In vitro analysis of partial loss-of-function ZIC2 mutations in holoprosencephaly: alanine tract expansion modulates DNA binding and transactivation

Lucia Brown¹, Melinda Paraso¹, Ruth Arkell² and Stephen Brown¹,*

¹Department of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, NY, USA and ²Early Development, Mammalian Genetics Unit, MRC, Harwell, Oxfordshire, UK

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Heterozygous loss-of-function mutations in ZIC2 result in the severe brain malformation known as holoprosencephaly (HPE), indicating that forebrain development is exquisitely sensitive to the activity of this poorly understood transcription factor. To identify the regions of ZIC2 that are essential for activity, we have assessed the ability of a variety of ZIC2 mutant proteins to function in in vitro assays. Two sources of information were used to design relevant mutations. First, phenotype producing mutations in human and in mouse ZIC2 were mimicked and secondly, a comparative sequence analysis of the C-terminal was carried out. Analysis of these mutations suggests that either a decrease or an increase in ZIC2 mediated transcriptional activity can produce a forebrain phenotype. In addition, the analysis reveals that the C-terminal of ZIC2 contains both activation and repression domains. This region of ZIC2 contains an alanine-tract, and expansion of this domain is associated with HPE. In vitro analysis of proteins with alterations in alanine-tract length illustrates that the C-terminal alanine-tract of ZIC2 influences the strength of DNA binding and alters transcriptional activity in a promoter-specific manner. This finding provides a possible mechanism by which alanine-tract expansion mutations could alter the function of other transcription factors.

INTRODUCTION

Holoprosencephaly (HPE) is a term used to describe a spectrum of malformations of the ventral midline of the forebrain. In its most severe form, there is a single holosphere instead of the normal paired cerebral hemispheres, and in its less severe forms, there are varying degrees of malformation of midline brain structures (1). Recently, there have been major advances in understanding the molecular underpinnings of HPE (2), and in particular, we have previously shown that heterozygous mutations in ZIC2 result in HPE in humans (3,4). The presence of ZIC2 mutations in HPE patients, taken together with the fact that chromosomal deletions which contain ZIC2 are associated with HPE, makes it clear that haploinsufficiency of ZIC2 can cause HPE. Supporting the conclusion that forebrain development requires a precise level of ZIC2 activity is the observation that mice with a targeted mutation resulting in diminished Zic2 expression have an HPE-like malformation (5).

In mammals, genome sequencing has confirmed that there are five homologous ZIC genes that have been numbered 1–5. The locus link ID numbers for these are 7545, 7456, 7547, 84 107 and 85 416, respectively. This family was originally defined by homology to the Drosophila pair rule gene odd-paired (opa). All of the ZIC genes are presumed to be C2H2 type transcription factors based on homology, although little is known regarding their downstream targets or upstream regulation. In the developing mouse embryo, all five Zic genes are expressed in the dorsal brain and in the spinal cord in a highly overlapping pattern (6–8), although close examination reveals that the Zic genes also have unique domains of expression during early neural development (9). Correspondingly, mutational analysis of mouse Zic genes reveals distinct roles for these genes during development of the nervous system. As discussed, a hypomorphic allele of Zic2 exhibits HPE. The expression of Zic1 and Zic4 is not initiated until the early somite stages of development (9 and

*To whom correspondence should be addressed. Email: sab8@columbia.edu
¹The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
unpublished data), and mutations in these genes result in cerebellar defects, whereas the forebrain appears unaffected (8,10). Mutation of mouse and human ZIC3 results in prominent abnormalities of left–right axis formation, whereas CNS malformations are an uncommon part of the phenotype (8,10). The expression pattern of Zic5 overlaps extensively with that of Zic2 and yet mutation of this gene results in hypoplasia of the cranial neural crest, but no forebrain phenotype has been reported (11). Thus, it is clear that despite amino acid homology and overlapping expression domains, the developmental functions of these proteins vary.

