Molecular Properties of Zic Proteins as Transcriptional Regulators and Their Relationship to GLI Proteins*

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Zic family genes encode zinc finger proteins, which play important roles in vertebrate development. The zinc finger domains are highly conserved between Zic proteins and show a notable homology to those of Gli family proteins. In this study, we investigated the functional properties of Zic proteins and their relationship to the GLI proteins. We first established an optimal binding sequence for Zic1, Zic2, and Zic3 proteins by electrophoretic mobility shift assay-based target selection and mutational analysis. The selected sequence was almost identical to the GLI binding sequence. However, the binding affinity was lower than that of GLI. Consistent results were obtained in reporter assays, in which transcriptional activation by Zic proteins was less dependent on the GLI binding sequence than GLI1. Moreover, Zic proteins activated a wide range of promoters irrespective of the presence of a GLI binding sequence. When Zic and GLI proteins were cotransfected into cultured cells, Zic proteins enhanced or suppressed sequence-dependent, GLI-mediated transactivation depending on cell type. Taken together, these results suggest that Zic proteins may act as transcriptional coactivators and that their function may be modulated by the GLI proteins and possibly by other cell type-specific cofactors.

The Zic genes encode zinc finger proteins that mediate diverse events in vertebrate development (1, 2). The genes are expressed in the developing or mature central nervous system, somites, and limb buds in a spatially restricted manner (3–8). A Zic1-deficient mouse shows cerebellar abnormalities and marked ataxia (9). Mutations in human ZIC2 or mouse Zic2 lead to a congenital brain anomaly known as holoprosencephaly (10–12). The human ZIC3 mutations result in a disturbance of the left-right body axis (13). In Xenopus, Zic genes play a role in the initial phase of neural and neural crest development (5, 7). The zinc finger domain was highly conserved not only in the vertebrate Zic family but also in Drosophila odd-paired, which regulates segmentation and midgut development (14, 15). All of these findings indicate that Zic genes exert an essential role in multiple aspects of development.

In addition to Zic orthologues in other species, the zinc finger domains of the Zic proteins, which consist of five tandemly repeated C2H2 motifs, show a notable homology to those of Gli family proteins (1). At present, Gli1, Gli2, and Gli3 have been reported in human, mouse, and frog (16–21) in addition to their nonvertebrate homologues Drosophila Cubitus interruptus (Ci; Ref. 22) and Caenorhabditis elegans Tra1 (23). Gli proteins are also known to regulate events in vertebrate development. In particular, it has been reported that Gli and Cubitus interruptus proteins function downstream of the Sonic hedgehog-Patched signaling pathway as transcriptional regulators (24–26). From here, in response to the Sonic hedgehog signal, activated Gli proteins bind directly to the enhancer element of a winged helix transcription factor, hepatocyte nuclear factor-3β, the expression of which is critical for floor plate development (27, 28).

The sequence-specific DNA binding of the GLI proteins has been studied in detail. A consensus nonamer target site, 5’-TGGTGGTGC-3’ (GLI-BS),1 has been identified for human GLI1 (29). A crystallographic study showed that GLI1 bound DNA through the last four fingers via extensive base contacts, which are highly conserved between GLI and Zic families (2, 30). Tra1 protein can also bind the GLI-BS possibly through the last three zinc fingers, because elimination of the first two fingers had little effect on its binding to DNA (23). Furthermore, the GLI3 zinc finger domain (GLI3-ZF) can also bind the GLI-BS (31). The target has been optimized to an extended 16-nucleotide sequence including the GLI-BS (GLI3-BS).

