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ORIGINAL ARTICLE

Isolation and characterization of *Xenopus laevis* homologs of the mouse *inv* gene and functional analysis of the conserved calmodulin binding sites

Yukuto Yasuhiko[1,*], Koichiro Shiokawa[1,*], Toshio Mochizuki[2,*], Makoto Asashima[3], Takahiko Yokoyama[4]

[1]Department of Biological Sciences, Graduate School of Science, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; [2]Department of Medicine, Kidney Center, Tokyo Women's Medical University, School of Medicine, Japan; [3]Department of Life Science, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 158-8902, Japan; [4]Department of Anatomy and Developmental Biology, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

The homozygous *inv* (*inversion of embryonic turning*) mouse mutant shows situs inversus and polycystic kidney disease, both of which result from the lack of the *inv* gene. Previously, we suggested that *inv* may be important for the left–right axis formation, not only in mice but also in *Xenopus*, and that calmodulin regulates this inv protein function. Here, we isolated and characterized two *Xenopus laevis* homologs (*Xinv-1* and *Xinv-2*) of the mouse *inv* gene, and performed functional analysis of the conserved IQ motifs that interact with calmodulin. *Xinv-1* expresses early in development in the same manner as mouse *inv* does. Unexpectedly, a full-length *Xenopus inv* mRNA did not randomize cardiac orientation when injected into *Xenopus* embryos, which is different from mouse *inv* mRNA. Contrary to mouse *inv* mRNA, *Xenopus inv* mRNA with mutated IQ randomized cardiac orientation. The present study indicates that calmodulin binding sites (IQ motifs) are crucial in controlling the biological activity of both mouse and *Xenopus* inv proteins. Although mouse and *Xenopus inv* genes have a quite similar structure, the interaction with calmodulin and IQ motifs of *Xenopus* inv and mouse inv proteins may regulate their function in different ways.

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Introduction

Although vertebrates are externally bilaterally symmetric, internal organs show a marked left–right (L–R) asymmetry, such as the left-sided presence of stomach and heart and occurrence of more lung lobes on the right side. This L–R asymmetry is genetically determined, and disturbance of the asymmetry often causes serious cardiovascular disease in humans [1, 2]. Recent progress suggested that primary cilia on the primitive node have an important role in the establishment of the L–R asymmetry. Nodal cilia rotate and make a leftward current [3-5]. This leftward flow subsequently induces asymmetrical gene expression such as *nodal* and *lefty*. The mouse mutant called *inv* (*inversion of embryonic turning*) shows a constant reversal of the L–R asymmetry in internal organs and develops cysts of the kidney [6]. In *inv* mutant mouse embryos, the left side expression of *nodal* and *lefty* is inverted [7, 8] and abnormal nodal flow was reported [4, 9], indicating that *inv* acts upstream of *nodal* and *lefty*. The mouse *inv* gene encodes 1062 amino acids containing 15 tandem repeats of

Correspondence: Takahiko Yokoyama
Department of Anatomy, Kyoto Prefecture University of Medicine, 465 Kajii-cho, Hirokoji- agaru, Kawaramachi-dori, Kamikyo-ku, 602-0841, Japan
Tel: 81-75-251-5303; Fax: 81-75-251-5304;
E-mail: tyoko@koto.kpu-m.ac.jp
*Present addresses: Yukuto Yasuhiko[1], Koichiro Shiokawa[2], Toshio Mochizuki[3]
[1]Cellular and Molecular Toxicology Division, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[2]Department of Biosciences, School of Science and Engineering, Teikyou University, 1-1 Toyosatodai, Utsunomiya-city, Tochigi prefecture 320-8551, Japan
[3]Department of Medicine II, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan
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the ankyrin motif, two IQ motifs and two nuclear localization signals [10]. In the *inv* mouse, only the first 91 amino acids of the inv protein remain, but the regions containing the ankyrin repeats as well as the IQ motifs were deleted [10, 11]. The introduction of *inv* cDNA completely rescued all the *inv* phenotypes, not only situs inversus but also cyst formation of the kidney, indicating that the loss of the *inv* gene caused these two unrelated abnormalities [10]. In humans, a mutation in the *inv* gene is also responsible for human laterality defects [12] and nephronophthisis (NPHP2) [13]. Thus, the *inv* gene is essential in establishing normal L–R asymmetry and kidney development in both mice and humans.

Previously, we reported that the mouse inv protein interacts with calmodulin at two sites, and that microinjection of mouse *inv* mRNA into the right blastomere of two-celled *Xenopus* embryos resulted in randomized L–R asymmetry in the orientation of visceral organs [14]. Furthermore, the calmodulin binding motif (the IQ motif) is critical for mouse *inv* mRNA in randomizing cardiac orientation, and Ca^{2+} regulates the interaction between calmodulin and the mouse inv protein.

In this study, we isolated two distinct full-length cDNAs for *Xenopus laevis inv* homologs (*Xinv-1* and *Xinv-2*, accession numbers AF321228 and AF321229 respectively), examined their expression and performed functional analysis of the conserved IQ motifs.

