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Biochemical and Biophysical Research Communications 324 (2004) 302–307
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Molecular properties of Zic4 and Zic5 proteins: functional diversity within Zic family

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Received 20 August 2004
Available online 25 September 2004

Abstract

The Zic-family proteins control various developmental processes. Previous studies have shown that Zic1, Zic2, and Zic3 can act as transcriptional regulators, and that their functions are repressed by I-mfa, which has been identified as a repressor for basic helix–loop–helix-type transcriptional factors. Here, we investigated the molecular properties of the Zic4 and Zic5 proteins. Zic4/Zic5 showed DNA-binding activity to the Gli-binding sequence, similar to Zic1/Zic2/Zic3 proteins. However, Zic4/Zic5 did not exhibit any significant transcriptional activation ability nor they bind to I-mfa differently from Zic1/Zic2/Zic3. The nuclear localization of Zic4/Zic5 was not affected by the presence of the I-mfa protein, whereas the Zic1/Zic2/Zic3 proteins were translocated to the cytoplasmic compartment in the presence of I-mfa. The difference may be attributable to the dissimilarity of the N-terminal region between the Zic1/Zic2/Zic3 and Zic4/Zic5 proteins, since the binding of the Zic1/Zic2/Zic3 proteins to I-mfa occurs through their N-terminal regions.

Keywords: Zic; Transcription; Zinc-finger protein; Nuclear localization; I-mfa; ZOC domain

Recent studies of the vertebrate Zic proteins have revealed they play essential roles in animal development. They control neuroectodermal differentiation, neural tube formation, cerebellar patterning, and axonal projection during the course of neural development (reviewed in [1]). They also play a critical role in the neural crest development [2,3], skeletal patterning [4,5], and left–right axis determination [6,7]. The Zic proteins are also considered to play a role in the regulation of gene expression, because the expression of a number of genes has been shown to be affected in gain-of-function and loss-of-function analyses of the Zic genes [1].

The Zic proteins appear to affect gene expression via transcriptional regulation. We previously reported the molecular properties of Zic1, Zic2, and Zic3, especially in terms of their DNA binding selectivity and transcriptional activation ability [8]. The Zic1/Zic2/Zic3 proteins are normally localized in the cell nuclei [8,9]. The zinc-finger domain can bind DNA sequences that are also bound by the Gli zinc-finger proteins (Gli-binding sequence, GBS). Mutation analysis showed that the Zic zinc-finger domains bind DNA in a sequence-specific manner. The results of a reporter gene assay revealed that the genes possess transcriptional activation ability, which was modulated by the Gli proteins, depending on the cell type [8,9].

To identify the essential functions of the Zic proteins, we analyzed the proteins interacting with Zic2 by yeast two-hybrid screening, and identified the Zic-binding protein, I-mfa, which has been identified as an inhibitor of the basic helix–loop–helix type of transcription factors [10]. I-mfa can bind Zic1, Zic2, and Zic3, based on the observations in an immunoprecipitation assay, and Zic2 N-terminal region to the zinc-finger domain

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.052

and the cysteine-rich C-terminal region of the I-mfa proteins have been shown to be essential for the physical interaction between the two proteins [10]. Based on their expression pattern and the phenotypes of Zic-mutant mice [4,5] and I-mfa-mutant mice [11], the Zic–I-mfa interaction has been predicted to have a role in skeletal development and patterning.

Previous studies have also characterized additional members of the Zic-family proteins, Zic4 [12] and Zic5 [3,13]. Zic4 and Zic5 homologues have been identified in mammalian and amphibian tissues, but not in invertebrates. Zic4 and Zic5 proteins have a highly conserved C_2H_2-type zinc-finger DNA-binding domain shared with the other Zic-family proteins. However, the deduced amino acid sequences of the Zic4 and Zic5 proteins, outside of their zinc-finger domain, are rather divergent from those of the Zic1/Zic2/Zic3 proteins. A phylogenetic tree analysis revealed Zic5 among the Zic-family proteins is the most closely related to the invertebrate homologue, Odd-paired [13,14]. Knockout analysis indicated that Zic5 plays a role in neural tube closure and in the generation of neural crest derivatives [3]. It is also known that Zic5 in the *Xenopus* species stimulates neural crest development [15]. Although there is accumulating evidence indicating the biological importance of the Zic4/Zic5 proteins, their functions at the molecular level remain to be clearly elucidated.