The fact that Zic proteins are nuclear proteins and have zinc finger domains highly similar to those of Gli proteins led us to infer that the Zic proteins might act as transcription factors in a manner similar to that of Gli proteins. However, little is known about the regulation of gene expression by the Zic proteins. The only finding in support of this to date is that the zinc finger domain of Zic1 (Zic1-ZF) can bind the GLI-BS (1). Therefore, the Zic proteins may regulate the expression of target genes by interacting with a GLI-BS-like sequence. Nevertheless, no methodological analysis of the DNA recognition sequence for Zic family proteins has been performed to date. We therefore undertook the task of empirically determining the Zic binding sequence and characterizing its binding properties. Furthermore, we examined transcriptional regulation by Zic proteins and tested whether the Zic proteins affected the GLI-

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1 The abbreviations used are: GLI-BS, GLI binding sequence; GLI3-BS, GLI3 binding sequence; Zics, Zic1, Zic2, and Zic3; ZF, zinc finger domain; PCR, polymerase chain reaction; GST, glutathione S-transferase; EMSA, electrophoretic mobility shift assay; TK, thymidine kinase; MLP, major late promoter; CMV, cytomegalovirus.
DNA interaction and GLI-mediated transcriptional regulation. This study should pave the way for a better understanding of the molecular mechanism of Zic-mediated transcriptional regulation.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of the Zinc Finger Domain of Zic1, Zic2, Zic3, and GLI3—cDNAs corresponding to the zinc finger domain of Zic1 (Zic1-ZF, amino acids 213–403), Zic2 (Zic2-ZF, amino acids 484–494), and Zic3 (Zic3-ZF, amino acids 495–505) were cloned into a pET-BOS vector (36). The expression constructs were driven by the lac promoter, and the proteins were overproduced in E. coli strain BL21 (DE3). The Zic1–Zic3 fragments were cleaved at the NdeI–SacI sites of the pET-BOS vector and purified by nickel-chelate affinity (Qiagen). The glutathione–S-transferase (GST) fusion proteins were purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) without denaturation. The histidine-tagged proteins were affinity-purified by nickel-chelate affinity (Qiagen), denatured by urea, and subsequently renatured in the purification process. The purified proteins were finally dialyzed against a solution consisting of 25 mM HEPES, pH 7.5, 50 mM MgCl2, 10 mM ZnSO4, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 12% glycerol and stored at −80 °C in small aliquots.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed in a binding buffer consisting of 25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM MgCl2, 10 μM ZnSO4, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 12% glycerol and stored at −80 °C in small aliquots.

Binding Site Selection and Mutational Analysis—Initially, DNA fragments consisting of random oligonucleotides flanked on each side by a known sequence were generated by PCR amplification of R62AB (Promega) into a pBLUEScript (Stratagene). Insertion–deletion sequences were aligned to conform to the GLI consensus sequence. Compromised sequences, which involve the nonrandom flank-sequences, are aligned to conform to the GLI consensus sequence.

The expression vector Flag-GLI3 was constructed by placing the Flag epitope sequence in frame to the N terminus of human GLI3. The expression vector Flag-Zic1 and Flag-Zic2 were constructed by placing the Flag epitope sequence in frame to the N terminus of human Zic1 and Zic2, respectively. The expression vector Flag-Zic3 was constructed by placing the Flag epitope sequence in frame to the N terminus of human GLI3. In addition to the full-length constructs, Zic1-FS and Zic3-FS were made, which included the entire cDNA fragments of mouse Zic1 and Zic3 with frameshift mutations to produce truncated proteins lacking all five zinc fingers and C-terminal regions (Zic1-FS, amino acids 1–153; Zic3-FS, amino acids 1–153).

**FIG. 1** Compilation of the Zic1 (30 clones), Zic2 (30 clones), and Zic3 (35 clones) binding sequences. DNA sequences that bound to the Zics were selected as described under “Experimental Procedures.” After five rounds of selection, shifted oligonucleotides were subcloned into pBluescript, and their sequences were determined. The cloned random sequences are aligned to conform to the GLI consensus sequence. Compromised sequences, which involve the nonrandom flank-sequence in the 9 positions, are removed. The table at the bottom summarizes the frequency with which each nucleotide is represented in the 9 positions. The bottom sequence in each table represents the compiled most favored sequence in each of the nine nucleotide positions.

