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Identification of Nuclear Localization, DNA Binding, and Transactivating Mechanisms of Krüppel-like Zinc Finger Protein Gli-Similar 2 (Glis2)*[S]

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Gli-similar 1–3 (Glis1–3) constitute a subfamily of Krüppel-like zinc finger (ZF) transcription factors that are closely related to the Gli protein family. Mutations in GLIS2 are linked to nephronophthisis, a chronic kidney disease characterized by renal fibrosis and atrophy in children and young adults. Currently, very little information exists about the mechanism of action of Glis2, its target genes, or the signaling pathways that regulate its activity. In this study, we show that a region within ZF3 is required for the nuclear localization of Glis2. Analysis of Glis2 DNA binding demonstrated that Glis2 binds effectively to the consensus Glis binding sequence (GlisBS) (G/C)TGGGGGGT(A/C). Although Glis2 was unable to induce transactivation of a GlisBS-dependent reporter, it effectively inhibited the GlisBS-mediated transactivation by Gli1. Mutations that disrupt the tetrahedral configuration of each ZF within Glis2 abolished Glis2 binding to GlisBS and also abrogated its inhibition of Gli1-mediated transactivation. In contrast, Glis2 was able to activate the murine insulin-2 (*Ins2*) promoter by binding directly to two GlisBS elements located at −263 and −99 within the *Ins2* promoter. Phosphomimetic mutation of Ser^{245} inhibited the binding of Glis2 to GlisBS and dramatically affected its transactivation of the *Ins2* promoter and its ability to inhibit GlisBS-dependent transactivation by Gli1. In this study, we demonstrate that Glis2 can function as a transcriptional activator and that post-translational modification within its DNA-binding domain can regulate its transcriptional activity. This control may play a critical role in the Glis2-dependent regulation of target genes and renal function.

Glis (Gli-similar) 1–3 are members of a subfamily of Krüppel-like zinc finger transcriptional regulators that are related to Gli and Zic proteins (1–6). These transcription factors share a highly conserved DNA-binding domain consisting of five C_2H_2-type zinc finger motifs that are involved in the recognition of specific DNA-binding sites in the promoter regulatory region of target genes. Glis proteins play a critical role in a number of different physiological processes and have been implicated in several pathologies (4). Glis2 has been reported to be critical for maintaining normal kidney architecture and function (7, 8). Mice deficient in Glis2 develop progressive tubular atrophy, severe interstitial fibrosis, glomerulosclerosis, and infiltration of inflammatory cells that ultimately result in renal failure and premature death. A recent study linked mutations in human GLIS2 to nephronophthisis, an autosomal recessive kidney disease that constitutes the most frequent genetic cause for end-stage renal failure in the first three decades of life (7). Gene expression profiling analysis indicated that Glis2 deficiency induces increased expression of many genes associated with proinflammatory and fibrotic responses (7, 8). In addition, the observed dysregulation of extracellular matrix homeostasis and induction of TGFβ and Slug in Glis2 knock-out mice suggested that loss of Glis2 function may promote epithelial-mesenchymal transition in renal tubule epithelial cells (7).

Glis3 is essential for the development of pancreatic β-cells and the maintenance of normal renal functions, whereas deficiency in Glis3 function leads to the development of neonatal diabetes and polycystic kidney disease (9–11). The optimal DNA binding site for Glis3 (GlisBS)[2] has recently been characterized (12), and Glis3 was shown to regulate *Fgf18* (fibroblast growth factor 18) and *Ins* (insulin) gene expression by binding to GlisBS within the respective promoters (13–15).

Although relatively little is understood about the various stages involved in Glis2-mediated transcriptional regulation, recent studies have provided some clues about the signaling pathways that may regulate its function (3, 7, 16). Both Glis2 and Glis3 localize to the primary cilium, suggesting that Glis proteins may be involved in signaling pathways that originate from the primary cilium (7, 8, 10). Moreover, several Glis2-interacting proteins have been identified, including CtBP1 (C-terminal binding protein 1) that functions as a co-repressor of Glis2 (3). Although other studies have demonstrated the interaction of Glis2 with β-catenin and p120 catenin (3, 16, 17), further analysis is required to establish the physiological significance of these interactions.

In this study, we demonstrate that Glis2 is able to activate the mouse insulin-2 (*Ins2*) promoter by binding two proximal GlisBS elements. We further characterize several other key

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[2] The abbreviations used are: GlisBS, Glis binding sequence; ZFD, zinc finger domain; ZF, zinc finger; GBS, Gli-binding site; EGFP, enhanced GFP; aa, amino acid(s); Luc, luciferase; MBP, maltose binding protein.

steps involved in Glis2-mediated transactivation, including Glis2 nuclear localization and DNA binding, and identify a critical role for Ser^{245} phosphorylation, a previously identified target of the EGF signaling pathway (18). Our data suggest that Glis2 can function as an activator of transcription and that its activity can be modulated by post-translational modifications. It is likely that these processes play an important role in regulating Glis2-dependent gene expression and functions in the kidney.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293, HeLa, and mIMCD3 (mouse inner medullary collecting duct) cells were purchased from ATCC. HEK293 and HeLa cells were grown in DMEM (Invitrogen) and mIMCD3 cells in DMEM/F12 (1:1) supplemented with fetal bovine serum, penicillin, and streptomycin.

Plasmids—N-terminal FLAG-tagged Glis2 and mutant Glis2 mammalian expression vectors were generated by cloning the respective cDNAs into the HindIII-BamHI sites of p3XFLAG-CMV10 (Sigma). Point mutations within p3XFLAG-CMV10 were generated by site-directed mutagenesis using the QuikChange Site-directed Mutagenesis kit (Stratagene) following the manufacturer's protocol. To generate the Glis2(237–264)-EGFP and Glis2(237–286)-EGFP expression plasmids, the respective cDNAs together with an upstream Kozak sequence were cloned into the EcoRI-BamHI sites of pEGFP-N2 (Clontech). A putative translation initiation site in the EGFP sequence downstream of BamHI was mutated to CTG. MBP-Glis2 fusion proteins were constructed by cloning the PCR product of the targeted region of Glis2 into the EcoRI-HindIII sites of pMAL-c2x (New England Biolabs). Mouse *Ins2* promoter constructs were generated by PCR amplification of the −696 to +8 region of the mouse *Ins2* gene by using mouse genomic DNA as template and cloned into the NheI-HindIII sites of pGL4.10 luciferase reporter vector. mouse *Ins2*(−696/+8) was used as template for the subsequent deletion constructs of mouse *Ins2*. Site-directed mutagenesis of the two GlisBS sites within the mouse *Ins2* promoter was carried out by site-directed mutagenesis. The mutated bases are underlined in the following: GlisBS(−99) (5′-CTG-CTGACCTACTTCACCTGGAGCCC) and GlisBS(−263) (5′-GGAACAATGTCTTCTGCTGTGAAC).

Transfection—Lipofectamine 2000 and OptiMEM (Invitrogen) were used in all transfections as per the manufacturer's protocol. Cells were processed for the required application between 24–40 h post transfection. For luciferase assays, 293T cells were transfected with 200 ng of luciferase reporter constructs per well of a 12-well plate and 100 ng, 5 ng of β-galactosidase, and 100 ng of each cotransfectant or as indicated. Cells were lysed with passive lysis buffer (Promega), firefly luciferase assayed with luciferase reagent substrate (Promega), and normalized with β-galactosidase activity assayed with luminescent substrate (Clontech).