Materials and methods

Cloning of Xenopus inv homologs

Degenerative oligonucleotides were designed from regions encoding conserved ankyrin repeats of mouse and human inv proteins. Total RNAs of adult *Xenopus* kidney and liver were used as a template for cDNA synthesis. These RNAs were reverse-transcribed with Superscript II (Invitrogen, Carlsbad, California), and used as PCR templates. An amplified product was subcloned into the pGEM T-vector (Promega, Madison, WI) and sequenced. The product encoded multiple ankyrin repeats that were 70% identical in amino-acid sequence to mouse *inv* ankyrin repeats 3-10. The fragment was labelled with ^{32}P-dCTP using an oligopriming kit (Amersham Pharmacia Biotech, Uppsala Sweden) and used as a probe to screen a *Xenopus* oocyte cDNA library (Clontech ZL5000a, Clontech, Mountain View, CA, USA). The first screening gave us four *inv*-related cDNA fragments. Sequences showed that three of these fragments encoded the same gene and were named *Xinv-1,* and the fourth cDNA was named *Xinv-2*. We screened at least half a million clones at each library screening.

To obtain a full coding sequence of the *Xinv* genes, we screened cDNA libraries, including two oocyte cDNA libraries (Clontech ZL5000a and ZL5001T), the kidney and liver cDNA libraries (Stratagene, La Jolla, CA, USA) and the stage 11.5 embryo cDNA library (a gift from Dr Bruce Blumberg). Rapid Amplification of cDNA Ends (RACE) was also performed to obtain the 3' end of *Xinv-1* using oocyte cDNA as a template.

Northern hybridization and RT-PCR

For Northern analysis, polyA RNAs were purified from total RNAs obtained from oocytes and liver. Five mg of polyA RNAs were electrophoresed and blotted onto nylon membranes (Amersham Pharmacia Biotech). Membranes were hybridized with a ^{32}P-labelled *Xenopus inv* probe (*Xinv-1*) and washed with 2×SSC, 0.1% SDS at 60 °C and exposed to X-ray film for 1-7 days.

For RT-PCR, RNA was extracted from embryos at various stages by the AGPC method. Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) following the manufacturer's protocol. Primers specific to each *Xinv* clone were as follows:

Xinv-1 forward: 5'-GCT GGT GGA AAA GGA GAA AGC TAG AA-3'

Xinv-1 reverse: 5'-CCT ACA TTA CAA CAC TGG AGA GCG CA-3'

Xinv-2 forward: 5'-CCT ACA TTA CAA CAC TGG AGA GCG CA-3'

Xinv-2 reverse: 5'-CTA CAC CAC ACC AAA CGT CAA TTG AAA-3'.

PCR reaction was performed using Ex Taq (Takara, Kyoto).

We amplified β-actin to verify the RT reaction. Primers for β-actin amplification were actinF: 5'-GGA CTT GGC TGG ACG TGA CCT GAC-3', actinR: 5'-CAG ACT CAT CAT ACT CCT GCT TGC TG-3' 18 cycles of PCR were performed.

Whole-mount in situ hybridization

The plasmid pYY94 contained 1453 bp of 3' terminal region of *Xinv-1* ORF (open reading frame) in pBluescript SK(-) (Stratagene, La Jolla, CA, USA). The plasmid was linearized at either the *Not* I site (for the antisense probe) or the *Xho* I site (for the sense probe) and subjected to *in vitro* transcription using T7 or T3 polymerase to produce an antisense or sense probe, respectively, in the presence of digoxigenin-modified UTP. Albino *X. laevis* embryos at various stages were fixed in MEMPFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1mM $MgSO_4$, 4% paraformaldehyde) and processed for whole mount *in situ* hybridization as described previously [15]. Coloring reaction was performed overnight at 4 °C, then overnight at room temperature using BM purple (Roche Diagnostics, Basel, Switzerland).

DNA construction, site-directed mutagenesis, in vitro transcription for mRNA injection and in vitro translation

The ORFs of *Xinv-1* and *-2* were PCR-amplified and ligated into the transcriptional vector pCS2+ [16] between the *EcoR* I and *Stu* I sites using a ligation kit (Takara). The sequence was confirmed not to have a mutation. For *Xinv-1* transcription, we substituted the T3 promoter for the SP6 promoter of pCS2+. The plasmids, pYY158 (*Xinv-1*) and pYY159 (*Xinv-2*), were linearized at the *Not* I site, and transcribed with T3 RNA polymerase using MEGAscript kit (Ambion).

Mouse *inv* cDNA (pGEM-mINV) [10] was digested with *EcoR* I (Takara) and the 3 kb fragment obtained was ligated into the *EcoR* I site of pCS2+. The resultant plasmid pYY34 was linearized at the *Not* I site and transcribed with SP6 RNA polymerase. Site-directed mutagenesis of *Xenopus inv* IQ motifs was performed using Gene-Editor kit (Promega). The mutated ORFs were subcloned to pCS2+ (modified its promoter from SP6 to T3) and transcribed with T3 RNA polymerase. To obtain GFP mRNA, pbGFP/RN3P [17] was linearized with SfiI and transcribed with T3 RNA polymerase. All

in vitro transcription was performed in the presence of a cap analog (New England Biolabs).

Two cDNA clones, *Xinv-1* and *-2*, were subjected to *in vitro* translation using rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine. Subsequently, lysates were analyzed with SDS-PAGE. The gel was dried and examined by autoradiography.