A detailed understanding of how the Zic genes in general, and ZIC2 in particular, function in brain development will ultimately require both in vivo and in vitro studies in which ZIC2 function is experimentally manipulated. A critical tool in such studies will be mutant alleles with characterized alterations in ZIC2 function. The majority of mutations that we and others have encountered are unlikely to provide insight as they are predicted to result in complete loss-of-function. However, a total of six human and mouse mutations that are likely to result in a stable but altered protein product have been reported. The fact that these mutations are associated with HPE argues that they result in altered ZIC2 function, and are therefore likely to be informative in future studies.

In an effort to better understand the effects of mutations on ZIC2 activity, we have constructed ZIC2 cDNA clones containing HPE-associated mutations, and we have used several in vitro methods to examine the functional effects of these mutations. Data presented show that two HPE-associated mutations result in a near-complete loss of transcriptional activity, whereas two other mutations result in a decrease to ~50% of the normal level. One missense mutation results in ~2-fold increase in in vitro activity, a result that raises the possibility that even subtle changes in ZIC2 functional activity can result in brain malformation and further bolsters the concept that a delicate balance of ZIC2 activity is necessary for normal brain development.

Of the six mutations studied, three affect the C-terminal of the ZIC2 protein. Given that Zic1, 2 and 3 are highly similar and that the most divergent part of the ZIC2 protein is the C-terminal, it is likely that this part of the ZIC2 protein permits specifically to its unique role in forebrain development. These observations led us to construct a series of deletion and truncation mutations designed to explore the functional role of the C-terminal of ZIC2. In vitro analysis of these constructs points to a complex role of this portion of the protein in modulating the transcriptional activity of ZIC2.

At least eight different human malformation syndromes (including HPE) can be caused by alanine-tract expansion in transcription factors (12). It is therefore of general interest to determine how alanine-tract expansion affects transcription factor function. We present evidence that alanine-tract expansion of ZIC2 is likely to affect transcriptional activity through altered DNA binding, a finding that provides a general mechanism through which this type of mutation may lead to altered function.

### RESULTS

A total of 20 ZIC2 mutations in humans with HPE have been previously reported (4,13,14). Of these, 10 are nonsense mutations, two are missense, one is an in-frame deletion and seven are alanine-tract expansions. In the mouse, a hypomorphic mutation that does not alter the coding sequence of Zic2 as well as a point mutation in the DNA binding domain has been reported in association with brain malformation (5,15). In the present study, we have focused our attention on all those mutations that are predicted to result in an altered but possibly functional protein (summarized in Table 1). Five mutations were discovered in humans with HPE, whereas the sixth was originally isolated in a screen for dominant point mutations in the mouse (15,16). Mice homozygous for this mutation have severe forebrain defects and die by E13.5, whereas heterozygotes have less severe malformations, such as spinal bifida and ventral spotting, and are fertile (15). We have constructed cDNA clones that mimic each of these six mutations and have used several in vitro techniques to analyze their effects.

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**Table 1. Summary of mutations and phenotypes**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>References</th>
<th>Inherited?</th>
<th>Phenotype</th>
<th>Number of patients</th>
<th>DNA binding</th>
<th>Luciferase result</th>
</tr>
</thead>
<tbody>
<tr>
<td>D152F</td>
<td>(4)</td>
<td>Yes</td>
<td>Lobar HPE</td>
<td>Isolated case</td>
<td>NT</td>
<td>60%; n = 3; SD = 0.085</td>
</tr>
<tr>
<td>Q36P</td>
<td>(13)</td>
<td>Yes</td>
<td>Semi-lobar HPE</td>
<td>Isolated case</td>
<td>NT</td>
<td>170%; n = 3; SD = 0.11</td>
</tr>
<tr>
<td>C370S</td>
<td>(15,16)</td>
<td>NA</td>
<td>Severe defect in</td>
<td>Murine mutation</td>
<td>Not detectable</td>
<td>5%; n = 3; SD = 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>forebrain development</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>in homozygous mutant mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 bp Deletion</td>
<td>(4)</td>
<td>Uncertain</td>
<td>Middle interhemispheric</td>
<td>Isolated case</td>
<td>NT</td>
<td>60%; n = 3; SD = 0.045</td>
</tr>
<tr>
<td>(amino acids 435–447)</td>
<td></td>
<td></td>
<td>fusion variant of HPE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frameshift at amino acid 441</td>
<td>(4)</td>
<td>No</td>
<td>Alobar HPE</td>
<td>Isolated case</td>
<td>NT</td>
<td>2%; n = 3; SD = 0.02</td>
</tr>
<tr>
<td>with stop at amino acid 530</td>
<td></td>
<td></td>
<td>Lob, semi-lobar and</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine-tract expansion</td>
<td>(4)</td>
<td>No</td>
<td>alobar HPE</td>
<td>Seven patients</td>
<td>Reduced</td>
<td>5%; n = 3; SD = 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>from six families</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