Confocal Microscopy—mIMCD3 or HeLa cells were transfected with 0.25 μg of the indicated FLAG-Glis2 or Glis2-EGFP plasmids using Lipofectamine 2000 and fixed 24 h later in 4% paraformaldehyde, quenched (20 mM glycine, 75 mM ammonium chloride), permeabilized, and blocked in 0.2% Triton X-100 in SuperBlock buffer (Thermo Scientific). Cells were washed in PBS, subsequently incubated for 1 h with anti-FLAG M2 mouse monoclonal antibody (Sigma) in SuperBlock buffer, washed with PBS, and incubated with goat anti-mouse Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) for 40 min. Cells were washed with PBS, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was observed with a Zeiss confocal microscope LSM 510 NLO (Zeiss, Thornwood, NY).

MBP Protein Purification—MBP-Glis2 fusion protein constructs were transformed in Rosetta cells. Bacteria were induced with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside at 16 °C for 24 h. After sonication in extraction buffer (20 mM Tris-HCl, pH 8.2, 200 mM NaCl, 0.05% Triton X-100, 5% glycerol, 1 mM β-mercaptoethanol, and 0.5 mM immidazole), bacterial lysates were centrifuged, and supernatant was mixed with prewashed amylose binding resin (New England Biolabs) for 30 min. Fusion proteins were eluted by incubation with 10 mM maltose for 10 min in extraction buffer twice, and purity was checked by SDS-PAGE (supplemental Fig. 1).

Electrophoretic Mobility Shift Assay—Annealed probes were labeled with [γ-^{32}P]ATP. Binding reactions were carried out with 2 μg of purified MBP-Glis2 fusion protein and 50,000 cpm of the labeled probe at room temperature in 10 mM Tris-HCl, pH 8.2, 0.2 mM EDTA, 100 mM KCl, 1 mM DTT, 5% glycerol, and 2.5 μg/ml BSA. DNA-protein complexes were then separated on a 6% tris-borate-EDTA polycacrylamide gel, dried, and exposed for autoradiography.

RNA Isolation and Quantitative RT-PCR—RNA from rat tissues and cell lines was isolated with an RNeasy mini kit as described previously (19). The RNA was reverse-transcribed using a high capacity cDNA archive kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Quantitative RT-PCR reactions were performed using the TaqMan system. The sequences of Glis2 primer and probe sets were as follows: forward primer (TGGCCACTT-TGTGTCACATGA), Glis2 reverse primer (CGCTGACAT-AGGAGCCACTGT), and Glis2 probe (CAGCTGCGC-CCACCCCCTAA). All results were normalized relatively to the 18 S RNA transcripts.

RESULTS

Nuclear Localization of Glis2 Is Dependent on Zinc Finger 3—Glis2, to exert its function as a transcription factor, requires translocation to the nucleus. Although Glis2 also localizes to the primary cilium in renal epithelial cells (7), it must translocate to the nucleus to regulate gene expression. To understand the domains involved in its nuclear translocation, we analyzed the effect of several deletions on the subcellular localization of Glis2 in mIMCD3 cells. C-terminal deletions up to aa 264 and N-terminal deletions up to aa 237 had little effect on the nuclear localization of Glis2, whereas further deletions at either end greatly affected the accumulation of Glis2 into the nucleus (Fig. 1*A*). These data suggested that the region required for nuclear localization of Glis2 resided in ZF3 spanning the residues 237–264. Analysis of additional N-terminal deletions within the ZF3 up to aa 246 and 256 con-

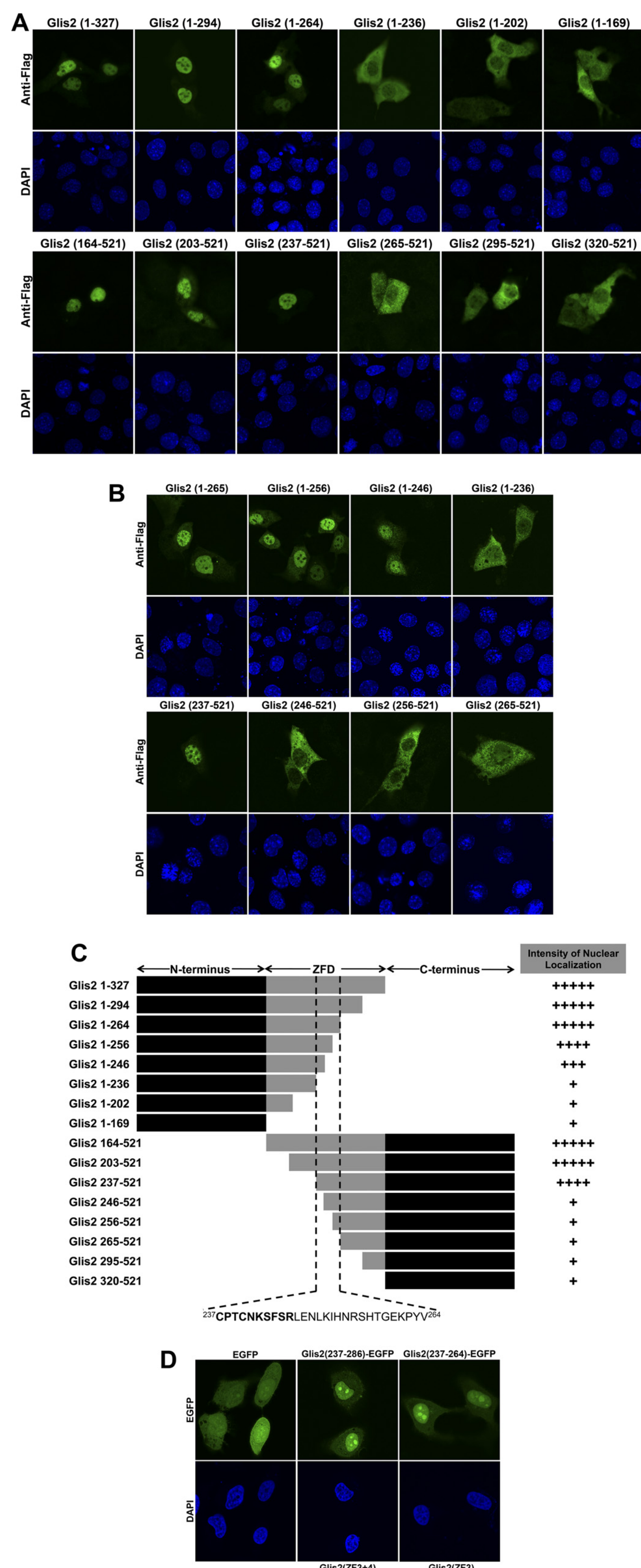


FIGURE 1. **Nuclear localization of Glis2 is dependent on residues 237–246 in ZF3.** *A*, representative confocal micrographs showing the localization of several FLAG-Glis2 C-terminal (*top*) or N-terminal (*bottom*) deletion mutants transiently expressed in mIMCD3 cells stained with anti-FLAG antibody (*green*) and DAPI staining (*blue*). *B*, further N- and C-terminal deletions within the ZF3 show that residues 237–246 play an important role in the nuclear localization of Glis2. *C*, a schematic overview of the deletion mutants used in to identify the region responsible for the nuclear localization of Glis2. Sequence of Glis2 required for the optimal nuclear localization of Glis2 spanning ZF3 is shown at the bottom where 237–246 (*boldface letters*) represents the critical region. *D*, the (237–264) region of Glis2 is able to promote the nuclear accumulation of EGFP. Plasmids expressing either EGFP, Glis2(237–286)-EGFP or Glis2(237–264)-EGFP were transfected in HeLa cells, and 24 h later, their subcellular localization was examined by confocal microscopy.