**Plasmid Construction**—A luciferase reporter plasmid driven by a thyminid kinase (TK) promoter, TK-0GBS-Luc, was constructed by ligating a herpes simplex virus TK promoter derived from plasmid pRL-TK (Promega) into the HindIII and XhoI sites of the pGL2-Basic vector (Promega). Six tandem copies of the GLI-BS (32) were inserted upstream of the TK promoter in TK-0GBS-Luc, giving rise to TK-6GBS-Luc. A luciferase reporter plasmid driven by a major late promoter of polyoma virus, MLP-Luc, was constructed by removing the GAL4 binding sites from pG5Luc (Promega), and a luciferase reporter plasmid driven by an SV40 promoter, SV40-Luc (pGL2 promoter vector), was purchased from Promega. A luciferase reporter plasmid driven by a cytomegalovirus (CMV) promoter, CMV-Luc, was constructed by inserting a luciferase gene downstream of the CMV promoter region of the pCS2+ vector (33, 34), and a luciferase reporter plasmid driven by a Zic1 promoter, Zic1-Prom-Luc, was constructed as described previously (SmaI (148); Ref. 35). The internal standard, pRL-EF, was constructed by inserting the renilla luciferase gene of a pRL-TK vector (Promega) into a pE-FOS-BOS vector (36).

Expression vectors were constructed by inserting the cDNA fragments containing the entire open reading frame of mouse Zic1, Zic2, and Zic3, human GLI1, or GLI3 into the XbaI sites of pE-FOS-BOS (36). The expression vector Flag-Zic1 was constructed by placing the Flag epitope sequence in frame to the N terminus of human GLI1. In addition to the full-length constructs, Zic1-FS and Zic3-FS were made, which included the entire cDNA fragments of mouse Zic1 and Zic3 with frameshift mutations to produce truncated proteins lacking all five zinc fingers and C-terminal regions (Zic1-FS, amino acids 1–153; Zic3-FS, amino acids 1–153).
Transfection—293T and C3H10T1/2 cells were maintained in Dulbecco’s modified Eagle’s medium and basal medium Eagle’s medium, respectively, each containing 10% fetal bovine serum. At 60–70% confluence, the cells in a 24-well dish were transfected with Effectene (Qiagen) or LipofectAMINE Plus (Life Technologies, Inc.) transfection reagent according to the manufacturer’s instructions. Cells were harvested 24 h after transfection and processed for reporter assay and Western blot analysis.

Reporter Assay—For 293T cells, cotransfection experiments were performed with 90 ng each of the luciferase reporters and 100 ng each of the expression constructs, together with 5 ng of pRL-EF as an internal standard. C3H10T1/2 cells were cotransfected with 180 ng of the luciferase reporters, 200 ng of the expression constructs, and 10 ng of pRL-EF. When the expression vectors for Zic and GLI were cotransfected, the total amounts of expression vectors were kept constant by adding an empty vector (pEF-BOS). Luciferase activities were measured according to the manufacturer’s recommendation (Promega) using a Minilumat LB 9506 luminometer (Berthold). The firefly luciferase activity was normalized to the renilla luciferase activity obtained by cotransfection. The relative fold-activation was presented as the ratio of the normalized value to that from empty vector transfectant.

Western Blot Analysis—293T cells were transfected with 1 μg of expression vector and harvested 24 h after transfection. The cellular proteins were separated by 5–10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes (Millipore). The membranes were blocked in 5% skim milk overnight and incubated with anti-Zic monoclonal antibody (ZC26; Ref. 3), anti-GLI1 polyclonal antibody (N16; Santa Cruz Biotechnology), anti-GLI3 polyclonal antibody (C20; Santa Cruz Biotechnology), and anti-Flag monoclonal antibody (M2; Sigma). The bound antibodies were detected using horseradish peroxidase-conjugated anti-mouse IgG (Zic and Flag) and anti-goat IgG (GLI1 and GLI3) and ECL reagents (Amersham Pharmacia Biotech).

