**Physical and Functional Interactions between Zic and Gli Proteins**

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Zic and Gli family proteins are transcription factors that share similar zinc finger domains. Recent studies indicate that Zic and Gli collaborate in neural and skeletal development. We provide evidence that the Zic and Gli proteins physically and functionally interact through their zinc finger domains. Moreover, Gli proteins were translocated to cell nuclei by coexpressed Zic proteins, and both proteins regulated each other's transcriptional activity. Our result suggests that the physical interaction between Zic and Gli is the molecular basis of their antagonistic or synergistic features in developmental contexts and that Zic proteins are potential modulators of the hedgehog-mediated signalizing pathway.

Zic and Gli transcription factors share a highly conserved zinc finger domain and have critical roles in multiple developmental processes. In human, mutations in ZIC2, ZIC3, and GLI3 genes result in various developmental abnormalities. ZIC2 results in malformation of the forebrain (holoprosencephaly), ZIC3 in a disturbance of the left to right body axis (heterotaxy), and GLI3 in complex anomalies of the brain and digits (cephalopolysyndactyly syndrome) (1–3). Studies in mice show that full-length mouse Zic1–(1–447) and mouse Zic2 and Zic3 (19, 21). The deletion series of Zic1 and GLI3 were also cloned. These deletions were as follows: Zic1-(1–384), (1–330), (1–298), and (1–447) with deletion of residues 299–329, Zic1-(1–447) with deletion of 299–359, and Zic1-(1–447) with deletion of 299–383; GLI3-(1–547), (1–829), and (1–1596) with deletion of 548–624. Flag-tagged full-length human GLI1 (22) and human GLI3 (3) constructs were as described by Dai et al. (23). For Flag-tagged mouse Gli2, a Flag epitope tag was introduced at the amino terminus of mouse Gli2 (24) by PCR, and Flag-tagged Gli2 was subcloned into pCAGGS (25). An Shh expression construct was made by inserting a chick Shh cDNA clone containing the entire open reading frame into pEF-BOS (26). The CBP-HA expression plasmid was kindly provided by Dr. S. Ishii. To clone the GST-GLI3 and GST-Zic1 fusion proteins, fragments were PCR-amplified using primers that introduced BamHI and SalI sites, the sequences verified, and the fragments cloned into the BamHI-SalI sites of the pGEX-4T3 vector (Amersham Pharmacia Biotech). The regions cloned in this manner were GLI3-(1–461), (400–(400–722), (705–1140), (1100–1350), (1296–1596), (1350–400–639), (1350–400–547), and (1350–547–639) as well as Zic1-(300–384). GST fusion proteins were affinity-purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). For bacterial expression of Flag-tagged GLI3-(461–639), the EcoRI fragment encoding GLI3-(461–639) was inserted into the EcoRI site of the pFLAG-ATS vector (Sigma). Bacterially expressed Flag-tagged GLI3-(461–639) protein was affinity-purified by anti-Flag M2 affinity gel (Sigma).

The following proteins were affinity-purified from bacterial expression cultures using anti-Flag M2 affinity gel (Sigma):

- GLI3-(461–639)
- Zic1-(300–384)
- GST fusion proteins were affinity-purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Bound material was detected by immunoblotting with anti-

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1 The abbreviations used are: HA, hemagglutinin; PCR, polymerase chain reaction; Shh, Sonic hedgehog; GST, glutathione S-transferase; TK, thymidine kinase; luc, luciferase; CBP, CREB (cAMP-response element-binding protein)-binding protein.

2 J. Aruga, unpublished observations.
Subcellular Localization Studies—NIH3T3 cells and 293T cells were transiently transfected with appropriate expression constructs. 24 h after transfection, cells were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 20 min at room temperature and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline for 2 min. The cells were incubated in blocking buffer (1% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline) for 1 h at room temperature and then incubated with the anti-HA antibody and the anti-Flag antibody. The bound antibodies were detected by Alexa 488-conjugated anti-rabbit IgG or Alexa 568-conjugated anti-mouse IgG antibodies (Molecular Probes, Inc.).