In this paper, we report on the functional characterization of the Zic4 and Zic5 proteins. Their DNA-binding specificity, transcriptional activation ability, and I-mfa protein-binding ability were examined in comparison with those of the Zic2 protein.

Materials and methods

Protein purification. The zinc-finger domains of Zic4 and Zic5 were cloned by PCR amplification of the mouse Zic4 [12] and Zic5 [3] cDNA using the following primers (Zic4ZF: 5′-CCGGATCCTTTTTCCGCTATATGC-3′, 5′-CCGTCGACGCGAGAGCAGACGTT-3′; Zic5ZF: 5′-CCGGATCCTTCCTGCGCTACATGC-3′, 5′-CCGTCGACGGGAGTCCCCACCGAC-3′). These DNA fragments were digested by *Bam*HI and *Sal*I, and inserted into pGEX4T1 (Amersham Pharmacia). The glutathione *S*-transferase (GST) fusion proteins GST-Zic4ZF and GST-Zic5ZF were expressed in *Escherichia coli* DH5α. Both proteins were prepared as soluble fractions in a buffer {50 mM Tris–HCl, pH 8.0, 20% glycerol, 100 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF)}. Glutathione–Sepharose beads were used for the purification of these proteins and eluted with the buffer containing 10 mM of reduced glutathione. The purified proteins were then dialyzed against a buffer (20 mM Hepes–KCl, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 12% glycerol, and 0.1% Nonidet P-40).

Gel shift assay. DNA fragments were prepared as described [8]. Protein–DNA complexes were formed in 20 μl of binding buffer (40 mM Tris–HCl, pH 8.0, 7 mM $MgCl_2$, 3 mM DTT, 0.1 mg/ml bovine serum albumin, 90 mM NaCl, and 150 ng poly(dI–dC)) with recombinant GST-Zic2, -4, or -5 zinc-finger-domain fusion proteins. The DNA–protein complexes were separated on 4% polyacrylamide gel under native conditions and analyzed with a phosphorimager (Fuji BAS2500).

Immunoprecipitation assay. Hemagglutinin epitope (HA)-tagged Zic4 and Zic5 were produced by inserting the cDNA fragment containing the entire open-reading frame of mouse Zic4 and Zic5 cDNAs into pHM6 (Roche). 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HA-tagged Zic2 [10], HA-tagged Zic4, HA-tagged Zic5, and FLAG-tagged I-mfa [10] were overproduced in 293T cells by transfection of several expression vectors. The cells were washed with PBS(−) containing 1 mM PMSF and disrupted in a lysis buffer (40 mM Hepes–KOH, pH 8.0, 10% glycerol, 150 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, 0.1 mM EDTA, and 1 mM PMSF). After centrifugation (20,000*g* for 15 min), the supernatant was mixed with anti-HA agarose beads (10 μl, Sigma) and incubated for 6 h at 4 °C. Bound proteins were eluted by the addition of SDS-sample buffer, loaded on for SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were transferred onto a PVDF membrane and analyzed by Western blotting.

Reporter assay. Luciferase reporter assays were performed as previously described [8]. Each luciferase reporter plasmid (90 ng) was co-transfected with the HA-Zic2, HA-Zic4 or HA-Zic5 expression vectors (100 ng), in the presence of varying amounts of the Flag-I-mfa expression vectors, and 5 or 100 ng (5 ng for 293T cells, 100 ng for 10T1/2 cells) of pRL-EF [8] was included in the transfection mixture as an internal control.

Immunofluorescence staining. NIH3T3 cells were transfected with an HA-tagged Zic (Zic2, Zic4, and Zic5) expression vector and/or a Flag-tagged I-mfa expression vector. After 24 h, the transfected cells were fixed and used for immunofluorescence staining, as described [10].

Homology analysis. Homology scores were determined using CLUSTALW DDBJ version (http://www.ddbj.nig.ac.jp/search/ex_clustalw-e.html).