firmed that the region between aa 237 and 246 was required for nuclear localization. Additional C-terminal deletions within the ZF3 up to aa 256 and 246 slightly enhanced cytoplasmic staining of Glis2, whereas a deletion up to aa 237 abolished the accumulation of Glis2 into the nucleus suggesting that the region between aa 237–265 is required for the optimal nuclear localization of Glis2 with the 237–246 sequence being the most critical (Fig. 1, *B* and *C*). Mutation of one of the cysteines within ZF3 did not affect the accumulation of Glis2 in the nucleus significantly (supplemental Fig. 2) indicating that the tretrahedral conformation of ZF3 was not required. Similar mutations in the other ZF motifs also did not affect the nuclear localization of Glis2 (supplemental Fig. 2).

To examine the ability of ZF3 to promote nuclear accumulation, we analyzed the subcellular localization of two chimeric proteins, Glis2(237–286)-EGFP and Glis2(237–264)-EGFP. In contrast to EGFP, which was distributed almost equally between the nucleus and cytoplasm, both Glis2(237–286)- and Glis2(237– 64)-EGFP fusion proteins were predominantly localized to the nucleus in both HeLa (Fig. 1*D*) and mIMCD3 cells (data not shown). These data suggest that ZF3 alone is capable of promoting nuclear retention of EGFP. In contrast to FLAG-Glis2 mutants (Fig. 1, *A* and *B*), both Glis2-EGFP fusion proteins also localized to the nucleoli. Shorter regions of ZF3 did not promote the nuclear accumulation of EGFP (data not shown) suggesting that under these conditions the entire ZF3(237–264) is required to induce nuclear retention.

Glis2 Binds GlisBS with Stronger Affinity than GBS—Glis proteins modulate gene transcription by binding to specific DNA binding sites in the promoter of target genes (4). Previous studies identified TGGGTGGTC (GBS) and (G/C)TGGGGGGT(A/C) (GlisBS) as the consensus optimal binding sites for Gli1 and Glis3, respectively (12, 20). Although the DNA binding element for Glis2 has not yet been clearly defined, we hypothesized that given the high degree of homology between the zinc finger domains, the binding site of Glis2 might resemble that of Gli1 and Glis3. To analyze the Glis2 DNA binding, we examined the binding of an MBP-Glis2(166–349) fusion protein containing the complete zinc finger domain of Glis2 to ^{32}P-labeled consensus GlisBS and GBS probes by EMSA. This analysis demonstrated that Glis2 was able to bind the ^{32}P-labeled GlisBS consensus sequence. Moreover, it revealed unlabeled GlisBS competed more efficiently with ^{32}P-labeled GlisBS for Glis2 binding than GBS suggesting that GlisBS has a stronger binding affinity for Glis2 than GBS (Fig. 2*A*). The latter was supported by data showing that unlabeled GlisBS competed also more efficiently with

GlisBS 5′: TCAGACG**TGGGGGGT**CCAACTT

GBS 5′: TCAGACG**TGGGTGGT**CCAACTT

D	
GlisBS	GTGGGGGGTCC
M1	GTGGGGGGACC
M2	GTGGGGGTTCC
M3	GTGGGGTGTCC
M4	GTGGGCGGTCC
M5	GTGTGGGGTCC
M6	GTTGGGGGTCC
M7	GAGGGGGGTCC
M8	GTGGGGGGCCC
M9	TTGGGGGGTCC
M10	GTGGGAGGTCC
M11	GCGGGGGGCCC

FIGURE 2. **Glis2 is able to bind GlisBS efficiently.** *A* and *B*, the binding of MBP-Glis2(166–349) and the Glis2 mutants C265G and C295G, containing a Cys-to-Gly mutation in either the fourth or fifth ZF to ^{32}P-labeled GlisBS or GBS, respectively, was examined. Competition was carried out with unlabeled GlisBS and GBS as indicated. *C*, shown is the competition of unlabeled GlisBS and mutant GlisBS with ^{32}P-labeled GlisBS for MBP-Glis2(166–349) binding. *D*, the sequences of GlisBS and mutant GlisBS used in *C* are shown. Mutations are *underlined*.

	1	2	3	4	5	6	7	8	9	10
6XGlisBS-Luc	+	+	+	+	+	+	+	+	+	+
Gli1	-	125	-	-	125	125	125	125	125	125
Glis2	-	-	125	-	15	50	125	-	-	-
Glis2 (164-327)	-	-	-	125	-	-	-	15	50	125

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GlisBS	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gli1	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Glis2	-	-	WT	WT	C170G	C170G	C204G	C204G	C237G	C237G	C265G	C265G	C295G	C295G

FIGURE 3. **Glis2 suppresses activation of the GlisBS-Luc reporter by Gli1.** *A*, HEK293 cells were transfected with the (GlisBS)$_6$-Luc reporter and Gli1 or Glis2 expression vectors as indicated. Luciferase values were normalized with β-galactosidase activity. *B*, mutation of the first Cys in each ZF of Glis2 abrogates the inhibition of Gli1-induced activation of (GlisBS)$_6$-Luc reporter. *Bars* represent the average + S.D. of three independent experiments. *Bottom*, Western blot analysis of Gli1 and Glis2 levels of samples used in *B*.

^{32}P-labeled GBS for Glis2 binding than GBS (Fig. 2*B*). Mutation of the first Cys to Gly in either the fourth (C265G) or the fifth (C295G) zinc finger domain (ZFD) of Glis2, which destroys the tetrahedral conformation of the ZF, completely abolished Glis2 binding to GlisBS (Fig. 2*A*) and GBS (Fig. 2*B*), thereby ascertaining the requirement of the structural integrity of the ZFD for Glis2 DNA binding.

To further analyze the binding specificity of Glis2, we examined the ability of a series of unlabeled GlisBS point mutants (M1–M11) to compete against the radiolabeled consensus GlisBS for Glis2 binding (Fig. 2, *C* and *D*). Fig. 2*C* shows that the GlisBS mutants M1–M4 did not efficiently compete with ^{32}P-labeled GlisBS for Glis2 binding indicating a low affinity of Glis2 for these sequences. In contrast, GlisBS mutants M5–M11 competed well with ^{32}P-labeled GlisBS for