Yeast two-hybrid assay

The following plasmids were used to examine the interaction with calmodulin in the MATCHMAKER yeast two-hybrid system (Clontech):

GBT-158 (*Xinv-1* full-length cDNA)
GBT-182 (IQ1 mutated in *Xinv-1*)
GBT-183 (IQ2 mutated in *Xinv-1*)
GBT-184 (IQ1 and IQ2 mutated in *Xinv-1*)

The vector was derived from the pGBT vector (Clontech). Plasmids were introduced into the Y187 yeast strain with pCaM8 that contained the calmodulin gene fused to an activating domain for the yeast two-hybrid assay. Colonies, grown on minimal media lacking tryptophan and leucine, were tested for β-galactosidase activity with a colony-lift filter assay.

Microinjection of synthetic mRNA

Unfertilized eggs of *X. laevis* were manually ovulated from gravid females that had been injected with a human chorionic gonadotropic hormone, gonatropin (Teikoku Zoki, Kawasaki Kanagawa). Eggs were artificially fertilized, dejellied in 2% cysteine-HCl (pH 8.0) and kept in 0.1×Steinberg's solution [18]. The mRNAs to be tested were microinjected at a dosage of 2000 pg/embryo unless otherwise noted, together with GFP mRNA (200 pg/embryo in all the experiments) in 10 nl of distilled water. As injection of 5 ng mRNA/embryo resulted in a frequent occurrence of malformed embryos (abnormal cleavage and gastrulation failure), we decided to inject 2 ng of *inv* mRNAs per embryo. Microinjection was performed at the late two-cell stage or the early four-cell stage. The mRNA solutions were injected into the right or left half of each embryo in the animal hemisphere at the presumptive dorsal and ventral regions. All of the embryos were placed in 1×modified Barth's solution (MBS), containing 3% Ficoll 400 and 50 μg/ml gentamycin during microinjection. Injected embryos were kept in 1×MBS until stage 7 [19], and subsequently transferred into 0.1×Steinberg's solution for further culture. Embryos were kept at 23 ºC in 0.1×Steinberg's solution, containing 50 μg/ml gentamycin, before further observations were made. Embryos were cultured until stage 22 [19], and grouped into left-side injected and right-side injected embryos according to the distribution of the GFP signal under a fluorescent microscope (Olympus Model BHS-RFK, with a filter set for FITC observation). At stage 45, embryos were paralyzed with Tricaine (0.1% in 0.1×Steinberg's solution) and the orientations of heart and gut were examined under a dissection microscope. Statistical analyses were performed using a standard χ^2 test, and $p<0.05$ was considered a significant difference.

Results

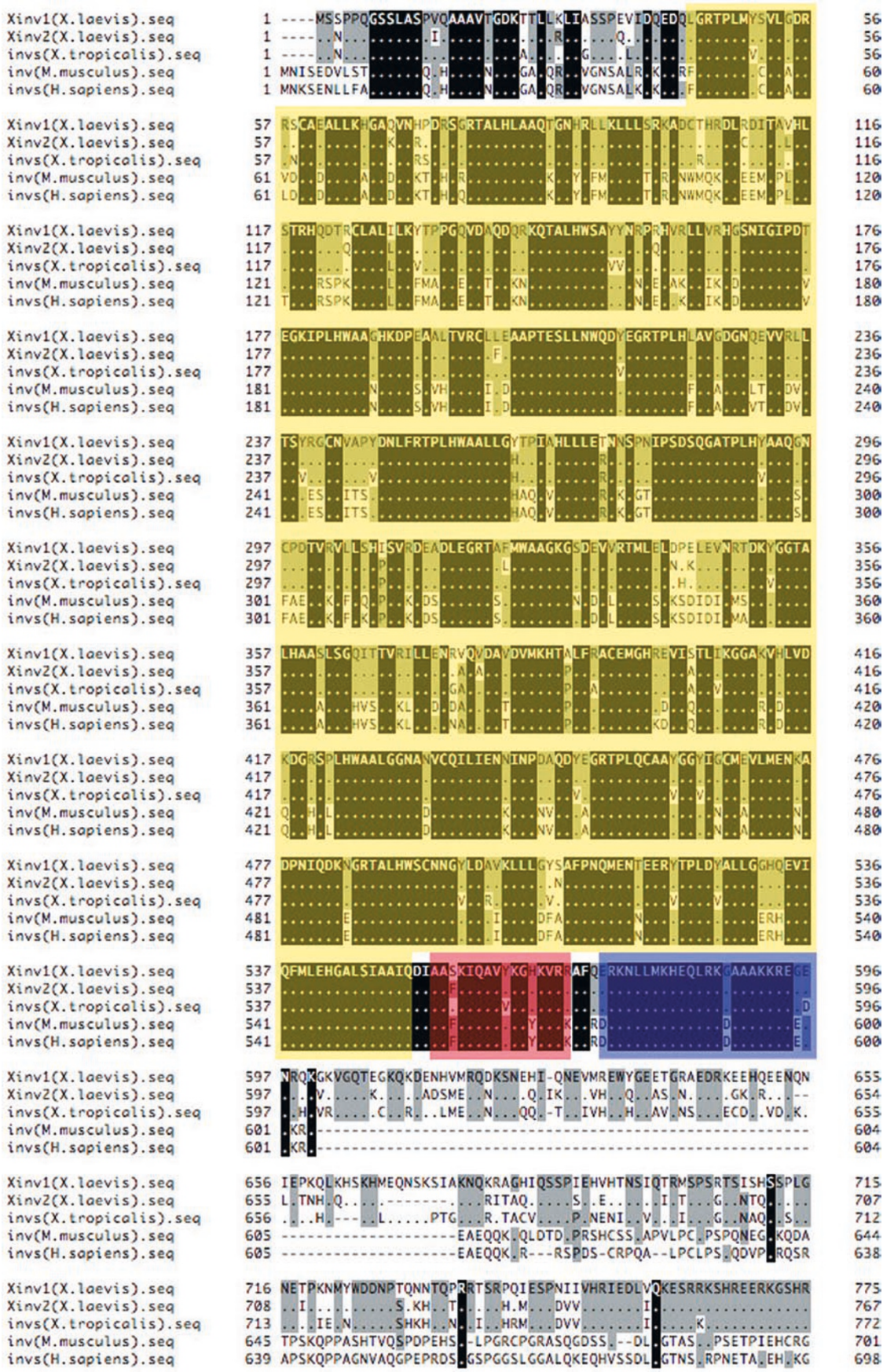
Two Xenopus inv homologs contain conserved ankyrin repeats and two IQ motifs