Luciferase Assay—Cells were cultured in 24-well dishes. NIH3T3 cells were transfected using Superfect with appropriate expression constructs. At 30 h after transfection, luciferase activities of the cells were measured as described (20). MNS70 cells (27, 28) were transfected using Fugene 6 (Roche Molecular Biochemicals) and assayed 48 h after transfection.

RESULTS AND DISCUSSION

Physical Interaction between Zic and Gli Proteins—To investigate the physical interaction between Zic and Gli, 293T cells were cotransfected with HA-tagged mouse Zic and Flag-tagged human or mouse Gli, and the resultant cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Flag antibody. Bands corresponding to GLI1, GLI2, and GLI3 proteins (Fig. 1A) were detected (Fig. 1A).

To identify the Zic1-binding domain in GLI3, we prepared glutathione S-transferase (GST) fusion proteins containing various parts of the GLI3 proteins (Fig. 1C). The fusion proteins were used for GST pull-down experiments with the lysates from 293T cells transfected with Zic1, and the precipitates were immunoblotted to detect Zic1 protein. The minimal region required for binding to Zic1 is amino acids 547–639 of GLI3 (Fig. 1, D and E), a region corresponding to the third through fifth zinc finger motifs (ZF3–5). It is the most conserved region between Zic and Gli proteins (19). Subsequently, the Gli-binding domain in Zic1 was determined by the GST pull-down assay using a series of deleted Zic1 mutants and GST-GLI3-(547–639) (ZF3–5) (Fig. 1F). The results revealed that a region (residues 300–384) corresponding to Zic1 ZF3–5 was involved in the Zic-Gli interaction (Fig. 1G). All three C2H2 zinc finger units in the Zic1 ZF3–5 domain were cooperatively involved in the interaction (Fig. 1G). GST-GLI3-(547–639) (ZF3–5) bound Zic1, Zic2, and Zic3 proteins (Fig. 1E), and GST-Zic1-(300–384) (ZF3–5) bound the GLI1, GLI2, and GLI3 proteins (Fig. 1H), suggesting that the association between the Zic and Gli proteins is conserved in other Zic-Gli combinations. Binding was also observed between purified GST-Zic1-(300–384) and bacterially expressed, purified Flag-GLI3-(461–639) (Fig. 1B), suggesting a direct physical interaction between Zic and Gli.

Translocation of Gli Proteins by Zic Proteins—Next we examined the subcellular localization of Zic and GLI proteins. Transfected HA-tagged Zic1, Zic2, and Zic3 were located in cell nuclei in all of the cell lines tested (NIH3T3, 293T, C3H10T1/2, COS7) (Fig. 2, A, B, I, and J, data not shown), whereas the subcellular localization of Flag-tagged GLI proteins has been found to vary in different contexts (8, 29). In NIH3T3 and 293T cells, both GLI1 and GLI3 proteins were located predominantly in the cytoplasm (Fig. 2, C, D, H, K, L, and P). Coexpression of Zic1 resulted in GLI1 and GLI3 proteins being translocated to the nucleus (Fig. 2, E, F, J, and P), suggesting an increase in the GLI levels or an increase in the nuclear localization of GLI proteins.

