriptional factor MEF2 induced by CaMKIV has been reported to be because of phosphorylation of histone deacetylases and their subsequent transport to the cytoplasm (45). Because the Glis1(ΔN544) mutant lacks the repressor domain, the transcriptional stimulation by CaMKIV does not appear to involve the repressor domain of Glis1, or the phosphorylation and release of co-repressors. Alternatively, the induction by CaMKIV may be because of phosphorylation and activation of a co-activator(s) resulting in an enhanced interaction with Glis1. It is interesting to note that CaMKIV has been reported to enhance the activity of the cAMP-response element-binding protein that serves as a co-activator for a number of different transcription factors (69).

Recent studies have indicated that members of the BMP and Wnt families of proteins, in addition of being downstream targets of Gli proteins, may also influence the expression or activity of Gli proteins (22–24). In light of the induction of Glis1-
mediated transactivation by CaMKs, it is interesting to note that certain Wnt signals cause an increase in intracellular Ca\(^{2+}\) and activation of calcium-dependent CaMKs (70). Therefore, it will be interesting to investigate whether Wnt signaling pathways act upstream from Glis1 activation.

The expression of Glis1 in adult mouse tissues was rather restricted. Glis1 was highly expressed particularly in placenta and kidney and at lower levels in testis, whereas it was barely detectable in other tissues. Although Northern analysis established high levels of Glis1 expression in adult kidney tissue, in situ hybridization studies of embryonic/fetal metanephroi showed moderate to low levels of expression and its distribution seemed relatively uniform or slightly ductular in these tissues. This suggests that Glis1 expression may not be significant to metanephric differentiation.

Studies of the embryonic expression of Glis1 show that this gene was both temporally and spatially regulated. Glis1 expression occurs first in extraembryonic tissues and lateral mesoderm during gastrulation, subsequently appears transiently in several defined structures of mesodermal lineage, including craniofacial regions, follicles, branchial arches, limb buds, somites and myotomes, genital tubercle, and tailbud. The temporal nature of Glis1 expression was demonstrated in tissues such as the limb in which expression occurred first anteriorly and posteriorly in mesenchyme at the junction of the limb and the trunk. Subsequently, this expression expanded to include mesenchyme beneath the apical ectodermal ridge at the distal end of the limb, which eventually disappeared leaving expression only at the anterior and posterior limits of the developing foot. Later, Glis1 was also up-regulated at the joint interzone of
the digits and in the footpads. The wave of expression and eventual site-specific disappearance suggests tight regulation in limited populations of primordial tissues.

Glis1 expression in presumptive dermomyotome suggests that this factor may function in myogenesis. Consistent with this role, Glis1 expression overlapped with genes such as Myf5 (71, 72), which are associated with myogenic differentiation, a target of Sonic hedgehog signaling, and positively regulated by Gli activation. However, Glis1 expression was not detected in myotome-originating skeletal muscle precursors migrating into the limb bud. Thus, it is possible that Glis1 functions transiently in the dermomyotome and is silenced in cells as they migrate out of the dermomyotome. In addition, because the mesenchyme of the branchial arch contributes to skeletal muscles of the face, it may also play a significant role in this process. The speculation that Gli1 may function in myogenesis is intriguing in light of the fact that the closely related Drosophila gene, glf/Lmd (Fig. 2, A and B), plays such a role during fly development (40, 41).

Although clearly distinct in sequence from members of the Gli family, the expression patterns of these related transcription factors overlap with those of Glis1. Gl1 expression occurs...
in early extraembryonic tissues, lateral mesoderm, and subsequent-ly in frontal nasal mesenchyme, and mesenchyme of the bran-chial arch, limb, and tail. Both Gli1 and -2 are expressed in the joint interzone regions (73), although we have noted spatial differences with Glis1 especially in the limb bud (19, 74). Also, Gli1 was largely associated with the epidermal component of the follicle and not with mesenchyme. Expressions of Gli2 and -3, however, were reported in craniofacial structures and mesenchyme surrounding the vibrissal follicle. The expression pattern of Glis1 differs considerably to that of the recently described family member Glis2, which is found predominantly in neural tissues and somites (50, 75).

One gene associated with an overlapping pattern of expression with Glis1 is dickkopf-1 (dkk-1), which encodes a soluble secreted inhibitor of Wnt signaling that is critical to proper anterior patterning (76). Similar to Gli1, dkk-1 expression has been observed in early anterior and posterior regions of limb mesenchyme just beneath the ectoderm and eventually expands into the mesenchyme underlying the AER. Furthermore, dkk-1 and Glis1 are both expressed in the first branchial arch in mesenchymal populations just beneath the surface ectodermal layer. This mesenchyme is involved in the formation of craniofacial structures, including facial muscles and vibrissal and hair follicles, where both genes are also expressed. Their expression in mesenchyme may indicate a role in the induction of surface ectoderm in the formation of structures such as the follicle.

In summary, in this study we describe the identification of the novel Krüppel-like zinc finger protein Glis1, which is closely related to members of the Gli and Zic subfamilies and to the Drosophila glf/Lmd. Glis1 appears to function as a transcription factor that may regulate transcription of target genes through interaction with GBS-like DNA-binding sites. Induction of gene expression may be mediated through activation of the potent activation function at the carboxyl terminus of Glis1. The transactivation activity is suppressed by a repressor domain at its amino terminus and can be enhanced by CaMKIV. The temporal and spatial pattern of Glis1 expression observed during embryonic development suggests that it may play a critical role in the regulation of specific developmental programs. Both the repressor and transactivator functions of Glis1 may be involved in this control.

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Identification of Glis1, a Novel Gli-related, Krüppel-like Zinc Finger Protein Containing Transactivation and Repressor Functions
Yong-Sik Kim, Mark Lewandoski, Alan O. Perantoni, Shogo Kurebayashi, Gen Nakanishi and Anton M. Jetten

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