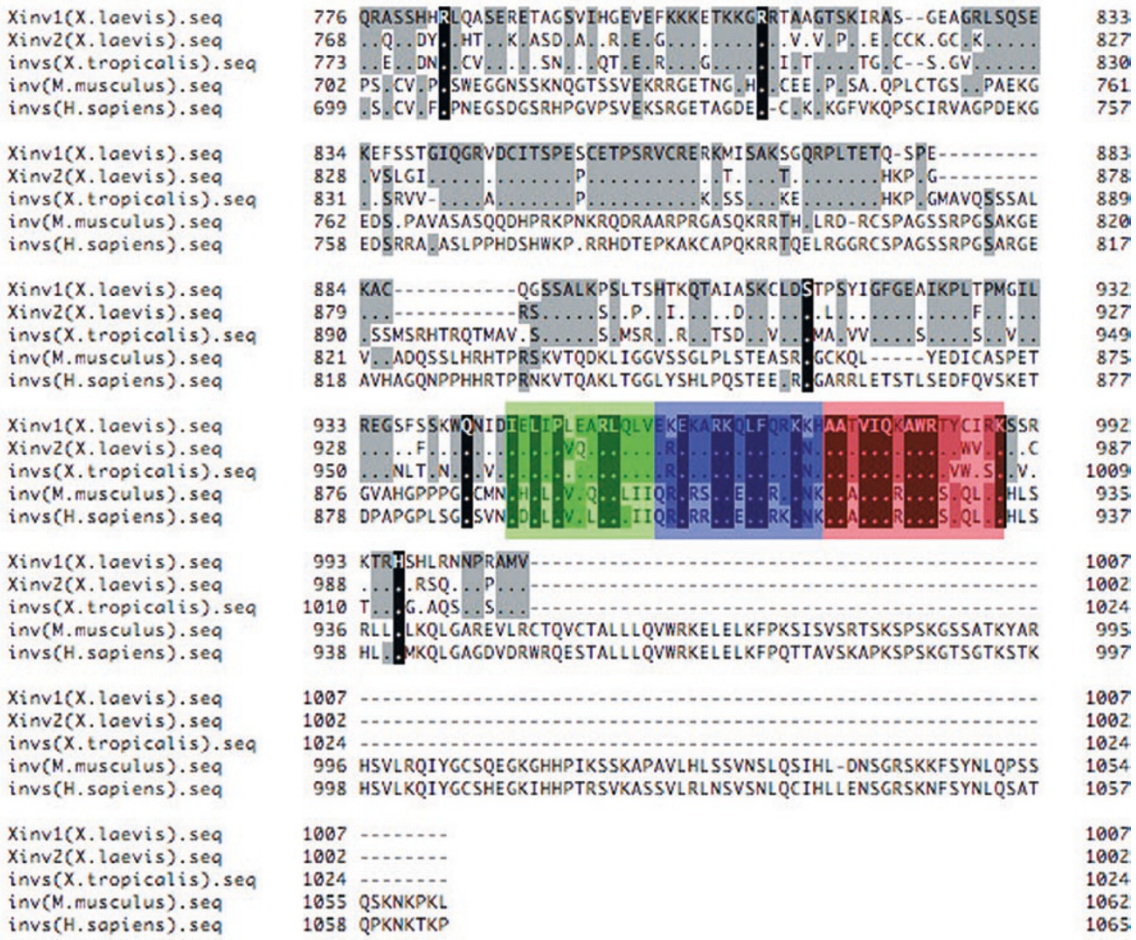
Two *Xenopus* cDNA clones, named *Xinv-1* and *-2*, contained an open reading frame (ORF) homologous to mouse *inv*, and encode 1007 and 1002 amino acids, respectively (Figure 1A). Both ORFs started with an ATG initiation codon that agrees with the Kozak rule [20], and have an in-frame stop codon in the 3' end. The clones are 89% identical to each other in amino-acid sequence. Nucleotide sequences of ORFs of *Xinv-1* and *Xinv-2* are 58.2% and 57.8% identical to that of the mouse *inv* ORF. At amino-acid level, *Xinv-1* and *Xinv-2* have 50% and 52% overall amino-acid identity to mouse *inv*. Both *Xinv-1* and X*inv-2* have 15 repeats of the ankyrin motif in the N-terminus (a yellow box in Figure 1A), and two calmodulin-binding IQ motifs, named IQ1 and IQ2 (16 amino-acid residues), that are highly homologous to those of mouse and human *inv* genes. Amino-acid sequences of the ankyrin repeats of *Xinv-1* are 73.4%, 71.8% and 74.5% identical to that of mouse, chick and zebrafish *inv*, respectively. Amino-acid sequences of the ankyrin repeats of *Xinv-2* are 74.7%, 75.5% and 72.7% identical to that of mouse, chick and zebrafish *inv*, respectively. A search in the *X. tropicalis* database identified a sequence that was homologous to *Xinv-1* and *-2*. The sequence encodes 15 repeats of the ankyrin motif and two IQ motifs. The sequence has 83.5% and 83.1% overall amino-acid identity to *Xinv-1* and *-2*, respectively. We could not find other related sequences in mouse, human and *X. tropicalis* databases. IQ1 is just behind the ankyrin repeats, and IQ2 is located near the C-terminus (red boxes in Figure 1A). We also found two basic amino-acid residues, one next to IQ1 and the other at the N-terminal side of IQ2, conserved between mouse and *Xenopus* (blue boxes), which are named Basic Region (BR)-1 and -2, respectively. There is a conserved hydrophobic region (designated HR) in the amino-terminal side of BR2 (green box in Figure 1A). Despite the general similarity of the deduced protein structure, *Xinv-1* has only one monopartite (SV40T antigen type) nuclear localization signal (NLS), and *Xinv-2* has no typical NLS, while there are two typical bipartite (nucleoplasmin type) nuclear localization signals (NLS) in mouse inv protein (Figure1B). These structures are well conserved among species (Figure 1B). Figure 2 shows a phylogenetic relationship among mouse, human, chick, zebrafish and *Xenopus* (*tropicalis* and two *laevis*) *inv* genes.