To investigate the physical interaction between Zic and Gli, 293T cells were cotransfected with HA-tagged mouse Zic and Flag-tagged human or mouse Gli, and the resultant cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Flag antibody. Bands corresponding to GLI1, Gli2, and GLI3 were detected (Fig. 1, top panel). Cell lysates were immunoprecipitated and immunoblotted using the antibodies indicated. Middle and bottom panels, expression analysis of the Flag- and HA-tagged proteins, respectively, by immunoblotting of the cell extracts. B, direct binding assay. Bacterially expressed Flag-tagged GLI3-(461–639) was incubated with GST-Zic1-(300–384), and a GST pull-down assay was performed. Bound material was detected by immunoblotting. C–H, mapping of the binding domains. C, mapping of the Zic-binding region in GLI3 protein. The various regions of the GLI3 protein indicated were prepared as GST fusion proteins. The numbers refer to amino acids. D and E, GST pull-down assays. Extracts from 293T cells expressing Flag-Zic1 (D) or Flag-Zic proteins (E) were incubated with the recombinant GST fusion proteins indicated. The elutions from glutathione-Sepharose beads were analyzed by immunoblotting. F, mapping of the GLI-binding region in Zic1 proteins. Expression vectors for Zic1 deletion proteins tagged with Flag epitope were constructed. The numbers refer to amino acids. G and H, GST pull-down assays. Cell lysates containing the indicated Flag-Zic1 deletion proteins (G) or Flag-Gli proteins (H) were subjected to GST pull-down assay using the GST fusion proteins indicated.

Fig. 1. Interaction of Zic proteins with Gli proteins. A, Flag-tagged GLI1, GLI2, and GLI3 were cotransfected with an empty HA-tagged expression vector, HA-Zic1 and HA-Zic2, in 293T cells (top). Cell lysates were immunoprecipitated and immunoblotted using the antibodies indicated. Middle and bottom panels, expression analysis of the Flag- and HA-tagged proteins, respectively, by immunoblotting of the cell extracts. B, direct binding assay. Bacterially expressed Flag-tagged GLI3-(461–639) was incubated with GST-Zic1-(300–384), and a GST pull-down assay was performed. Bound material was detected by immunoblotting. C–H, mapping of the binding domains. C, mapping of the Zic-binding region in GLI3 protein. The various regions of the GLI3 protein indicated were prepared as GST fusion proteins. The numbers refer to amino acids. D and E, GST pull-down assays. Extracts from 293T cells expressing Flag-Zic1 (D) or Flag-Zic proteins (E) were incubated with the recombinant GST fusion proteins indicated. The elutions from glutathione-Sepharose beads were analyzed by immunoblotting. F, mapping of the GLI-binding region in Zic1 proteins. Expression vectors for Zic1 deletion proteins tagged with Flag epitope were constructed. The numbers refer to amino acids. G and H, GST pull-down assays. Cell lysates containing the indicated Flag-Zic1 deletion proteins (G) or Flag-Gli proteins (H) were subjected to GST pull-down assay using the GST fusion proteins indicated.
the nucleus in varying levels (Fig. 2, E and M). This tendency was clearest in the case of GLI3 in NIH3T3 cells and GLI1 in 293T cells (Fig. 2, H and P). A mutant GLI3 protein lacking residues 548–624 (GLI3ZF3–5) was not translocated by the coexpressed Zic1 (Fig. 2H). Enhancement of the nuclear translocation of GLI proteins was also observed with any combinations of Zic-/Gli proteins (data not shown) and in other cell lines (C3H10T1/2, HeLa, and COS7, data not shown).