Results and discussion

To analyze the protein–DNA binding specificity, the Zn-finger DNA-binding domains of Zic4 and Zic5 were tested in terms of their binding sequence and with mutated series. GST-Zic Zn-finger fusion proteins (GST-Zic2ZF, GST-Zic4ZF, and GST-Zic5ZF) and GST proteins were formed with ^{32}P end-labeled DNA fragments. The sequence was originally characterized as a Gli-binding sequence (GBS), which can be also bound by Zic1, Zic2, and Zic3 [8] (Fig. 1A). After incubation of the DNA with the proteins, these DNA–protein complexes were subjected to the gel mobility shift assay. The GST-Zic4ZF and GST-Zic5ZF Zn-finger domains, but not GST, showed distinctly shifted single bands at the same position as GST-Zic2ZF (Fig. 1B). Although Zic4ZF showed weak binding to an M4 DNA sequence, differently from Zic2ZF and Zic5ZF, the overall binding specificities were similar among GST-Zic2ZF, GST-Zic4ZF, and GST-Zic5ZF (Fig. 1B). Taken together with the results of a previous study that showed similar binding specificities of the Zic1, Zic2, and Zic3 zinc-finger domains [8], our current results suggest that the DNA-binding properties of the Zic zinc-finger domains are highly conserved in all the five mouse Zic proteins.

In a previous study, we showed that the transcriptional activity of the Zic2 protein lies in the N-terminal

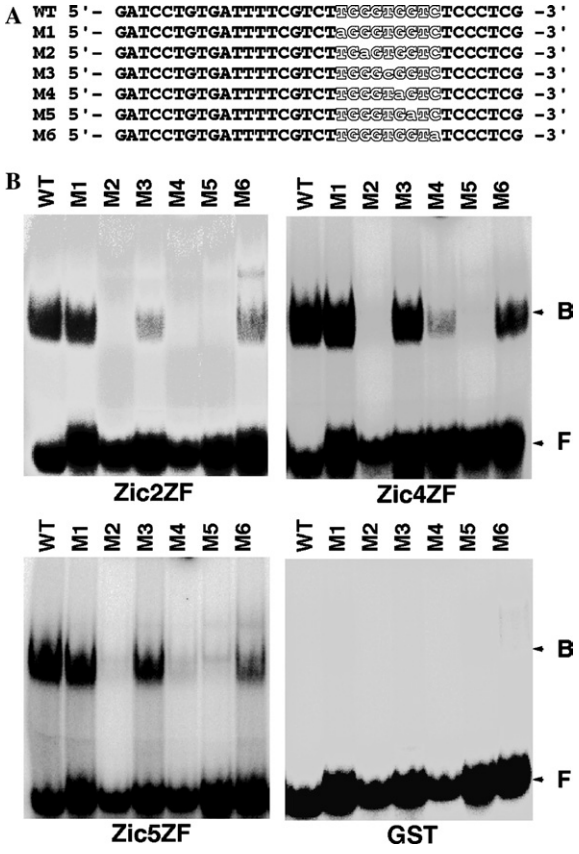

Fig. 1. Mutation analysis of the Zic-binding sequence. ^{32}P end-labeled oligonucleotides were mixed with GST-fused Zic Zn-finger proteins. Several complexes were separated by 4% polyacrylamide gel electrophoresis and the detection was conducted with a phosphorimager. (A) Target binding sequences of the Zic proteins. The lowercase letters of M1–M6 oligonucleotides show mutated nucleotides. (B) Gel shift assay. Wild-type (WT) and six mutated oligonucleotides were analyzed with GST-fused Zic Zn-finger proteins (GST-Zic2ZF, GST-Zic4ZF, GST-Zic5ZF, and GST as control). Arrows B show DNA–protein complexes, and arrows F show free oligonucleotides.