‘Luciferase result’ refers to luciferase activity expressed as a percentage of wild-type activity as described in the text and shown graphically in Figures 1 and 2. NA, not applicable; NT, not tested.
Report constructs and controls

Previous reports have shown that Zic genes can stimulate reporter constructs in transient transfection assays, providing a method for the evaluation of transactivation activity. In one such report, a PCR-based screening method was used to determine a DNA sequence that binds Zic proteins (17). However, when multiple copies of this sequence were placed upstream of a basal promoter and luciferase reporter, there was little or no effect on luciferase activity, raising the possibility that the DNA sequence determined is not physiologically relevant. Another report showed that a 70 bp segment of the apolipoprotein E gene promoter results in a strong response to transfected Zic plasmids when it was placed upstream of a luciferase reporter (18). In addition, DNA elements from the 5'-UTR of the apoE gene form stable complexes with Zic proteins in vitro, providing evidence of a specific DNA–protein interaction as well as the possibility to experimentally correlate in vitro DNA binding with transcriptional activity of mutant alleles. In addition, in our own preliminary experiments, we found that the background luciferase activity level associated with the apoE promoter was low when compared with that seen with reporters containing common basal promoters, further arguing that the interaction of Zic proteins with this promoter is likely to be relatively specific. In light of these observations, we chose to use the apoE promoter for the experiments reported subsequently, except when stated otherwise. Because wt-ZIC2 as well as many of the mutant alleles stimulated several standard basal promoters such as HSV-tk, the transfection control plasmid used in all cases was promoterless (see Materials and Methods).

Missense mutations

The first three mutations in Table 1 result in single amino acid substitutions and are therefore considered together. Each mutation along with luciferase assay results is shown in Figure 1A. Immunoblot of transfected cell lysates confirms that all three mutant proteins are stably produced (Fig. 1B). Because the C370S mutation alters a cysteine within one of the predicted zinc fingers, it is not surprising that this allele has lost all activity in this transactivation assay. To confirm the loss of DNA binding associated with this mutation, we produced purified protein from the C370S allele and tested DNA binding with an electrophoretic mobility shift assay (EMSA) (Fig. 1C).

The other two missense mutations affect the N-terminal proximal to the DNA binding domain. One of these (D152F) results in a reduction of activity of ∼50%. As the DNA binding domain is not affected, this effect is most likely due to altered protein–protein interactions. The substitution of Q for P at position 36 results in ∼2-fold increase in luciferase

Figure 1. Analysis of ZIC2 missense mutations. (A) Cartoon showing wt-ZIC2 and point mutations indicated by arrows. The green area indicates the DNA binding domain and the yellow indicates the C-terminal alanine tract. Next to each allele is the mean luciferase activity expressed as a percentage of wt activity. Error bars indicate 1 SD based on three measurements. (B) Immunoblot of lysates of cells transfected with wt and mutant ZIC2 alleles. (C) Electrophoretic gel shift analysis showing that the C370S mutation results in loss of DNA binding.
activity, demonstrating that this non-conservative change has significant functional consequences. Again, this is presumably due to altered interactions with other proteins and raises the possibility that increased ZIC2 activity might have consequences similar to loss of activity.