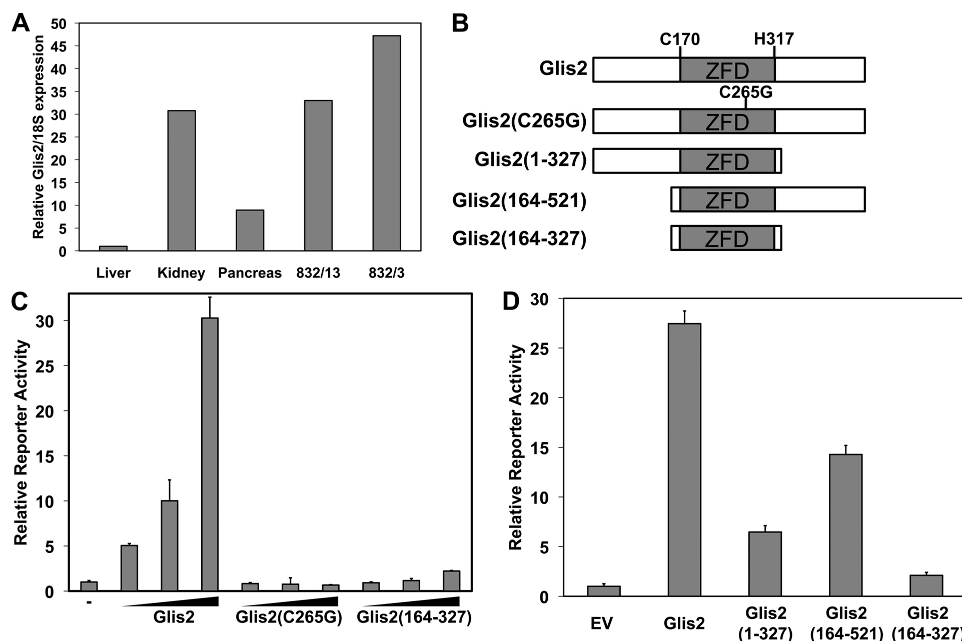


FIGURE 4. **Activation of the *Ins2* promoter by Glis2.** *A*, relative Glis2 expression in various rat tissues and INS-1 832/13 and INS-1 832/3 pancreatic cell lines. *B*, schematic representation of Glis2 point mutant and deletion constructs used in the study. *C*, Glis2, but not Glis2(C265G) or Glis2(164–327) (ZFD alone), is able to activate the *Ins2*(−696/+8) promoter. HEK293 cells were co-transfected with the *Ins2*(−696/+8)-Luc reporter and the Glis2 expression vectors as indicated. The relative luciferase activity normalized against β-galactosidase is plotted. *D*, deletion of either the N or C terminus of Glis2 reduced the activation of the *Ins2*(−696/+8) promoter compared with the full-length Glis2. *EV* refers to empty vector.

Glis2 binding. Substitution of the T in the GlisBS consensus for A, as in M1, abolished the ability to compete, whereas mutation to C, as in M8, did not affect its competition. Similarly, substitution of the fifth G for a C or A, as in M4 and M10, respectively, either abolished or had little effect on the interaction with Glis2. These data suggest that single nucleotide changes in the GlisBS sequence can significantly influence the binding of Glis2. However, none of the mutants competed more effectively than the GlisBS consensus indicating that among the binding sites tested, Glis2 exhibits the highest affinity for the consensus GlisBS.

Glis2 Inhibits Gli1-mediated Activation of GlisBS—The finding that Glis2 could bind the GlisBS led us to analyze its ability to activate a luciferase reporter under the control of six tandem repeats of GlisBS (12). In contrast to Gli1, which effectively induced GlisBS-dependent activation of the reporter, Glis2 had little effect on Luc reporter activity in HEK293 cells (Fig. 3*A*). Cotransfection of Glis2 with Gli1, however, greatly suppressed Gli1-induced reporter activity in a dose-dependent manner. The fact that the deletion mutant Glis2(164–327), containing only the DNA binding domain, was also able to repress Gli1-mediated transactivation suggested that the observed suppression is likely attributable to the competition between Glis2 and Gli1 for GlisBS binding (Fig. 3*A*).

To determine whether the binding of Glis2 to DNA is required for suppression of Gli1 transactivation function, the effects of several single Cys-to-Gly mutations, which destroy the tetrahedral configuration of each of the five zinc fingers, were examined. As shown in Fig. 3*B*, each ZF mutation abolished the ability of Glis2 to suppress Gli1-mediated activation of the GlisBS reporter. Western blot analysis of the protein lysates used in the reporter assay showed that co-expression with Glis2 did not significantly affect the level of Gli1 protein suggesting that Glis2-mediated suppression of Gli1 activation was unrelated to changes in Gli1 protein levels. These data further suggest that this suppression requires the intact structure of each zinc finger motif of Glis2 and likely involves competition between Glis2 and Gli1 for GlisBS binding. This conclusion is consistent with EMSA results showing that the Cys-to-Gly mutations within zinc finger 4 and 5 totally abolished the binding of Glis2(166–349) to GlisBS (Fig. 2, *A* and *B*).

Glis2 Activates Murine Insulin-2 Promoter by Binding to GlisBS—Although many genes have been reported to be aberrantly regulated in the kidney as a result of the loss of Glis2 expression, the mechanisms by which Glis2 controls these genes still remains poorly understood (7, 8). This is in part due to the lack of our understanding of the transactivation function of Glis2. Although Glis2 binds the consensus GlisBS efficiently (Fig. 2*A*), it fails to induce $(GlisBS)_6$-mediated transcriptional activation in HEK293 cells (Fig. 3*A*).

Recent studies showed that Glis3 strongly activated the *Ins2* promoter through two GlisBS in the proximal (−300 nt) promoter region (10, 15). Because Glis2 was able to bind GlisBS elements, we investigated whether Glis2 was able to mediate transcriptional activation through this promoter. We first established that Glis2 was expressed in two different pancreatic β-like cell lines, INS-1 832/13 and INS-1 832/3, at a level comparable with that of kidney, whereas its expression was significantly lower in whole pancreas and very low in liver (Fig. 4*A*). As shown in Fig. 4*C*, coexpression of Glis2 with a reporter plasmid in which luciferase is under the control of the −696/+8 region of the mouse *Ins2* promoter, showed that Glis2 strongly activated transcription through this promoter in HEK293 cells in a dose-dependent manner. This is the first