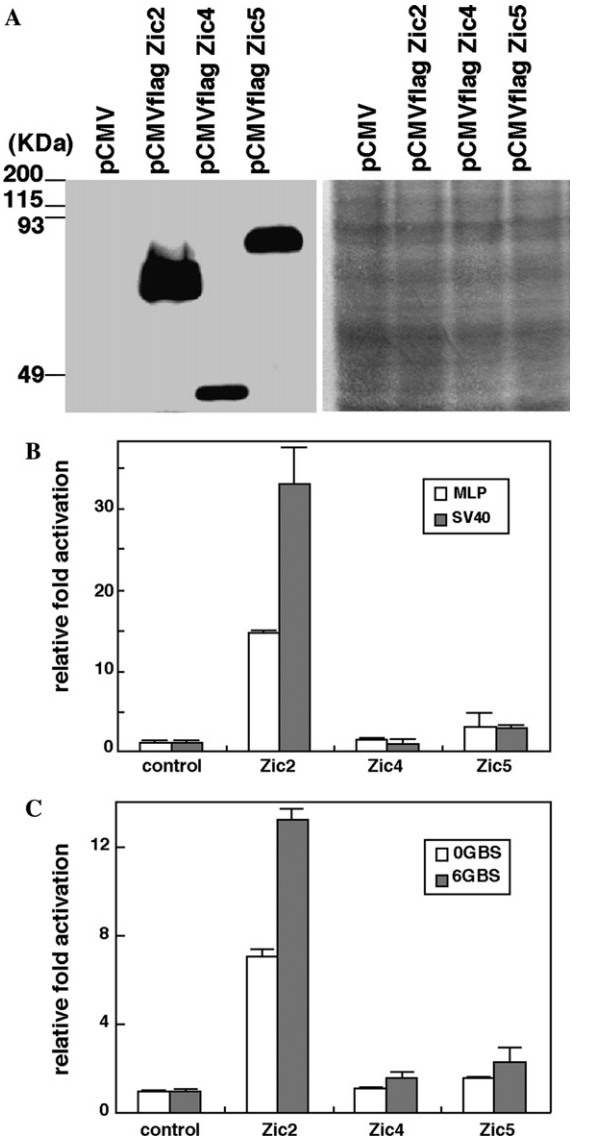

Fig. 2. Transcriptional activations by the Zic proteins. (A) Overproduction of Zic proteins in 293T cells. HA-tagged Zic2, -Zic4, and -Zic5 expression vectors or empty vector (pCMV2A) were transfected and these proteins were detected by immunoblotting (left panel). The right panel shows the protein staining of the membrane with amide black. (B) 293T cells were transfected with Zic2, Zic4, and Zic5 expression vectors with the reporter vectors, adenovirus major-late promoter–luciferase gene (MLP) or SV40 promoter–luciferase gene. (C) 10T1/2 cells were transfected with Zic2, Zic4, and Zic5 expression vectors with a reporter vector that has six additional Gli1-binding sequences as compared to the TK promoter–luciferase gene (6GBS) or TK promoter lacking the binding sequence (0GBS). All luciferase activities were normalized to the activity of an internal control (*Renilla* luciferase). The luciferase activities are indicated as relative values compared to that in the case of co-transfections of the reporter vector and the empty expression vector. The averages and standard error of the mean from three experiments are shown in (B) and (C). Overproduced Zic4 and Zic5 proteins do not increase the transcriptions mediated by the MLP, SV40, and TK with Zic/Gli-binding sequence promoters.

region of the protein [10]. Since Zic4 and Zic5 have divergent sequences of the N-terminal region relative to the Zic1/Zic2/Zic3 proteins, we were prompted to investigate whether Zic4 and Zic5 possess comparable transcriptional regulatory ability to the other Zic proteins. We utilized the same reporter assay system that we used to show the transcriptional activation ability of Zic1, Zic2, and Zic3 [8]. Overproduction of Zic1, Zic2, and Zic3 increased the SV40-promoter-, herpes simplex virus thymidinekinase (TK)-promoter-, mouse Zic1-promoter, and adenovirus major-late promoter (MLP)-driven transcriptions in the 293T cells. Although all the proteins were adequately produced by transfection (Fig. 2A), no obvious activation of Zic4 and Zic5 was observed in the 293T cells, even when MLP and the SV40 promoter, that are markedly activated by Zic2, were used (Fig. 2B). Next, we assessed the transcription-regulatory function of Zic4 and Zic5 in 10T1/2 cells using the TK promoter (0GBS) and TK6GBS (6GBS), which has six additional Gli-binding sequences as compared to the TK promoter. Both the promoters were activated very weakly by

overproduction of the Zic4 and Zic5 proteins, with no evident differences between 0GBS and 6GBS (Fig. 2C). These results imply that the transcriptional-regulatory function of Zic4 and Zic5 is not similar to that of the Zic1/Zic2/Zic3 proteins.