C-terminal mutations, deletions and truncations

The remaining three mutations (Table 1) occurred in the C-terminal of ZIC2. Two of these (fs441 and del 435–447) are shown schematically along with luciferase assay results in Figure 2A, whereas the third, the recurrent alanine-tract expansion, is discussed subsequently. Immunoblot and in situ immunohistochemistry provide evidence that both proteins are stable and localized to the nucleus (Fig. 2C and D). Deletion of amino acids 435–447 from a highly conserved part of the protein terminal to the DNA binding domain results in a decrease of ~50% in luciferase activity, which is consistent with the idea that this part of the protein interacts with other proteins necessary for activation. Frameshift at amino acid 441 results in complete loss of activity suggesting that the C-terminal is critical for activity. Of course, it remains possible that one or both of these alleles could have dominant-negative activities that cannot be determined with this assay.

The fact that three HPE-associated mutations with differing effects on ZIC2 activity occurred in the C-terminal of the protein led us to further investigate its functional role. To this end, we created a series of deletion mutations summarized in Figure 2A. In choosing which deletion and truncation alleles to construct, we were guided by a cross-species comparison of the C-terminal of the ZIC2 gene (Fig. 2D). The C-terminal portion of the human, mouse, Xenopus and zebrafish Zic2 genes can be conceptually divided into three types of amino acid sequences: first is a stretch of about 40 amino acids immediately following the DNA binding domain that is unique and highly conserved in Zic genes across species. Then, in human and mouse, this is followed by a tract of 15 alanines. Following the alanine-tract (in human and in mouse), there is a stretch of about 40 glycine and serine rich residues and then, just prior to the stop codon, there is a run of 8–10 very highly conserved amino acids. The alleles we constructed were designed to delete various combinations of these motifs in order to assess their respective roles in ZIC2 function.

As with the other constructs, the proteins encoded by deleted and truncated cDNAs were apparently stable and, with one exception, localized to the nucleus (Fig. 2C). Deletion of amino acids 341–441, which includes part of the DNA binding domain as well as a segment of conserved sequence that follows, as expected, results in a protein with no activity. In contrast, deletion of 430–472 does not reduce activity despite the fact this deletion includes the 12 amino acid HPE-associated deletion (amino acids 435–447) discussed earlier. This suggests that there are both activating and repressive domains located within the deleted segment. Truncation of the protein at amino acid 430 results in a 50% reduction of activity, whereas, paradoxically, truncation at amino acid 472 decreases activity by ~75%, again pointing to the idea that the alanine-tract and sequences close to it repress transactivation. This is in keeping with the finding (presented subsequently) that deletion of the alanine-tract results in a marked increase in transactivation. Finally, deletion of 436–526, which is very similar to the 430-ter truncation but retains the final eight amino acids, results in a ~90% reduction in activity. This implies that the final 8–10 amino acids form part of a repressive region as well. The long glycine-serine tract seems to be necessary for full activity, despite its simplicity. Taken as a whole, these data support the idea that the portion of the protein from the end of the DNA binding domain to the C-terminal has complex modulating effects on transactivation activity.

Expansions and contractions of the C-terminal alanine-tract

The final type of mutation we have analyzed is expansion of the C-terminal alanine-tract. An expansion from 15 alanines to 25 (25A), mimicking the naturally occurring HPE-associated mutation, results in a near-complete loss of transactivation (Fig. 3A). Transfection of this allele into 293 cells indicates that it is produced and stable (Fig. 3B). Because previous reports have raised the possibility that proteins with expanded alanine-tracks may form aggregates, we considered this possibility. Cells transfected with both the wt and the 25A allele were fixed and stained with ZIC2 specific anti-serum and shown to observers who were blinded to the experimental conditions (data not shown). No difference between the two conditions was noted, and in both cases the protein was localized to the nucleus.