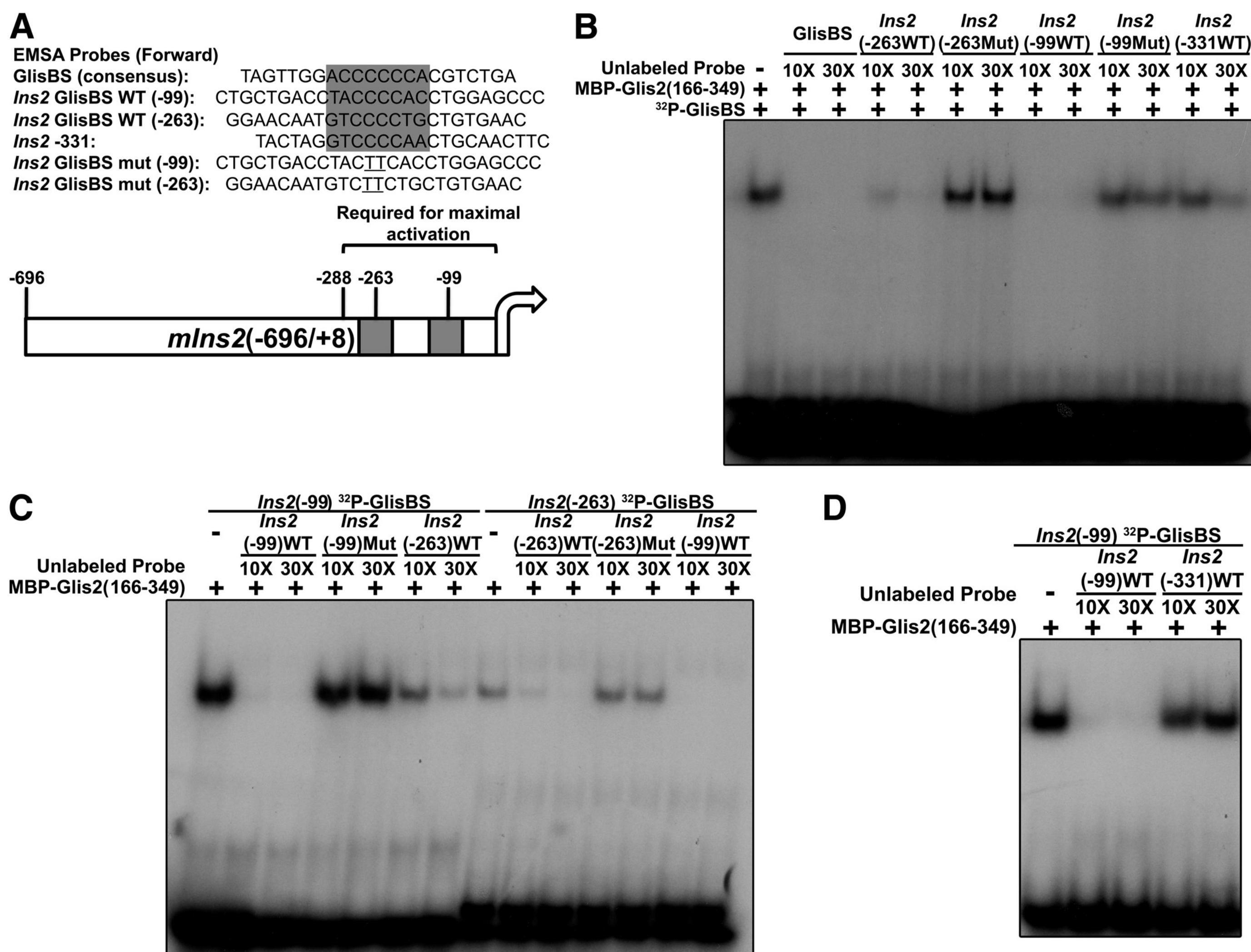


FIGURE 5. **Glis2 binds to GlisBS in the *Ins2* promoter.** *A*, sequences of GlisBS(−99), GlisBS(−263), and GlisBS(−331) in the *Ins2* promoter. A schematic of the *Ins2* promoter containing the GlisBS and the minimal region required for maximal activation by Glis2 is shown at the *bottom*. *B*, competition of unlabeled wild type and mutated (*mut*) GlisBS(−99), GlisBS(−263), and GlisBS(−331) with radiolabeled GlisBS for MBP-Glis2(166–349) binding. EMSA was carried out as described under "Experimental Procedures." *C* and *D*, EMSA of MBP-Glis2(166–349) binding to radiolabeled GlisBS(−99) and GlisBS(−263) and competition with unlabeled GlisBS(−99), GlisBS(−263), or GlisBS(−331).

evidence that clearly demonstrates the transactivation potential of Glis2, rather than its suggested role as transcriptional repressor as reported previously (3, 16). In contrast to wild type Glis2, the mutants Glis2(C625G), containing a point mutation within the fourth ZFD that destroys Glis2 binding to GlisBS, and Glis2(164–327), containing only the ZFD of Glis2, did not activate the *Ins2* promoter (Fig. 4, *B* and *C*). Additionally, the C- and N-terminal deletion mutants, Glis2(1–327) and Glis2(164–521), respectively, were both able to modestly activate the *Ins2* promoter, albeit less efficiently than full-length Glis2 (Fig. 4*D*). Taken together, these data suggest that Glis2-mediated *Ins2* transactivation is dependent upon DNA binding and that both the N and C termini of Glis2 are essential for maximal transactivation.

To further demonstrate that the activation of *Ins2* by Glis2 involves binding of Glis2 to the two GlisBS, GlisBS(−99) (TACCCCAC) and GlisBS(−263) (GTCCCCTGC), located at −99 and −263 of *Ins2*, respectively (Fig. 5*A*), we analyzed by EMSA the ability of these GlisBS sites to compete with radiolabeled consensus GlisBS for Glis2 binding (Fig. 5*B*). Both *Ins2* GlisBS(−99) and GlisBS(−263) competed very effectively with the consensus GlisBS for Glis2 binding, whereas mutated GlisBS(−263) or GlisBS(−99) were not very efficient in competing for Glis2 binding (Fig. 5*B*). Upon screening the promoter for possible additional GlisBS sites, a third putative GlisBS element at −331 (GlisBS(−331), GTCCCCAAC) was considered. However, GlisBS(−331) failed to compete with the consensus GlisBS for Glis2 binding (Fig. 5*B*). We next examined the binding of Glis2 to ^{32}P-labeled GlisBS(−99) or GlisBS(−263). The EMSA analysis in Fig. 5*C* supported the conclusion that Glis2 bound to these sites directly. GlisBS(−99) competed better than GlisBS(−263) suggesting that Glis2 might have a higher affinity for GlisBS(−99) than GlisBS(−263). Mutant GlisBS(−99) and GlisBS(−263), and GlisBS(−331) did not compete effectively for with GlisBS(−99) for Glis2 binding (Fig. 5, *C* and *D*).

To ascertain whether one or both of the GlisBS elements were involved in the activation of the *Ins2* promoter by Glis2,

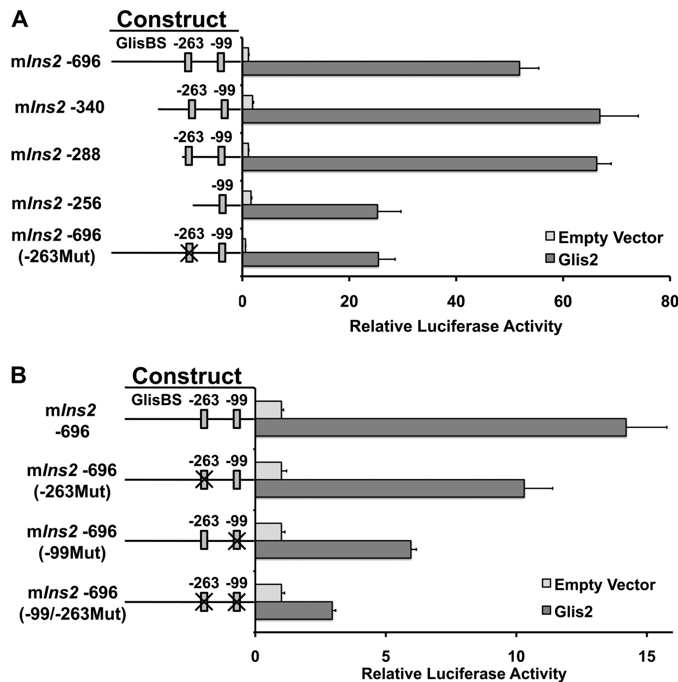


FIGURE 6. **The optimal activation of the *Ins2* promoter by Glis2 was dependent on both GlisBS(−99) and GlisBS(−263).** *A*, effect of a series of 5′ deletions within the *Ins2* promoter on its Glis2-mediated *Ins2*-Luc activation in HEK293 cells. *B*, effect of point mutations in GlisBS(−99) and GlisBS(−263) on Glis2-mediated activation of *Ins2*(−696/+8)-Luc. A schematic presentation of the various deletion and point mutations within *Ins2*(−696) is shown to the *left*. *mIns2*, mouse Ins2.