To further investigate the functional differences between Zic1/Zic2/Zic3 and Zic4/Zic5, we examined their relationship to the I-mfa protein, which can repress the transcriptional activation ability of the Zic1/Zic2/Zic3 proteins through physical interaction and blockage of their nuclear transport. We first performed an immunoprecipitation assay, which did not reveal any physical interactions, even when I-mfa and Zic4 or Zic5 were overexpressed (Fig. 3A). Although increasing the amount of the I-mfa expression vector strongly reduced the transcriptional activation ability of Zic2, no such effect could be observed on the transcriptional regulatory activity of Zic4 and Zic5 (Fig. 3B). However, these experiments of co-expression were not sufficient to explain why Zic4 and Zic5 were not affected by I-mfa; since these activations themselves occurred at very low levels, it was very difficult to detect any differences.

To confirm the deficient interaction ability of the Zic4 and Zic5 proteins, Zic4 and I-mfa, or Zic5 and I-mfa co-transfected cells were analyzed by immunofluorescence staining. By transfecting expression vectors, HA-tagged Zic proteins and Flag-tagged I-mfa fusion-protein were produced in NIH3T3 cells. When overproduced in cells, most of the I-mfa is retained in the cytoplasm (Fig. 4A-a). When only the Zic expression vectors transfected, all the Zic proteins were detected in the nucleus (Fig. 4A-b–d). The nuclear localization of Zic2 was inhibited with I-mfa co-expression (Fig. 4B-e–g), consistent with our observation in a previous study [10]. Although, more than 100 cells producing both Zic and I-mfa proteins were analyzed, no inhibition of the nuclear localization was observed in the Zic4–I-mfa (Fig. 4B-h–j) or Zic5–I-mfa co-transfected cells (Fig. 4B-k–m). These results support the idea that Zic4 and Zic5 do not have any significant I-mfa-binding ability.

What is the role of Zic4 and Zic5 in transcriptional regulation? It is possible that they work in tandem with Zic1, Zic2, and Zic3 to regulate the expression of the same target genes, considering their conserved DNA-binding properties and partially overlapping phenotype to the Zic2- and Zic5-mutant mice. However, the nature of the potential interaction between Zic4/Zic5 and Zic1/Zic2/Zic3 is still not clear at the present time point. The zinc-finger domains of the Zic and Gli proteins have been reported to physically interact with each other [9]. The Zic and Gli proteins counteract each other or cooperate depending on the cell type. Thus, the molecular mechanisms underlying Zic4/Zic5-mediated transcription should be clarified by revealing the relationship with the members of the Zic/Gli zinc-finger protein superfamily.