Xinv-1 is expressed maternally and in early embryos of Xenopus

The Northern hybridization analysis detected two signals of *Xinv* mRNA (about 4.4 and 5.8 kb) in the ovary. Only a larger band (about 5.8 kb) was detected in the liver (Figure3A). As we obtained two *Xinv* cDNAs, we made specific primers in their 3' ends to distinguish *Xinv-1* from *Xinv-2* mRNAs, and examined their expression. The signal for *Xinv-1* was constantly obtained in RNA from the ovary,

A


Xinv1(X.laevis).seq
Xinv2(X.laevis).seq
invs(X.tropicalis).seq
inv(M.musculus).seq
invs(H.sapiens).seq



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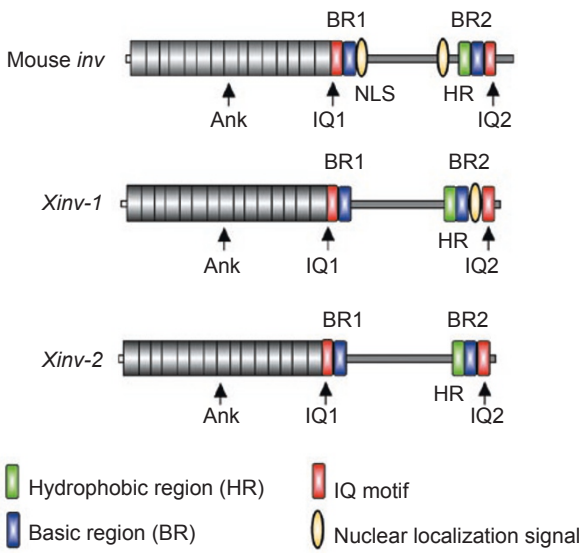

Figure 1 (A) Alignment of putative *Xenopus laevis* inv amino-acid sequences along with mouse, chick (*Gallus gallus*) and zebrafish (*Danio rerio*), and putative *X. tropicalis* inv protein sequences. *Xinv*-1, *Xinv*-2, *X. tropicalis* inv, zebrafish inv, chick inv and mouse inv proteins are 1007, 1002, 1020, 1021, and 1269 and 1138 amino acids, respectively. The conserved motifs are indicated as follows. Yellow boxes: ankyrin repeats, red boxes: IQ motifs, blue boxes: conserved basic regions (BRs), green box: conserved hydrophobic region (HR). IQ motifs and BRs were numbered from the N-terminal (IQ1, 2 and BR1, 2, respectively). **(B)** Schematic description of the structure of mouse and *Xenopus* inv proteins.

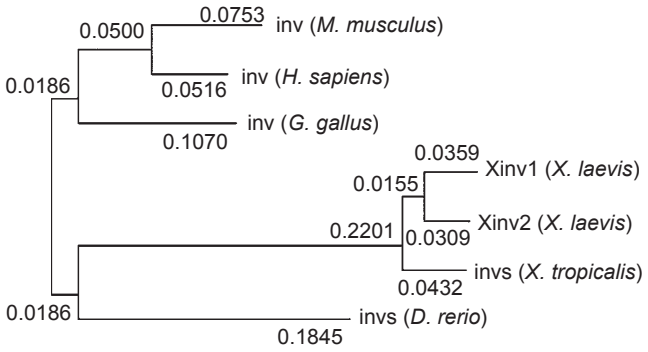

Figure 2 A phylogenic tree of inv related genes. The tree is constructed by the neighbor-joining method [21] using the amino-acids sequences of ankyrin repeat domains. Values indicate expectation of nucleotide substitution on each residue.

cleavage stage embryos through stages 3–27 (Figure 3B). Although the signal for β-actin was detected at 18 PCR cycles, the signal for *Xinv-2* was not detected from the unfertilized egg (ovary) to the hatching stage (St.33), even at 50 PCR cycles (Figure 3B). The detection of *Xinv* signals in the ovary suggested a possibility that the *inv* message is maternally provided in the egg.