we examined the effect of a series of deletions and point mutations within the *Ins2* promoter on the transactivation of the Luc reporter. Deletions up to −288 had no significant effect on the *Ins2* promoter activity (Fig. 6*A*). However, deletion of up to −256, which removed the GlisBS(−263), greatly diminished promoter activity. We next examined the effect of point mutations within GlisBS(−263) and GlisBS(−99) on *Ins2*(−696/+8)-Luc activation. Mutations in a single binding site resulted in a significant reduction in Glis2-induced activation of *Ins2*(−696/+8)-Luc, whereas mutations in both sites reduced activation by 80% (Fig. 6*B*). The GlisBS(−263) mutation reduced the activity to a similar extent as the −256 deletion consistent with the loss of the GlisBS(−263) site in both constructs (Fig. 6*A*). The *Ins2* promoter has been well studied and shown to be regulated by other transcription factors, including Pdx-1, MafA, and NeuroD, that bind to their respective DNA response elements within the −300 proximal *Ins2* promoter region. Activation mediated by these or other transcription factors might explain why the mutation of the two GlisBS sites did not totally abolish *Ins2* promoter activity.

Role of Ser^{245} Phosphorylation in Controlling GlisBS Binding and Transactivation—An *in vivo* global phosphoproteomic analysis of proteins isolated from HeLa cells by mass spectrometry identified phospho-Ser^{245} in Glis2 as an EGF-dependent phosphorylation site (18) (Fig. 7*A*). Ser^{245} is within a region conserved between mouse and human Glis2 and is localized in the loop of ZF3 within the DNA binding domain of Glis2. This led us to hypothesize that the phosphorylation status of Ser^{245} may play a critical role in regulating Glis2 activity either at the level of its nuclear localization, DNA binding, protein-protein interaction, and/or transactivation activity. We utilized a phosphomimetic strategy to study the effect of phospho-Ser^{245} on the activity of Glis2 whereby mutation of Ser^{245} to Asp (Glis2(S245D)) would mimic phospho-Ser^{245} in Glis2 and mutation to Ala (Glis2(S245A)) would mimic a nonphosphorylated residue. As shown in Fig. 7*B*, Glis2 and the Glis2(S245A) mutant were able to activate the *Ins2* promoter equally well. In contrast, the transactivation activity was completely lost with the Glis2(S245D) mutant. Because ZF3 is important for the accumulation of Glis2 into the nucleus, the loss of transactivation activity by Glis2(S245D) could be due to an effect on its nuclear translocation. However, both Ser^{245} Glis2 mutants predominantly localized to the nucleus suggesting that the loss of transactivation activity is not due to the cytoplasmic retention of Glis2(S245D) (Fig. 7*C*).

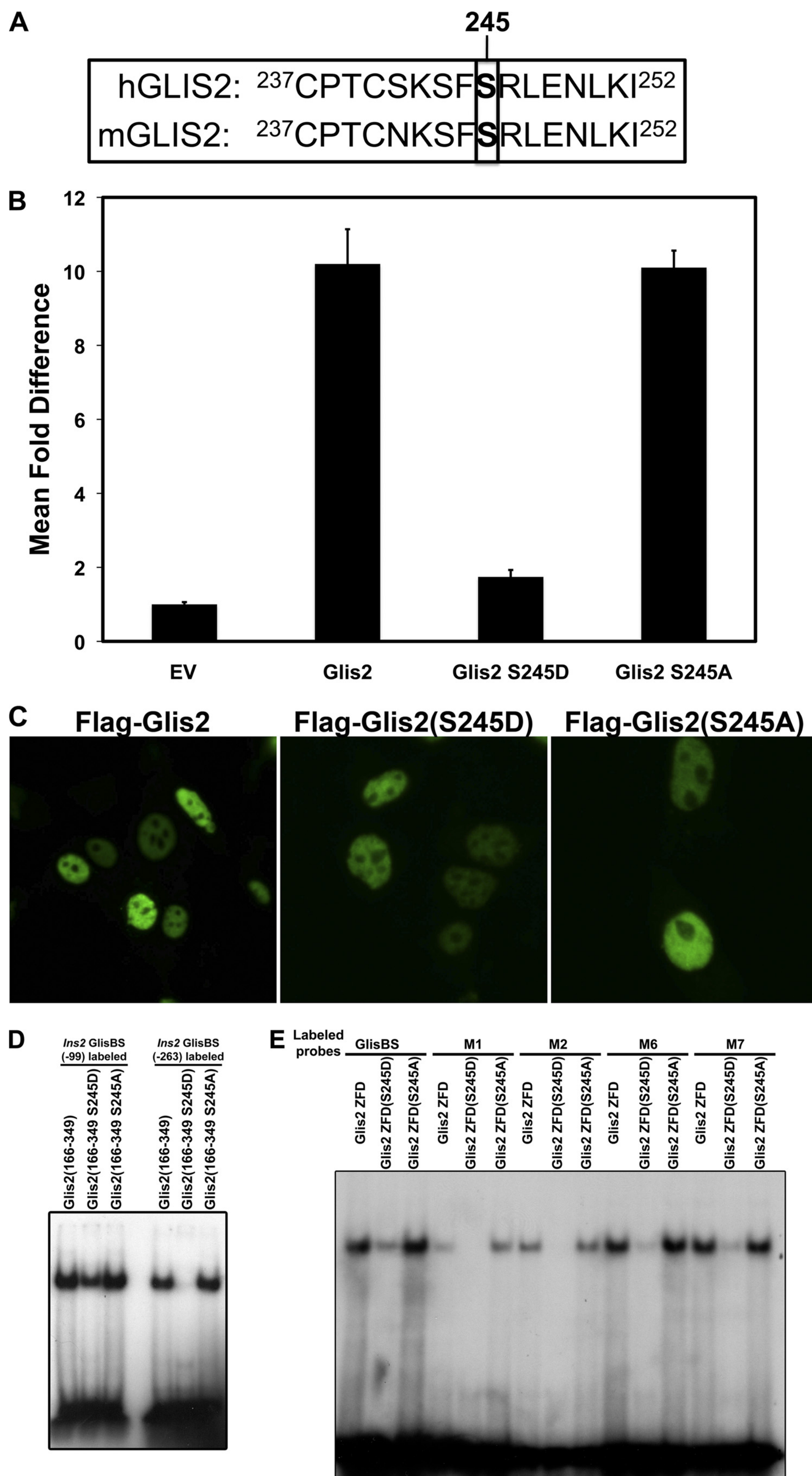
To test the possibility that the loss of transactivation activity was due to the inability of Glis2(S245D) to bind DNA, EMSA was carried out with radiolabeled GlisBS(−99) and the GlisBS(−263), MBP-Glis2(166–349), and MBP-Glis2(166–349) mutants containing either the S245D or S245A mutation. Both wild type Glis2 and Glis2(S245A) were able to bind the GlisBS(−99) and GlisBS(−263) efficiently; however, binding of Glis2(S245D) to GlisBS(−263) was completely lost and that to GlisBS−99) greatly reduced (Fig. 7*D*). These data suggest that phosphorylated Glis2 at Ser^{245} markedly affects its ability to bind GlisBS.