Results

Binding Site Selection of Zic Family Proteins—To determine the DNA sequence to which Zic proteins bind, we synthesized oligonucleotides containing 30 contiguous random nucleotides flanked on either side by a known sequence to facilitate PCR amplification and subsequent cloning of the selected DNA sequence. The radiolabeled random oligonucleotides were incubated with purified GST fusion protein containing the zinc finger domain of three mouse Zic proteins (Zic1-ZF, Zic2-ZF, and Zic3-ZF), DNA-protein complexes were separated on native polyacrylamide gels, and the bands shifted by the proteins were excised. After elution, the DNA was amplified by PCR and subjected to four additional rounds of DNA-protein binding, elution, and PCR amplification to enrich the Zic binding sequences. After a total of five rounds of selection, the bound
oligonucleotides were cloned and sequenced (Fig. 1). The core sequence most favored by Zic1-ZF, Zic2-ZF, and Zic3-ZF was nearly identical to the 9-nucleotide GLI consensus sequence (GLI-BS), i.e. 5'-TGGGTGTC-3'. The relative frequency of the appearance of each nucleotide in the 9 positions indicated that all 5 guanosine residues, 2 thymidine residues at the fifth and sixth positions, and a cytidine residue at the 3'-most position were preferred in common, whereas a thymidine residue at the 5'-most position was not.

However, there seemed to be no absolute requirement for any specific nucleotide position. So we asked whether a different target sequence might exist in strongly bound sequences. To test this, we selected target sequences that gave stronger shifted bands than GLI3-BS. Among 13 sequences with high binding affinity to Zic1-ZF, there was no conserved sequence other than GLI-BS (data not shown). This result indicated that the GLI-BS-like sequence was uniquely preferred by the Zic Family Proteins—although all of the selected sequences could be bound by the Zics-ZF, it was unclear whether the target nucleotide sequence, there was an apparent difference in binding affinity. To determine the relative binding affinity of the Zics-ZF and GLI3-ZF, both proteins were incubated simultaneously with the GLI3-BS probe, and the resultant complexes were analyzed by EMSA. To discriminate the two kinds of fusion proteins, different tags were used. The mixture consisting of GST-GLI3-ZF (10 nM) and His-Zic2-ZF (0–2000 nM; Fig. 3A) or His-GLI3-ZF (20 nM) and GST-Zic2-ZF (0–500 nM; Fig. 3B) was reacted with the GLI3-BS probe and analyzed by EMSA. Even though an excess concentration of Zic2 protein was used, the intensity of the band shifted by the GLI3 protein (the complex formed between GLI3-ZF and GLI3-BS) was not markedly changed in both experiments. In contrast, the bands shifted by the Zic proteins were almost completely removed by a lower concentration of GLI3 protein (Fig. 3C). These findings confirmed that, although the Zic family proteins specifically bound the GLI3-BS, the binding affinity was much lower than that of GLI3-ZF, and Zics-ZF do not compete with GLI3-ZF for the target sequence.