Zic and Gli Proteins Regulate Each Other’s Transcriptional Activity through the Zinc Finger Domains—Zic proteins activate transcription from the thymidine kinase (TK) promoter in a process that is partially dependent on GLI-Bs (20, Fig. 3). However, Gli proteins specifically require GLI-BS for transcriptional regulation and have essentially no effect on the promoter in the absence of GLI-Bs (Ref. 20; Fig. 3). To clarify the significance of the Zic-Gli association in transcriptional regulation, we performed Zic-Gli cotransfection experiments using TK promoter-luciferase reporter constructs with and without GLI-BS (pGBS-TK-luc and pTK-luc, respectively) in NIH3T3 cells. When GLI1 and Zic1 (Fig. 3A) or GLI1 and Zic2 (Fig. 3D) were cotransfected, reporter gene expression was synergistically activated both in the presence and absence of GLI-BS. This synergistic activation was also observed in a Shh-responsive cell line (MNS70) (Fig. 3C). The level of synergistic increase was not influenced by the presence of an Shh signal (Fig. 3C), suggesting that the Shh signal does not regulate the Zic-Gli interaction. By contrast, full-length GLI3 enhanced reporter gene expression when coexpressed with a general transcription cofactor, CBP (Ref. 23; Fig. 3D). When Zic1 was coexpressed with GLI3 and CBP, a marked increase was observed in comparison with GLI3 and CBP coexpression (Fig. 3D). Conversely, when Zic1 was coexpressed with a carboxyl-terminally truncated GLI3 protein (Ref. 30; GLI3ΔZF3–5, 350 ng), Flag-carboxyl-terminally truncated GLI3 (GLI3ΔZF3–5, 350 ng), Flag-carboxyl-terminally truncated GLI3 lacking ZF3–5 (GLI3 1–547, 350 ng), and CBP-HA (100 ng) in the indicated combinations. The numbers refer to amino acids. The total amount of DNA was adjusted to 500 ng with control vector pCMVtag2. D, NIH3T3 cells were transfected with expression vectors for Flag-Zic1 (50 ng), Flag-GLI1 (50 ng), and for Flag-GLI1 (150 ng) and Flag-Zic2 (100 ng) together (B). C, luciferase activity in MNS70 cells transfected with expression vectors for Shh (100 ng), Flag-GLI1 (50 ng), Flag-Zic1 (50 ng), and Flag-Zic2 (50 ng) in the indicated combinations. A total of 200 ng of reporter plasmid (pGBS-TK-luc, black; pTK-luc, white) was included in each transfection experiment. The total amount of DNA was adjusted to 400 ng with control vector pCMVtag2. A–G, A–G and I–O, subcellular localization of Zic and Gli in NIH3T3 (A–G) and 293T cells (I–O). The cells were transfected with HA-Zic1 alone (A, B, I, and J), Flag-GLI3 alone (C and D), Flag-GLI1 alone (K and L), HA-Zic1/Flag-GLI3 (E–G), and HA-Zic1/Flag-GLI1 (M–O). Nuclei were counter-stained with DAPI (4’,6-diamidino-2-phenylindole) (blue) (B, D, G, J, L, and O). Note that GLI3 and GLI1 translocated to the nucleus when coexpressed with Zic1 (E and M). H and P, summaries of the subcellular localization of GLI1 and GLI3 proteins in NIH3T3 (H) and 293T cells (P) in the presence and absence of coexpressed Zic1. Cells were classified depending on the expression of the GLI protein in the nucleus (N), cytoplasm (C), or both the nucleus and cytoplasm (N+C). The numbers of cells counted are indicated by n. The percentages are shown.
The Zic-Gli associations may be involved in transcriptional regulation as well as in synergistic or antagonistic effects in different developmental contexts (13, 17). Synergistic activation can be explained in part by enhancement of the nuclear localization of Gli proteins by Zic proteins. In addition, the physical interaction may contribute to the recruitment of both proteins onto the GLI-BS or core promoter, resulting in elevation of the local concentrations of both proteins. On the other hand, if we assume that the protein-to-protein association interferes with the ability of Gli or Zic to regulate transcription, then the presence of Zic would reduce the effect of Gli and vice versa. This could be the molecular basis of the Zic-Gli counter-activity. The presumptive molecular machinery of synergistic activation or inactivation by the Zic-Gli interaction may vary according to cell type and the developmental context.

Gli/Ci proteins function downstream of the hedgehog (hh) signaling pathway as both transcriptional activators and repressors (30–33). On the other hand, the signal transduction pathway mediated by Zic proteins has not been well understood. Our results suggest that Zic proteins can interact with every Gli protein including the repressive form. We therefore speculate that Zic proteins are a potential modulator of the hh signaling pathway in various situations in animal development. Interestingly, expression of the Zic family grossly over-laps that of GLI3 in neural tube, somites, and limb buds (17, 34, 35). Further clarification of the role of Zic proteins in the hh signaling pathway should help clarify the molecular mechanisms of body pattern control.

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