To further compare the abilities of Glis2(166–349)(S245A), Glis2(166–349)(S265D), and Glis2(166–349) to bind distinct GlisBS sequences, their capacities to bind to labeled consensus GlisBS and four GlisBS mutants (M1, M2, M6, and M7) were compared by EMSA. Glis2(166–349) and Glis2(166–349)(S245A) bound GlisBS and GlisBS mutants to a similar degree (Fig. 7*E*), whereas Glis2(166–349)(S245D) showed a greatly reduced binding to the consensus GlisBS, very poor binding to M6 and M7, and no binding to M1 and M2. The poor binding of Glis2(166–349) to radiolabeled M1 and M2 is consistent with our results in Fig. 2*C* showing that M1 and M2 mutants competed poorly with the consensus GlisBS for Glis2 binding.

In Fig. 3*A*, we showed that Glis2 was able to repress the Gli1-dependent activation of $(GlisBS)_6$-Luc. We, therefore, were interested in determining the ability of Glis2(S245D) and Glis2(S245A) to inhibit this activation. Both Glis2 and Glis2(S245A) repressed Gli1-dependent activation of $(GlisBS)_6$-Luc to a similar degree, whereas the S245D mutation greatly diminished the repression of Gli1-mediated activation by Glis2 (Fig. 8). These data support the concept that phosphorylation of Ser^{245} can modulate the transcriptional activity of Glis2 and, therefore, Glis2-dependent gene expression and functions.

DISCUSSION

In this study, we characterized several features of the Glis2 signaling pathway, including the nuclear localization, DNA binding, transactivation activity, and the modulation of Glis2 activity by phosphorylation. Translocation of Glis2


A
245
hGLIS2: 237CPTCSKSFSRLENLKI252
mGLIS2: 237CPTCNKSFSRLENLKI252
B
Mean Fold Difference
12
10
8
6
4
2
0
EV
Glis2
Glis2 S245D
Glis2 S245A
C
Flag-Glis2
Flag-Glis2(S245D)
Flag-Glis2(S245A)
D
Ins2 GlisBS (-99) labeled
Ins2 GlisBS (-263) labeled
Glis2(166-349)
Glis2(166-349 S245D)
Glis2(166-349 S245A)
E
Labeled probes
GlisBS
M1
M2
M6
M7
Glis2 ZFD
Glis2 ZFD(S245D)
Glis2 ZFD(S245A)

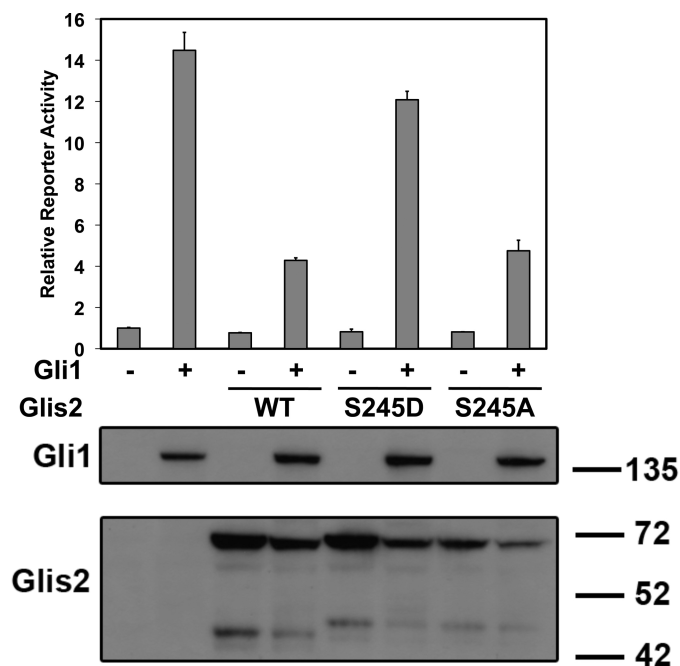


FIGURE 8. **The S245D mutation greatly reduced the repression of Gli1-mediated transactivation by Glis2.** $(GlisBS)_6$-Luc reporter, Gli1, Glis2, and mutant Glis2 expression plasmids were transfected into HEK293 as indicated. Relative reporter activity was measured 48 h after transfection. Western blot at the bottom of the graph shows the level of expression of Gli1 and Glis2 proteins.

to the nucleus is essential for its transcriptional activity and regulation of nuclear import and export provides an important mechanism by which cells can control the activity of nuclear proteins (21). We previously reported that exogenously expressed Glis2 predominantly localizes to the nucleus (1). The classical import mechanism involves binding of the importin-α/β heterodimer to a nuclear localization signal consisting of one or two basic regions, thereby targeting it for transport through nuclear pore complexes. Analysis of the Glis2 protein sequence for the presence of putative nuclear localization signal sequences did not identify any putative nuclear localization signal site. Examination of a series of N- and C-terminal deletions on the subcellular localization of Glis2 indicated that the region between residues 237 and 265 contained within the ZF3 is required for the optimal accumulation of Glis2 into the nucleus, with the 237–246 sequence being the most critical. The role of ZF3 in the nuclear accumulation was supported by data showing that the ZF3 is sufficient to promote nuclear retention of a Glis3(ZF3)-EGFP chimeric protein. Previous studies of other Krüppel-like ZF proteins have indicated the importance of several ZF domains in nuclear import. In Zic proteins, nuclear localization depends on dispersed positive residues in ZF2–3, whereas Glis3 nuclear localization requires ZF4 (12, 22, 23). Unlike Glis3, in which the structure of ZF4 is required for nuclear localization, the loss of the tetrahedral configuration of ZF3 had little influence on the nuclear localization of Glis2, suggesting that a specific aa sequence rather than the tetrahedral structure is important.

After entering the nucleus, Glis proteins function as transcriptional regulators by binding to specific DNA elements in the promoter of target genes (4). The recognition of specific DNA response elements is mediated by the ZFs of the Glis proteins. Recently, (G/C)**TGGGGGGT**(A/C) was identified as the consensus binding sequence (GlisBS) of Glis3 (12). To date, the Glis2 DNA binding sequence was not yet clearly defined. Since the ZFD of Glis2 shows 59% homology with that of Glis3 (4), we hypothesized that Glis2 might bind DNA elements similar to GlisBS. We demonstrated that Glis2 was able to bind effectively to both GlisBS and GBS; however, competition analysis suggested that Glis2 has a higher affinity for GlisBS than GBS. Moreover, none of the GlisBS mutants tested competed more effectively with ^{32}P-labeled GlisBS for Glis2 binding than GlisBS itself. To determine the role of the tetrahedral conformation of ZFs on Glis2 activity, we examined the effect of single Cys-to-Gly mutations that abolish the ZF tetrahedral configuration, on the binding of Glis2 to GlisBS. Our results showed that disruption of the tetrahedral configuration of the fourth or the fifth ZF destroyed the ability of Glis2 to interact with GlisBS (Fig. 2*A*). Moreover, disruption of tetrahedral configuration in any of the five ZFs abolished the ability of Glis2 to inhibit the GlisBS-dependent transactivation by Gli1 (Fig. 3*B*). These data are consistent with the conclusion that the conformation of each ZF of Glis2 is required for optimal GlisBS binding and GlisBS-dependent transactivation. Previously, crystal structure analysis of Gli1- and Gli3-DNA complexes have indicated that ZF2–5 bind in the major groove and wrap around the DNA and that ZF4 and ZF5 make extensive base contacts (24, 25). Although these studies suggested that ZF1 does not make contact with DNA, our data clearly show that the tetrahedral configuration of ZF1 is required for DNA binding. The ZF1 may play a role in maintaining the proper structure of the entire ZFD.