To more accurately determine the binding affinities of the Zics-ZF and GLI3-ZF, we performed EMSA with a fixed concentration of the GLI3-BS probe (1 nM) and increasing concentrations (1 nM–1 μM) of the fusion proteins (Fig. 4). Further-
more, we performed EMSA with a fixed concentration (20 nM) of the fusion proteins and increasing concentrations (0.75-200 nM) of the probe (Fig. 5). Then the intensities of the bands corresponding to the protein-DNA complexes were measured by densitometry, and the binding curves were plotted (Fig. 6). In these assays, the GLI3-ZF showed considerably higher binding affinity to the GLI3-BS ($K_d$, $8.5 \times 10^{-9}$ M; $B_{\text{max}}$, $9.3 \times 10^{-9}$ M) than the Zics-ZF ($K_d$ of Zic1-ZF, $5.2 \times 10^{-8}$ M; Zic2-ZF, $4.8 \times 10^{-8}$ M; and Zic3-ZF, $7.1 \times 10^{-8}$ M; $B_{\text{max}}$ of Zic1-ZF, $7.9 \times 10^{-9}$ M; Zic2-ZF, $5.4 \times 10^{-9}$ M; and Zic3-ZF, $7.7 \times 10^{-9}$ M).

To test whether some of the DNA-protein complexes might dissociate during electrophoresis in the gel, we also electrophoresed the DNA-protein complexes for various times. However, there was no significant alteration in the dissociation rate during gel electrophoresis (data not shown), indicating that the different $K_d$ values reflect the differential affinities in the binding mixture.

**Different Transcriptional Activities between Zic and GLI Proteins Mediated through the Selected Sequence**—We next asked whether the Zic proteins were capable of activating or repressing transcription mediated by the selected binding sequence (Fig. 7). For this purpose, we prepared Zic, GLI1, and GLI3 expression vectors under the control of an EF1α promoter. We used the firefly luciferase genes as reporters, controlled by the major late promoter of the adenovirus (MLP-Luc), which consists of minimal promoter elements (a TATA box and a transcription initiation site), the Zic1 promoter (Zic1-prom-Luc), the SV40 promoter (SV40-Luc), and the CMV promoter (CMV-Luc). Luciferase activity was measured after cotransfection into 293T cells. The firefly luciferase activity was normalized to the activity of the cotransfected EF1α-driven renilla luciferase, because the Zic and GLI expression vectors failed to transactivate the EF1α promoter (data not shown). All Zic proteins activated the reporter genes driven by the MLP, the Zic1 promoter, and the SV40 promoter but failed to activate that driven by the CMV promoter (Fig. 7A). On the other hand, neither GLI1 nor GLI3 proteins activated any of the promoters.

To examine whether the GLI-BS affected the transcriptional activation by Zic proteins, we used the firefly luciferase genes, controlled by the TK promoter with or without six copies of the GLI-BS (TK-6GBS-Luc and TK-0GBS-Luc). Zics always activated transcription driven by the TK promoter irrespective of the presence of the GLI-BS in both 293T and C3H10T1/2 cells (Fig. 7, B and C). The presence of GLI-BS enhanced the reporter gene expression to 1.5-2.0-fold in C3H10T1/2 cells, whereas in 293T cells, Zic1 or Zic2 enhanced expression to a lesser extent. In contrast, GLI1 markedly enhanced reporter expression, dependent on the presence of GLI-BS in both cell lines (14-fold (293T) or 8-fold (C3H10T1/2) elevation in comparison with TK-0GBS-Luc). GLI3 completely failed to activate the reporter gene expression mediated by the GLI-BS in either cell type.
The fact that Zic proteins activated reporter gene expression independent of the GLI-BS prompted us to ask whether the Zic gene or mRNA may indirectly affect the transcriptional activation. To address this possibility, we transfected Zic1-FS and Zic3-FS, which contained the entire cDNA sequence of Zic1 and Zic3, respectively, but produced truncated proteins without all five zinc fingers. However, these constructs were not capable of activating any of the reporter genes tested, demonstrating that Zic proteins themselves could activate transcription (Fig. 7).

These findings show that the Zics have transcriptional activation capacity, but this activity is less dependent on the GLI-BS than is GLI1 activity. The different binding affinities to the target sequence can account for the differential dependence on GLI-BS between the Zic proteins and GLI1.