Although we have never encountered any alanine-tract alterations other than the 25-alanine expansion, we were curious to explore the effects of other alanine-tract alterations. To this end, we constructed cDNAs containing 35 alanines (35A) and 2 alanines (a 13 alanine deletion) (Fig. 3A). Again, both proteins were produced in transfected cells (Fig. 3B); however, in the case of the 35A allele, there was punctate staining suggestive of aggregate formation in both the nucleus and the cytoplasm (Fig. 3C). This effect was obvious to blinded observers as it was present in a high percentage of cells. In luciferase assays, the 2A allele exhibited a 2.5-fold increase in activity when compared with wt-ZIC2, suggesting that the alanine-tract may function as part of a repressive domain. Paradoxically, however, the 35A allele exhibited activity consistently, slightly higher than the 25A allele, making it impossible to conclude that there is a simple relationship between alanine-tract length and transcriptional activation activity (Fig. 3A).

Alanine-tract expansion mutations in HoxD13 result in a gain of function, whereas other alanine-tract expansions appear to result in loss-of-function. This observation led us to consider the possibility that the alteration in transcriptional activity we observed might vary according to the promoter sequence being used. To this end, we compared the transcriptional activation of wt-ZIC2 with the alanine-expanded allele using the SV40 immediate-early promoter (pGL3-promoter Promega). In comparison with the apoE promoter, the observed level of transcriptional activation attributable to wt-ZIC2 was only ~10-fold higher than background (~(50–100-fold seen with the apoE promoter). Lengthening of the alanine-tract reduced transcriptional activation, but less dramatically than that seen with the apoE
promoter. On the other hand, shortening the alanine-tract resulted in decreased rather than increased activity (Fig. 3A). Altogether, these results argue that the effects of alanine-tract length on DNA binding and presumably on transcriptional activity depend to some extent on the DNA sequence being queried.

Gel shift assays to directly assess DNA binding of mutant Zic proteins

Alanine-tract expansions in transcription factors have not been thought to have an effect on DNA binding as alanine-tracts do...
not form parts of recognized DNA binding domains. However, co-transfection experiments reported subsequently suggested that alanine expansion might reduce DNA binding. We tested this possibility directly using EMSA. To this end, His-tagged, purified ZIC2 proteins containing the wild-type, 15 alanines as well as altered forms containing two and 25 alanines, respectively, were incubated with end-labeled double-stranded oligonucleotides and analyzed with gel electrophoresis (Fig. 3D). Although we attempted to produce protein with the 35A expansion, we were unable to recover enough soluble protein to examine the effect of this large expansion on DNA binding. Reduction of the alanine-tract

Figure 3. Analysis of alanine-tract alterations. (A) Luciferase assay results showing that alanine-tract length has strong and promoter-specific effects on the transactivation activity of ZIC2. (B) Immunoblot of transfected cell lysates. (C) Immunohistochemical analysis of CHO cells transfected with the 35A allele. Note the apparent intranuclear and intracytoplasmic aggregates. (D) EMSA demonstrating the effect of alanine-tract alteration on DNA binding. The comassie stained gel below the autoradiogram indicates the amount of input protein used for that lane. (E) Densitometric analysis of the adjacent autoradiogram indicates the inverse relationship between alanine-tract length and in vitro DNA binding of ZIC2 protein.
to two resulted in an ~5-fold gain in DNA binding when compared with the wild-type protein, whereas expansion resulted in a reduction to approximately half (Fig. 3E). Consistent with this, the 2A allele also resulted in a marked increase in transactivation activity (Fig. 3A). The complex formed between the alanine-expanded protein and the oligonucleotide consistently ran as a more diffuse smear of higher apparent molecular weight, a result that also suggests that the DNA–protein complex adopts a markedly different conformation when the alanine-tract is expanded. These results are consistent with the idea that the alanine-tract can modulate transcriptional activation by modulating DNA binding.