Whole-mount *in situ* hybridization (Figure 3C) showed that the *Xinv* mRNA signal was present in the morula (St. 7) to the tailbud stage. After the neurula stages, *Xinv* signals were detected in the dorsal side of embryos. In the tailbud stage (St. 22), *Xinv* signals were also in the dorsal side with a little anterior bias. No obvious asymmetrical pattern was observed in the examined embryos. Throughout the stages, the signal was not detected using a sense probe.

A single amino-acid substitution of isoleucine to glutamic acid eliminates calmodulin-inv protein interaction

We reported that the region encoding the IQ motifs plays a crucial role in the activity of mouse *inv* mRNA to randomize the L–R asymmetry of *Xenopus* embryos by right-sided mRNA microinjection [14]. Sequence analysis shows that *Xenopus* inv proteins have IQ motifs that are homologous to mouse inv protein (Figure1A and 4A), and yeast two-hybrid assay showed that Xinv-1 protein also

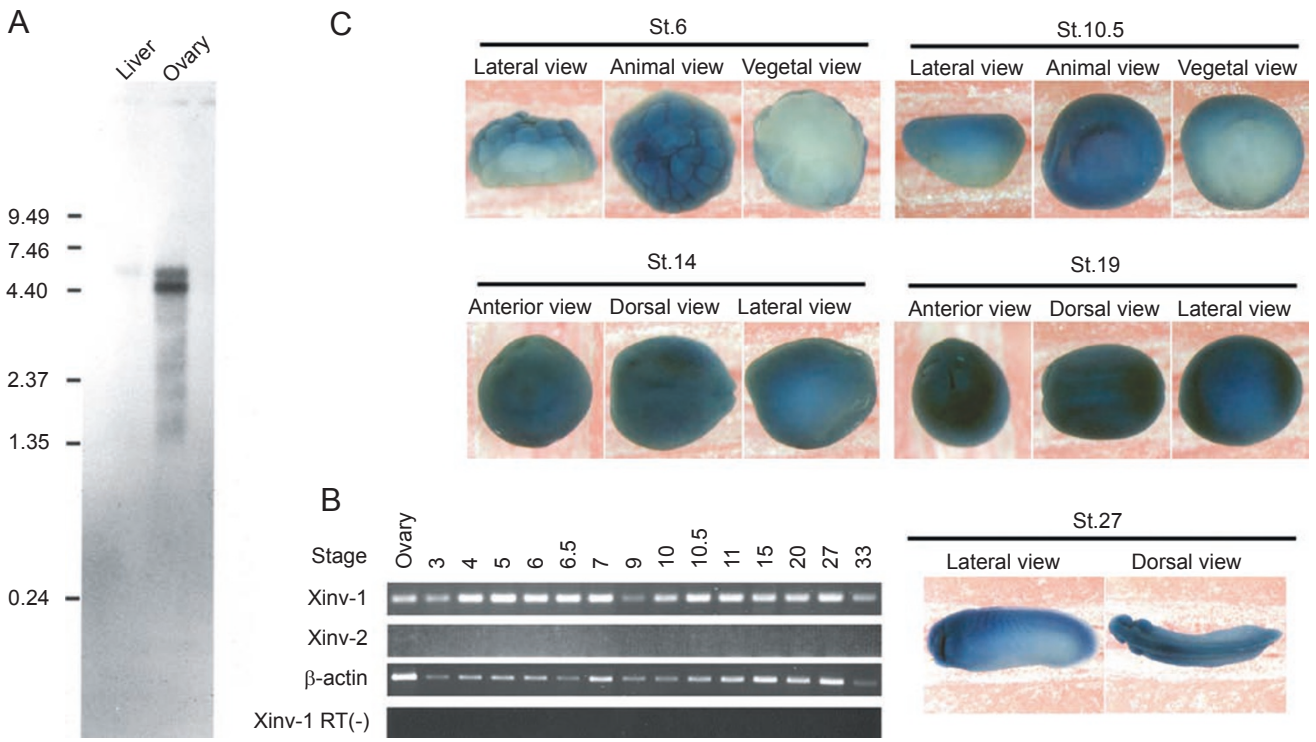

Figure 3 (A) Northern hybridization for *Xenopus* inv cDNA expression. In all, 2 μg of polyA+RNA is loaded in each lane, and exposed for 7 days at –80 °C. A 4.4 kb transcript (arrowhead) was detected in ovary while not in liver. Size markers (in kb) are shown in the left side of the autoradiograph. **(B)** Temporal expression patterns of *Xenopus* inv examined with RT-PCR. *Xinv-2* is not detected by PCR with up to 50 cycles, while *Xinv-1* is detected at 46 cycles. RT-PCR with β-actin specific primers was carried out in parallel to control the amount of input RNA. RT (-), RT-PCR without reverse transcriptase. Stages are shown over each lane. **(C)** Expression pattern of *Xinv* from stage 6 to stage 27 shown by whole-mount *in situ* hybridization using digoxigenin-labeled *Xinv-1* cRNA as a probe. No significant unilateral expression of *Xinv* is detected.