The Zic Family Proteins Cooperate or Interfere with the GLI in Regulating Transcription—We investigated whether Zic proteins could affect the GLI-BS-dependent transactivation by GLI1. Different relative amounts of the Zic and GLI1 expression vectors were transfected into C3H10T1/2 or 293T cells to titrate this interaction (Fig. 8). When Zics were coexpressed with GLI1 in C3H10T1/2 cells, the GLI-BS-TK-driven luciferase expression was significantly increased beyond that predicted by the summed luciferase activities induced separately by the Zics and GLI1 (Fig. 8A). Increasing amounts of cotransfected Zics enhanced reporter gene expression in a Zic dose-dependent manner. For example, cotransfection with 100 ng of Zic2 increased the GLI1-mediated transactivation by 3-fold. In contrast, when Zics and GLI1 were cotransfected into 293T cells, there was a reduction in reporter gene expression to less than that of Zic or GLI1 single transfectants, possibly because of the interaction between Zics and GLI1 (Fig. 8B).

We also carried out Zic and GLI3 cotransfection, although GLI3 failed to activate or repress the TK-6GBS-Luc reporter gene (Fig. 9). Unexpectedly, even in this case, we obtained results essentially similar to those of Zic-GLI1 cotransfection experiments. When cotransfection was performed in C3H10T1/2 cells, the luciferase activities were increased. However, the increased activities were nearly equal to the addition of the luciferase activities induced separately by the Zics and GLI3 (Fig. 9A). On the other hand, in 293T cells, luciferase activities were lower in Zic-GLI3 cotransfectants than in Zic single transfectants (Fig. 9B). At a certain ratio of expression vectors (Zics:GLI3 = 10:50 ng), the activities were even lower than that from transfection of the empty vector. As a consequence, it became clear that the synergistic activation or repression of reporter gene expression is dependent on cell type for both GLI1 and GLI3. Comparable protein expression levels were confirmed by Western blot analysis for all of the transfections (Fig. 10), indicating that the observed transcriptional synergism and interference were not attributable to different efficiencies of expression of the transfected genes.

DISCUSSION

Zic Proteins as General Transcription Activators—In the present study, we were able to establish a consensus binding sequence for Zics by EMSA-based target selection and mutational analysis. The Zic binding sequence was essentially identical to the GLI-BS, 5'-TGGTGGTGGTC-3', and had a minimum consensus sequence of 5'-GGGTGGTC-3'. The binding affinities for this sequence were very similar among the three Zic proteins examined. However, the Zics-ZF bound the GLI-BS much more weakly than GLI3-ZF, as shown by competition experiments and the calculated binding constant. The Kd values of Zics were much higher than those of other transcription factors that function in a sequence-specific manner (37, 38). Therefore, it is unlikely that Zic proteins compete with GLI for the GLI-BS.

The binding properties were consistent with the results of the reporter assay, in which the dependence of Zic proteins on the GLI-BS for transcriptional activation was much less than that of GLI1. Interestingly, Zic activated transcription even in the absence of the GLI-BS via various promoters (TK promoter, adenovirus major late promoter, SV40 early promoter, and Zic1 promoter). On the basis of these facts, rather than being the transcription factors that regulate transcription by direct binding to DNA, Zic family proteins may function as transcriptional coactivators, which potentiate the activity of other transcription regulatory factors. It is possible that Zics interact with the transcription machinery or other factors that regulate tran-
scriptional efficiency. An alternative possibility is that Zic proteins might function in the post-transcriptional gene expression processes. Because Zic proteins are localized in the cell nuclei (1, 3), they could be involved in RNA processing or transport from the nucleus to the cytoplasm.

Although the Zic-GLI proteins activated a variety of promoters, they had little activity on the EF1α and CMV promoters. This may be related to promoter strength, because of the six promoters we tested, these two promoters had the strongest activities (~10–1000-fold stronger than other promoters tested). The transcriptional machinery, which is required for activation by Zic proteins, might have already used by the EF1α or CMV promoters alone. Further study is needed to elucidate the domain required for the transcriptional activation in the Zic proteins and to identify factors that interact with that domain to understand the regulatory mechanism of gene expression by Zic proteins.