**Co-transfection of mutant and wt-ZIC2 alleles**

Mutations in transcription factors are expected to result in loss of DNA binding or altered protein–protein interactions, or both. We reasoned that if a mutant allele of ZIC2 had the capability to antagonize the activity of the wild-type protein (dominant-negative activity), then we might be able to see this effect in co-transfection experiments in which both the mutant and the normal alleles could interact. To investigate this, we performed mixing experiments in which each of the ZIC2 alleles that exhibited very low activity in the luciferase assay was co-transfected along with wt-ZIC2 in varying ratios (Fig. 4). As expected, the del 341–441 allele, in which the DNA binding domain is partially deleted, had little or no effect on the activity of wt-ZIC2 even when it was present in 10-fold molar excess. On the other hand, the other three inactive alleles (25A, fs441 and C370S) did suppress the activity of normal ZIC2, suggesting either that these proteins are capable of binding DNA, but lack the ability to transactivate or that they can bind to other proteins necessary for transcriptional activation. In the case of fs441, it is reasonable to assume that the protein is capable of binding DNA, but cannot transactivate. In the case of the C370S mutation, we have shown that DNA binding is severely reduced if not completely absent. Therefore, we assume that this allele inhibits the normal allele by binding other components of the transcription complex and rendering them unavailable. Interestingly, co-transfection with the 25A allele resulted in less suppression of ZIC2 induced luciferase activity than did the fs441 or C370S alleles. Even when present in a 10-fold molar excess, the 25A allele did not suppress the activity of wt-ZIC2 to <35% of normal, a result which suggested that the alanine-tract expansion mutation might result in diminished DNA binding as demonstrated earlier.

**Lack of homodimerization of ZIC2**

DNA binding transcription factors of various classes are known to form both hetero and homodimers, and this property is likely to be critical to the combinatorial regulation of gene expression (reviewed in 19). With respect to Zic proteins, there are no published data to suggest the formation of homodimers, but others have shown that Zic proteins interact physically with members of the Gli family of transcription factors and have suggested that this physical interaction is critical to the proper regulation of gene expression (17,20). In the context of the preceding mixing experiments, where a ZIC2 allele with no ability to bind DNA can antagonize the function of the wt protein, we considered the possibility that ZIC2 might form homodimers and that dimerization between mutant and wt-ZIC2 proteins could explain the diminished activity of the wt allele. To investigate this, we performed co-immunoprecipitations using ZIC2 molecules with two different epitopes (Flag and myc). We found no evidence for physical interaction of ZIC2 with itself (data not shown). In addition, we also performed a yeast two-hybrid analysis to directly assay ZIC2 physical interaction with itself. When yeast carrying ZIC2 in the activation domain vector were mated with yeast carrying ZIC2 in the bait vector, there was no growth on either triple or quadruple drop out media, indicating that ZIC2 does not functionally interact with itself (data not shown).
DISCUSSION

In vitro analysis of HPE-associated mutations in ZIC2

The regulation of transcription by developmental transcription factors such as ZIC2 is likely to be complex, involving many interactions that are tissue and developmental stage-specific. In vitro assays of transcriptional activity such as the one we have employed are highly simplified and are likely to detect only severe alterations in transcription factor function. The finding that a mutation results in near-total loss of activity in such an assay implies that the protein must be altered such that very basic components of the transcriptional machinery do not function correctly. Of the six mutations we tested, three result in a near-total loss of activity in the in vitro assay, and we conclude that these mutations are likely to behave as a nulls in vivo as well. In all three cases, we provide some explanation of why activity is lost. As predicted, the C370S mutation abolishes DNA binding. In the homozygous mutant state, ZIC2 activity is predicted to be essentially nil. In the heterozygous state, it is possible that the mutant protein antagonizes the normal allele as seen in co-transfection experiments. Interestingly, mice heterozygous for this mutation frequently have malformations such as a neural tube defect. This could be either due to haploinsufficiency or due to dominant-negative effect of the mutant allele. As there is no simple null mutation available for comparison, this point remains unresolved. The fs441 mutation is likely to severely alter the tertiary structure of the protein as a simple truncation of the terminal amino acids results in much less striking reduction of transactivation. As discussed subsequently, the alanine expansion mutation is likely to affect activity through altered DNA binding.