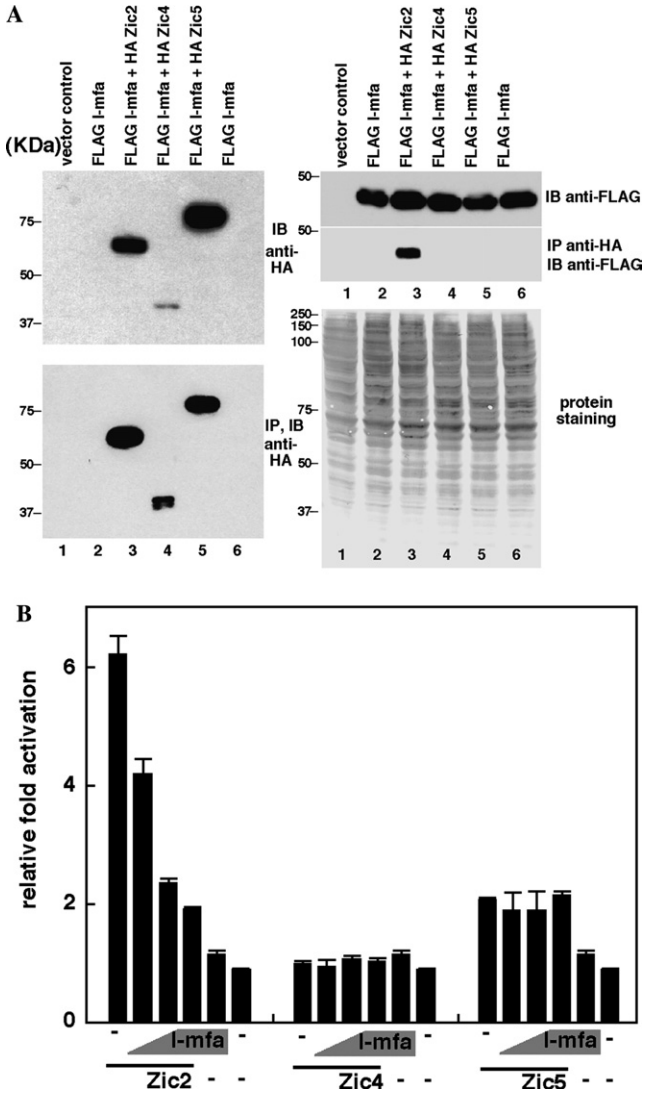

Fig. 3. Interactions between I-mfa and the Zic proteins. (A) The upper two panels (IB) show the results of immunoblotting using 10% input for immunoprecipitation, and the lower two panels (IP) show the results of immunoprecipitation with anti-HA antibody. After 10% SDS–PAGE, the proteins were detected by immunoblotting. The IB membrane was stained with amide black (lower right panel). Although FLAG-tagged I-mfa was co-immunoprecipitated with HA-Zic2, it was not co-immunoprecipitated with HA-Zic4/Zic5. (B) Repression of transcriptional activation by I-mfa. 10T1/2 cells were transfected with 100 ng of the Zic expression vectors and different amounts of the I-mfa expression vector (100–300 ng), along with TK-luciferase, a reporter gene. The luciferase activities were normalized to that of an internal control. The averages and standard error of the mean from three experiments are shown. No inhibition of transcriptional activation was observed for the I-mfa and Zic4/Zic5 co-expressions.

This study showed functional differences between Zic4/Zic5 and Zic1/Zic2/Zic3 in terms of the transcriptional regulatory and I-mfa-binding ability. These differences may be related to the functional diversity previously shown in the *Xenopus* Zic family [15]. It is also possible that the phenotypic differences between the Zic1/Zic2/Zic3- and Zic5-mutant mice [1,3] were