The ability of Glis2 to bind GlisBS is shared with other Glis proteins and members of the Gli and Zic families (4). It is likely that these proteins bind with different affinities to overlapping sets of GlisBS sequences and when expressed in the same cell, compete with each other for GlisBS binding thereby influencing gene expression. Although Glis2 binds GlisBS, it was unable to induce $(GlisBS)_6$-dependent transcription of the Luc reporter in several cell lines. Moreover, we showed that Glis2 inhibits the induction of $(GlisBS)_6$-dependent transactivation of the Luc reporter by Gli1 likely by

FIGURE 7. **GlisBS binding and transcriptional activity was greatly reduced in Glis2(S245D).** *A*, sequence comparison of ZF3 of human and mouse Glis2. Ser^{245} of human Glis2 (*boldface text*) has been previously identified as a target of EGF-induced phosphorylation (18). *B*, mutation of Ser^{245} to Asp resulted in loss of activation of the *Ins2* promoter by Glis2 in HeLa cells, whereas mutation to Ala had little effect. *C*, predominant nuclear localization of Glis2(S245D) and Glis2(S245A) suggests that the loss activity is not due to retention of the Glis2(S245D) mutant in the cytoplasm. *D*, EMSA showed that the binding of MBP-Glis2(S245D) mutant to radiolabeled GlisBS(−263) and GlisBS(−99) is greatly reduced, whereas binding of MBP-Glis2(S245A) is not changed significantly. *E*, comparison of the binding of MBP-Glis2(166–349) and its S245D and S245A mutants to radiolabeled GlisBS (consensus), or mutants M1, M2, M6, and M7 (see Fig. 1*D*) by EMSA.

competing for the same DNA binding site. This is supported by data showing that the deletion mutant Glis2(164–327) containing the ZFD only was also able to inhibit Gli1-induced transactivation (Fig. 3*A*), whereas Cys-to-Gly mutations in any of the ZFs abolished this ability (Fig. 3*B*). These data suggest that binding of the Glis2(ZFD) to the GlisBS is required for this inhibition and rule out the possibility that the inhibition involves squelching of co-activators by the N- or C-terminal domain of Glis2.

Previous studies reported that Glis2 is most highly expressed in the kidney (1, 7, 8, 26). Although the expression of Glis2 in the pancreas is significantly lower than the kidney, the pancreatic β-like cell lines, INS-1 832/13 and 832/3, expressed Glis2 levels comparable with that of kidney. We believe that the low expression in the pancreas suggests that Glis2 may be expressed in specific pancreatic cell types. We recently reported that Glis3, which is highly expressed in β-cells, activates the *Ins2* promoter through two GlisBS within its proximal promoter region (10). In this study, we show that as Glis3, Glis2 was also able to bind GlisBS(−99) and GlisBS(−263) of the *Ins2* promoter. We then investigated whether Glis2 was able to activate the *Ins2* promoter. Our data demonstrate that in contrast to $(\text{GlisBS})_6$-Luc, Glis2 effectively induced activation of the *Ins2* promoter. A Glis2 mutant containing a mutation in ZF4 that abolished GlisBS binding, was unable to activate the *Ins2* promoter suggesting that an intact ZFD and binding of Glis2 to GlisBS was required for the activation.

Several studies have shown that the binding of transcription complexes to their respective DNA response elements depends on the context of the promoter. Analysis of the activation of the mouse *Ins2* promoter by Glis2 revealed that the two GlisBS element are essential for maximal activation, thereby postulating a possible synergism between the two Glis2-GlisBS motifs. The cooperation between the two GlisBS binding elements may involve an interaction between the Glis2 transcriptional activation complexes bound at the two sites and additional cofactors that bridge these two complexes. The Glis2 protein complexes might be brought to a close proximity via a loop in the proximal promoter. Recent studies have reported that Glis2 can interact with β-catenin, p120 catenin, and CtBP1 (3, 16, 17). Although, Glis2 has been shown to be able to form a repressor complex through its interaction with the co-repressors CtBP1 and HDAC3, the physiological significance of such interactions has yet to be elucidated. Moreover, future studies are required to identify additional transcriptional mediators that interact with Glis2 and regulate the transcriptional activity of Glis2.

A global proteomic analysis of EGF-treated HeLa cells identified a Glis2 peptide phosphorylated at Ser^{245}, which resides in the loop of ZF3 (18). To study the possible impact of this phosphorylation on Glis2 signaling, we examined the effect of Ser^{245} phosphorylation on the nuclear localization and DNA binding ability of Glis2, as well as its ability to activate the *Ins2* promoter. Our data shows that the phosphomimetic mutation Glis2(S245D) had no significant influence on the nuclear localization of Glis2 but greatly diminished the binding of Glis2 to the GlisBS sites within the *Ins2* promoter. The binding of the Glis2(S245D) mutant to GlisBS(−263) was almost totally abolished, whereas that to GlisBS(−99) was greatly reduced suggesting a certain degree of selectivity to what extent the phosphorylation of Ser^{245} affects Glis2 binding. In addition, the transcriptional activity of the Glis2(S245D) was significantly reduced, correlating with its reduced ability to bind GlisBS. These data suggest that phosphorylation of Ser^{245} may be an important modulator of the transcriptional activity of Glis2.

Glis2 plays a critical role in the maintenance of normal renal functions. In mice and humans, loss of Glis2 function leads to the development of nephronophthisis, an end-stage renal disease characterized by renal atrophy and fibrosis (7, 8). Studies have indicated that induction of epithelial-mesenchymal transition in renal tubule cells is a contributing mechanism by which Glis2-null mice develop fibrosis. Interestingly, in a number of cell systems, including kidney epithelial cells, EGF has been shown to promote epithelial-mesenchymal transition (27–29). Therefore, phosphorylation of Glis2 by an EGF/TGFα-dependent signaling pathway and the subsequent loss of Glis2 activity may be part of the mechanism by which EGF promotes epithelial-mesenchymal transition. Such a mechanism would be consistent with the epithelial-mesenchymal transition-related fibrosis observed in Glis2-null mice. Therefore, post-translational modifications of Glis2, such as described at Ser^{245}, may play an important role in the development of renal fibrosis.

In summary, this report provides further insights into several features important in the Glis2 signaling pathway. Our study identifies a region in ZF3 between aa 237 and 246 that is critical for the nuclear retention of Glis2. Moreover, we demonstrate that Glis2 can bind GlisBS effectively and that the tetrahedral configuration of all five ZFs is essential for optimal binding to GlisBS. We demonstrated for the first time that Glis2 can activate transcription by binding specific GlisBS elements in the mouse *Ins2* promoter and that both Glis-binding sites are required for the maximal activation by Glis2. We further provide evidence that posttranslational modification of Glis2 can modulate the transcriptional activity of Glis2. This post-translational modification may play an important role in the regulation of gene expression by Glis2 and in the development of renal fibrosis.

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