The two mutations with partial activity are more difficult to interpret. In the case of the D152F mutation, the mother of the child affected with HPE actually carried the same mutation, but did not have HPE. Although it is possible that this single amino acid change is an incidental finding and did not cause HPE, it seems unlikely in light of the fact that this amino acid is highly conserved, both within the ZIC family members and in the ZIC2 across several species. In the case of the del 435–447 allele, the deleted amino acids (SAEPQSSSNLSP) are part of a conserved region that is present in most known Zic proteins including those from Xenopus, zebrafish (Fig. 3) and Drosophila, making it likely that their presence is important for function. Interestingly, the patient with this mutation has an unusual variant form of HPE, known as middle-interhemispheric-fusion defect (21), raising the possibility that the altered transcriptional activity of this allele of ZIC2 has slightly different developmental consequences than complete loss-of-function. Again, an in vivo functional assay will be needed to resolve this issue.

Patients with ZIC2 mutations are always heterozygous and therefore, in the case of null mutation, expected to have 50% of normal activity. Heterozygosity for a mutation that results in a 50% loss of activity has the net effect of reducing total activity by 25%. Our results suggest that either forebrain development is extremely sensitive to ZIC2 activity or the two mutations with partial loss-of-function in the luciferase assay result in a more complex dysfunction that is not detected by this assay. There are few, if any, examples where loss of only 25% of the activity of a transcription factor has caused developmental abnormalities. Interestingly, in a recent report of ZIC3 mutations in patients with heterotaxy type malformations of the heart, several of the mutations analyzed demonstrated only slightly reduced transcriptional activation (22); however, ZIC3 is X-linked, and the malformations occurred in males who therefore had a low level of residual activity.

We were surprised to find that one of the HPE-associated mutations (Q36P) increased the transactivation activity of ZIC2, a result that raises the intriguing possibility that increased activity could have the same ultimate effect as decreased activity. Unfortunately, this is an isolated case and the mutation was present in the unaffected mother making it impossible to conclude that the mutation caused the malformation. Nonetheless, the Q at this position is conserved in both the Xenopus and the zebrafish ZIC2 genes, making it reasonable to think that alteration to P could have deleterious consequences. Resolution of this issue will require a relevant in vivo assay.

Alanine-tract alterations

Alanine-tract expansions in transcription factors have been implicated as the molecular basis of at least eight different human diseases and malformation syndromes (12). This raises the possibility that alanine-tracts have a common role in multiple transcription factors and that the disease causing mechanism might be similar in all the conditions. The details of how expanded alanine-tracts disrupt protein function have not been fully defined in any of these diseases or syndromes, although in the case of HoxD13 there is evidence that alanine-tract expansion results in a dominant gain of function that involves the abnormal expression of other Hox genes (23).

Our data indicate that the 25A allele of ZIC2 is stable in transfected cells and that, in vitro, it exhibits altered DNA binding. With respect to the apoE promoter, it has lost most its transcriptional activation activity. Paradoxically, with respect to the SV40 promoter, the 25A allele of ZIC2 shows a less marked decrease in activity, and reduction of the alanine-tract to two results in diminished rather than increased activity. This points to the possibility that alanine-tract length can alter DNA binding in a sequence-specific manner. If confirmed, this could explain why alanine-tract expansions affect so many different transcription factors and can result in both gain- and loss-of-function. It could also provide an evolutionary mechanism for fine-tuning of transcription factor activity. It will be interesting to see whether altered DNA binding is a consequence of alanine-tract expansion in other transcription factors, and this will be particularly true in those cases where the true DNA binding target is well characterized.