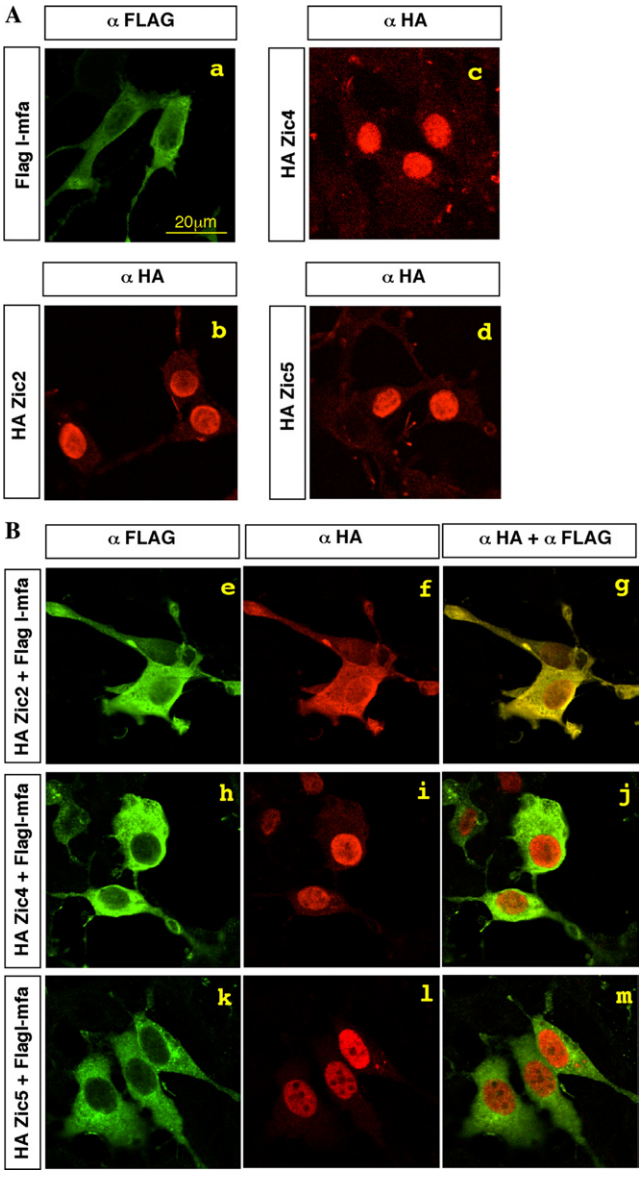

Fig. 4. Cellular localization of the Zic proteins. NIH3T3 cells were transfected with HA-Zic, FLAG-I-mfa, or both. The produced proteins were detected using anti-HA (red) and anti-FLAG (green) antibody. (A) Overproduced I-mfa is detected in the cytoplasm (a). Zic2, Zic4, and Zic5 proteins are localized in the nucleus (b, c, and d). (B) Co-transfection of Zic2–I-mfa (e, f, and g), Zic4–I-mfa (h, i, and j), and Zic5–I-mfa (k, l, and m). I-mfa was detected using anti-Flag antibody (e, h, and k), HA-tagged Zic proteins were detected using anti-HA antibody (f, i, and l) and both HA-Zic and Flag-I-mfa proteins were detected using both antibodies (g, j, and m). The scale bar is shown in the lower right corner in panel (a).

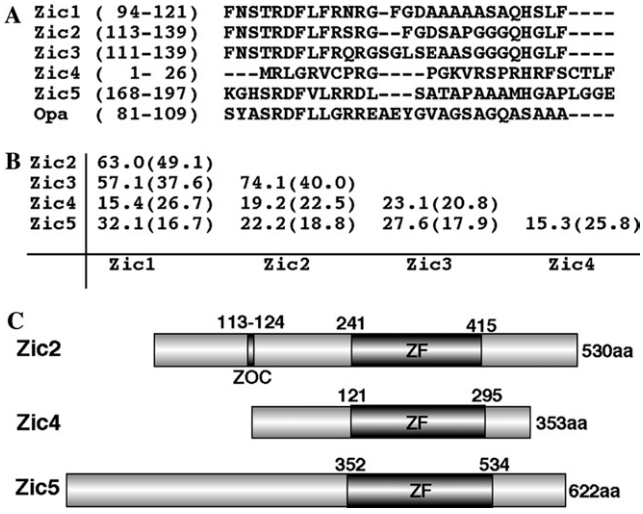

Fig. 5. Structural differences among the Zic-family proteins. (A) Alignment of the mouse Zic1-5 and *Drosophila* Opa amino acid sequences of the N-terminal region containing the ZOC domain. The letters in parentheses indicate the amino acid number of the indicated sequence. (B) Homology scores of the N-terminal region containing the ZOC domain. The numbers indicate the scores for the ZOC-containing region (A) and the numbers in parentheses indicate those in the N-terminal half without the zinc-finger domains. (C) Structural mapping of the mouse Zic2, Zic4, and Zic5 proteins. While the ZOC domain is conserved in the Zic1, Zic2, and Zic3 proteins, Zic5 possesses an incomplete ZOC domain and Zic4 does not possess a ZOC domain at all. ZF indicates the zinc-finger domain that is highly conserved among all the Zic-family proteins.

due, at least in part, to the differences in the functions of these proteins.

A clear structural difference between the Zic1/Zic2/Zic3- and Zic4/Zic5 proteins was observed in the N-terminal region (Figs. 5A and B). In particular, Zic4/Zic5 had a missing clear ZOC domain (conserved among the Zic and Odd-paired domains) (Figs. 5A–C) that is conserved in Zic1/Zic2/Zic3. The ZOC domain is located at their N-terminus, and is functionally required for the transcriptional activation ability of the protein and partially responsible for the direct binding with I-mfa [10]. Our results raise the possibility that the presence or absence of the ZOC domain may be related to the generation of functional diversity among the Zic-family proteins. Interestingly, the clear ZOC motif is present in the vertebrate Zic1/Zic2/Zic3 and *Drosophila* Odd-paired protein, but not in ascidians [1].