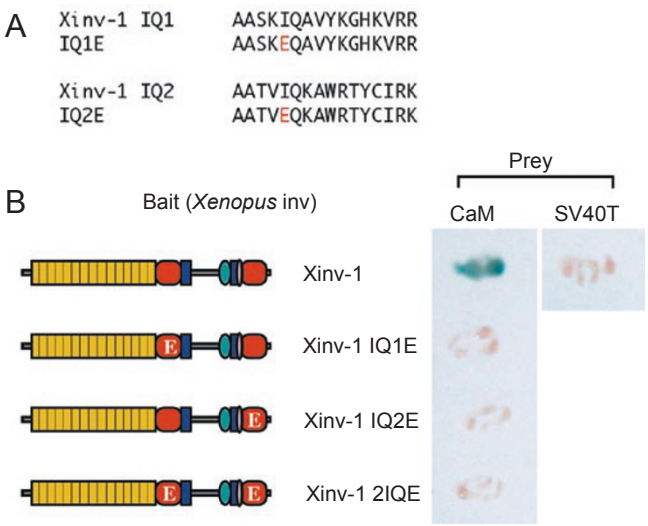

Figure 4 (A) A single amino-acid substitution of isoleucine (I) by glutamic acid (E) eliminates calmodulin–inv protein interaction. **(B)** Description of the mutated Xinv IQ motifs. Substitution of isoleucine (I) with glutamic acid (E) is indicated in red. IQ motif mutated *Xenopus* inv's lose calmodulin-binding activity. The interaction between a wild-type Xinv protein or mutated Xinv proteins and calmodulin or SV40 large T antigen (CaM and SV40T, shown in the upper row as 'prey') was assayed using a yeast two-hybrid system. A schematic description of the constructs indicated in the left column as 'bait'. The white letters 'E' in IQ motifs (depicted as red blocks) represent the amino-acid substitution. In this experiment, wild-type Xinv proteins or mutated Xinv proteins were used as baits. Blue staining indicates the interaction between bait and prey. Only wild-type Xinv-1 shows calmodulin binding activity. SV40 large T antigen was a prey as a negative control of the yeast two-hybrid assay, and no interaction was observed.

interacts with calmodulin (Figure 4B).

To verify that *Xenopus* inv protein interacts with calmodulin at the IQ motifs, we performed site-directed mutagenesis at the regions encoding IQ motifs (Figure 4A). We made three mutant constructs of *Xinv-1*, containing a single amino-acid substitution from isoleucine (I) to glutamic acid (E) in one of their IQ motifs or both (Figure 4A). Xinv-1/IQ1E, Xinv-1/IQ2E and Xinv-1/2IQE contain the mutation in IQ1, IQ2 and in both IQ1 and IQ2 regions, respectively (Figure 4B, left column). A full-length ORF of these mutant constructs were cloned into yeast expression vector, and a two-hybrid assay was performed using mouse calmodulin (CaM) as prey. The yeast two-hybrid assay indicated that all these mutants had lost their calmodulin-binding activity (Figure 4B). Interestingly, mutation in either one of the IQ motifs sufficiently eliminated the whole calmodulin binding activity of the Xinv protein, indicating that the Xinv protein–calmodulin interaction needs two intact IQ motifs.

IQ-mutated but not wild-type Xinv mRNA injection reverses cardiac orientation

Microinjection of mouse *inv* mRNA into the right blastomere of two-cell stage *Xenopus* embryos causes randomization of L–R asymmetry and *Xnr-1* expression pattern [14]. To examine whether *Xinv* mRNA has the same effect on L–R asymmetry, we injected synthetic mRNA of *Xinv-1* (total 2 ng per embryo) into one of the blastomeres of two-cell-stage *Xenopus* embryos with GFP mRNA (Figure 5). Unexpectedly, the microinjection of *Xinv-1* mRNAs did not cause an L–R inversion (Figure 6). We performed *in vitro* translation experiment of *Xinv*-1 and *Xinv*-2 to verify that *Xinv* mRNAs were translated, using rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Resulted lysates were analyzed with SDS-PAGE and autoradiograph, giving signals at the expected molecular size (not shown).

However, when the IQ mutated *Xinv* mRNAs were microinjected into the right blastomere of two-cell stage *Xenopus* embryos, the reversal of cardiac orientation was seen in a significant number of the embryos (Figure 6), though the percentage of cardiac reversals was lower than that caused by mouse *inv*, 18% (Xinv-1/2IQE) to 22% (Xinv-1/IQ2E). Throughout the experiment, left-sided microinjection had no effect on the cardiac reversal rate when compared with noninjected siblings (not shown).

Discussion

In the present study, we identified two *X. laevis* genes that were homologous to the mouse *inv* gene. The overall similarity between mouse and *Xenopus inv*'s seems to be relatively low, but there are some distinct conserved domains between them: 15 ankyrin repeats, two CaM-binding motifs and NLSs (though NLS is absent in Xinv-2). These features are highly conserved among previously identified inv homologs of various kinds of vertebrates (mouse, chick and zebrafish). In addition, a search in the *X. tropicalis* database identified a sequence that was more than 80% identical to the two *X. laevis* genes. Although pylogenetic analysis showed that the distance between mouse and *Xenopus inv* genes is larger than that between mouse and zebrafish *inv* genes, this is not significant. Therefore, it is unlikely that the cloned genes are psedoalleles of the *inv* gene. These results suggest that the genes are *X. laevis inv* genes, and a possibility that their biological functions are shared.