Effect of alanine-tract length on solubility

Others have reported that proteins with long alanine-tracts can form apparent aggregates in transfected cells, and this has been proposed as a mechanism of protein malfunction and disease causation (24,25). With this in mind, we attempted to assess whether alanine expanded alleles of ZIC2 behave...
differently from wt protein in transfected cells using fluorescence microscopy. Although we did not attempt to develop a quantitative assay for the presence of apparent aggregates, observers were unable to detect any difference between wt-ZIC2 and 25A-ZIC2 in immunostained, transfected cells, and in neither case was punctate staining (suggestive of aggregate formation) visibly present. On the other hand, punctate staining suggestive of aggregate formation was present in both the cytoplasm and the nucleus in a high percentage of cells transfected with the 35A-ZIC2. Similarly, 35A-ZIC2 protein was insoluble in lysates of Escherichia coli, even when expressed at very low levels. These data support the idea that alanine-tracts beyond some critical length result in the formation of insoluble aggregates; however, it seems unlikely that aggregate formation is the cause of protein dysfunction in the spontaneously occurring 25A expansion found in patients with HPE.

C-terminal of ZIC2

The C-terminal of ZIC2 is part of the protein that is the most divergent from other Zic family members and also the most variable among Zic2 genes from different species. The human and mouse genes have a nearly identical stretch of glycine and serine rich residues that is not present in either the fish or the frog raising the possibility that this part of the molecule relates specifically to mammalian development. Our data indicate that removing this non-conserved part of the C-terminal does not have a large impact on the basic transcriptional activation activity of the protein. However, when one considers that a loss of only 50% of ZIC2 activity has profound consequences for CNS development, subtle alterations in activity are likely to be important. Clearly, it will be important to re-test the function of all of the deletion alleles in the setting of a relevant bioassay.

MATERIALS AND METHODS

Plasmids and mutations

A plasmid containing the full-length coding sequence of ZIC2 was modified in various ways to create all of the described mutations. In summary, single base alterations were created using the Quick-Change site directed mutagenesis system (Stratagene). The alanine-tract expansion mutations were created by the insertion of double-stranded oligonucleotides (Stratagene). The alanine-tract expansion mutations were created by restriction digest filling in the overhang with 32P-labeled dGTP. The dried gel was imaged using a Molecular Dynamics phosphor imager and an anti-rabbit secondary conjugated to alkaline phosphatase and detection was chemiluminescent (Amersham).

Protein transfer blots were prepared as described previously (26) and immunodetection of ZIC2 was accomplished with a ZIC2 specific serum (27) diluted 1:2000. The primary antibody was detected with a goat anti-rabbit secondary conjugated to alkaline phosphatase and detection was chemiluminescent (Amersham).

For immunohistochemistry, CHO cells were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Cells were fixed and incubated with anti-ZIC2 anti-serum in situ and an anti-rabbit secondary conjugated to Cy3 (Jackson Immunochromicals) was used for detection. Photographs were taken with a Nikon fluorescence microscope and a Diagnostic Instruments digital camera. The images were not enhanced to alter appearance, size or contrast.

Electrophoretic mobility shift assays

Proteins were prepared by subcloning an MscI–PstRI fragment of each ZIC2 construct into the pQE81 vector (Qiagen) that creates an N-terminal RGS-6His tag and allows for expression in E. coli. Bacterial cultures were induced with IPTG and, 2 h following induction, were lysed according to a standard protocol (Qiagen). After purification on a nickel resin, proteins were analyzed and quantitated by running on 12% polyacrylamide denaturing gels. For each DNA binding assay, 100 ng of protein (as determined by Bradford assay and confirmed by Comassie stained PAGE) and 1 pmol of double-stranded oligo were used. The oligos used (CCCTCTCCCTGCCCCTGCT and CAGCAGGGCAG AGGGA) correspond to –139 to –124 of the apoE promoter (18). Labeling was accomplished by annealing and filling in the overhang with 32P-labeled dGTP. The dried gel was imaged using a Molecular Dynamics phosphor imager and the densitometric determinations were performed using the accompanying ImageQuant software package.

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REFERENCES