A phylogenetic tree analysis revealed that the Zic5 protein retained a structural feature that has been preserved from ancestral Zic genes. It is likely that the vertebrate Zic1/Zic2/Zic3 proteins acquired a specialized function during the course of evolution. However, it is still possible that specific binding partners exist for the N-terminal region of Zic4 and Zic5. Further characterization of the binding partners of the Zic4/Zic5 proteins, as well as of the Zic1/Zic2/Zic3 proteins is necessary for a comprehensive understanding of the biological significance of the functional diversity observed among the vertebrate Zic proteins.

Acknowledgments

We thank Miyuki Ogawa for technical advice and helpful discussions. This work was supported by grants

from the Nakabayashi Trust For ALS Research and Grants-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology.

References

[1] J. Aruga, The role of Zic genes in neural development, Mol. Cell. Neurosci. 26 (2004) 205–221.
[2] K. Nakata, T. Nagai, J. Aruga, K. Mikoshiba, *Xenopus* Zic3, a primary regulator both in neural and neural crest development, Proc. Natl. Acad. Sci. USA 94 (1997) 11980–11985.
[3] T. Inoue, M. Hatayama, T. Tohmonda, S. Itohara, J. Aruga, K. Mikoshiba, Mouse Zic5 deficiency results in neural tube defects and hypoplasia of cephalic neural crest derivatives, Dev. Biol. 270 (2004) 146–162.
[4] J. Aruga, K. Mizugishi, H. Koseki, K. Imai, R. Balling, T. Noda, K. Mikoshiba, Zic1 regulates the patterning of vertebral arches in cooperation with Gli3, Mech. Dev. 89 (1999) 141–150.
[5] T. Nagai, J. Aruga, O. Minowa, T. Sugimoto, Y. Ohno, T. Noda, K. Mikoshiba, Zic2 regulates the kinetics of neurulation, Proc. Natl. Acad. Sci. USA 97 (2000) 1618–1623.
[6] M. Gebbia, G.B. Ferrero, G. Pilia, M.T. Bassi, A. Aylsworth, M. Penman-Splitt, L.M. Bird, J.S. Bamforth, J. Burn, D. Schlessinger, D.L. Nelson, B. Casey, X-linked situs abnormalities result from mutations in ZIC3, Nat. Genet. 17 (1997) 305–308.
[7] S.M. Purandare, S.M. Ware, K.M. Kwan, M. Gebbia, M.T. Bassi, J.M. Deng, H. Vogel, R.R. Behringer, J.W. Belmont, B. Casey, A complex syndrome of left–right axis, central nervous system and axial skeleton defects in Zic3 mutant mice, Development 129 (2002) 2293–2302.
[8] K. Mizugishi, J. Aruga, K. Nakata, K. Mikoshiba, Molecular properties of Zic proteins as transcriptional regulators and their relationship to GLI proteins, J. Biol. Chem. 276 (2001) 2180–2188.
[9] Y. Koyabu, K. Nakata, K. Mizugishi, J. Aruga, K. Mikoshiba, Physical and functional interactions between Zic and Gli proteins, J. Biol. Chem. 276 (2001) 6889–6892.
[10] K. Mizugishi, M. Hatayama, T. Tohmonda, M. Ogawa, T. Inoue, K. Mikoshiba, J. Aruga, Myogenic repressor I-mfa interferes with function of Zic family proteins, Biochem. Biophys. Res. Commun. 320 (2004) 233–240.
[11] N. Kraut, L. Snider, C.M. Chen, S.J. Tapscott, M. Groudine, Requirement of the mouse I-mfa gene for placental development and skeletal patterning, EMBO J. 17 (1998) 6276–6288.
[12] J. Aruga, A. Yozu, Y. Hayashizaki, Y. Okazaki, V.M. Chapman, K. Mikoshiba, Identification and characterization of Zic4, a new member of the mouse Zic gene family, Gene 172 (1996) 291–294.
[13] K. Furushima, T. Murata, I. Matsuo, S. Aizawa, A new murine zinc finger gene, Opr, Mech. Dev. 98 (2000) 161–164.
[14] M.J. Benedyk, J.R. Mullen, S. DiNardo, Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in *Drosophila* embryos, Genes Dev. 8 (1994) 105–117.
[15] K. Nakata, Y. Koyabu, J. Aruga, K. Mikoshiba, A novel member of the *Xenopus* Zic family, Zic5, mediates neural crest development, Mech. Dev. 99 (2000) 83–91.