Context-dependent Regulation of GLI Function by Zic Proteins

We also examined the relationship between Zic and GLI proteins. In C3H10T1/2 cells, Zic-GLI1 or Zic-GLI3 coactivated reporter gene expression, whereas in 293T cells, coexistence of the Zic and GLI proteins had a reverse effect. These results suggest a significant regulatory relationship between Zic and GLI proteins; however, the nature of this interaction remains unclear.

The interaction between Zic and GLI proteins may be en-

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**Fig. 8.** Coactivation or suppression of reporter gene expression by Zics and GLI1 cotransfection depending on cell context. C3H10T1/2 cells (A) or 293T cells (B) were cotransfected with the GLI1 expression vector and increasing amounts of Zic expression vectors along with a reporter gene, TK-6GBS-Luc. In C3H10T1/2 cells, 100 ng of GLI1 and 20–100 ng of Zic expression vectors were cotransfected (A), whereas 50 ng of GLI1 and 10–50 ng of Zic were cotransfected in 293T cells (B). Total amounts of expression vectors were kept constant by adding the pEF-BOS vector. Shown at the bottom are different combinations of expression vectors. Each value for luciferase activity was normalized to the activity of an internal control (renilla luciferase). Normalized values for luciferase activities (arbitrary units) in pEF-BOS-transfected cells were 34 (C3H10T1/2) and 57 (293T). Normalized luciferase activities from duplicate samples are presented relative to the empty vector pEF-BOS. Error bars represent S.D.

**Fig. 9.** Suppression of reporter gene expression by Zic and GLI3 cotransfection in 293T cells. C3H10T1/2 cells (A) or 293T cells (B) were cotransfected with the GLI3 expression vector and increasing amounts of Zic expression vectors along with a reporter gene, TK-6GBS-Luc. In C3H10T1/2 cells, 100 ng of GLI13 and 20 and 100 ng of Zic expression vectors were cotransfected (A), whereas 50 ng of GLI3 and 10 and 50 ng of Zic were cotransfected in 293T cells (B). Total amounts of expression vectors were kept constant by adding the pEF-BOS vector. Shown at the bottom are different combinations of expression vectors. Each value for luciferase activity was normalized to the activity of an internal control (renilla luciferase). Normalized values for luciferase activities (arbitrary units) in pEF-BOS-transfected cells were 62 (C3H10T1/2) and 54 (293T). Normalized luciferase activities from duplicate samples are presented relative to the empty vector pEF-BOS. Error bars represent S.D.
In conclusion, Zic1, Zic2, and Zic3-ZF specifically recognized and bound the GLI-BS but with a much lower binding affinity than that of the GLI3-ZF. Zic proteins activated a wide range of promoters. These results suggest that Zic proteins may function as transcriptional coactivators or as factors generally involved in the gene expression process. How can such general factors regulate specific developmental processes, including the patterning of forebrain, cerebellum, axial skeleton, vasculature, and visceral organs? A clue to solving this problem may be the relationship with Gli family proteins as shown in this study. To clarify the regulatory networks under a broad range of developmental process, the relationships between Zic proteins and other molecules in the hedgehog signaling pathway and transforming growth factor β superfamily, which are closely related to each other, should also be examined in both in vitro and in vivo studies.

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FIG. 10. Western blot analysis of Zic and GLI proteins in 293T cells. A, 293T cells were transfected with Zic or GLI1 expression vectors or both. A portion of cell lysates was subjected to Western blot analysis with anti-Zic and anti-GLI1 antibodies. The positions of molecular size markers are shown on the left. B, 293T cells were transfected with Zic or Flag-tagged GLI3 expression vectors or both. The Western blot analysis was performed with anti-Zic and anti-Flag antibodies.
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