The temporal expression pattern of *Xinv-1* indicated that *Xinv* mRNAs exist maternally and are expressed constantly during embryogenesis, similar to that of mouse *inv* [22]. Whole-mount *in situ* hybridization showed that *Xenopus inv* mRNA expresses evenly in both left and right sides of embryos. These results are also consistent with the previous observation in mouse embryos [10]. Although the

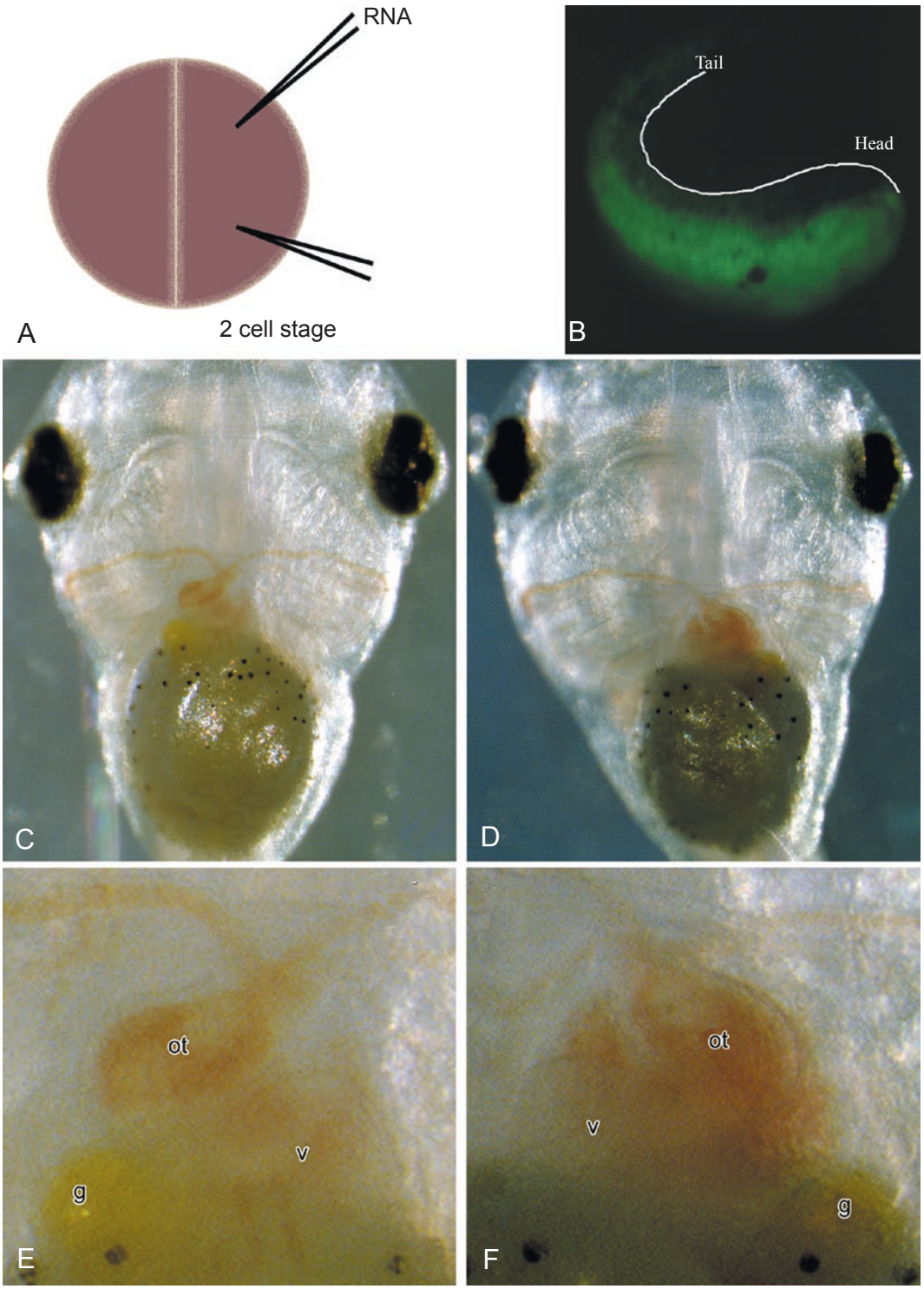

Figure 5 Injection of synthetic mRNAs into the blastomere of the two-cell-stage *Xenopus* embryo and randomization of the L–R asymmetry. **(A)** Microinjection was performed at two-cell or early four-cell stage, and mRNA solutions were injected into the left or right half of the embryos. **(B)** Dorsal view of injected embryo observed under a fluorescent microscopy. GFP fluorescence occurs in the right half of the embryo. The injected embryos were divided into right- and left-injected groups according to the occurrence of GFP fluorescence at St. 23–24. **(C)** A noninjected embryo showing normal visceral L–R asymmetry. **(D)** An example of reversal of L–R asymmetry. **(E)** and **(F)** The outer appearance of normal heart and gall. Abbreviations: ot, outflow tract; v, ventricle; g, gall bladder. Inverted L–R asymmetry of heart and gall in the embryo. Note that the positions of outflow tract and gall bladder are completely inverted.

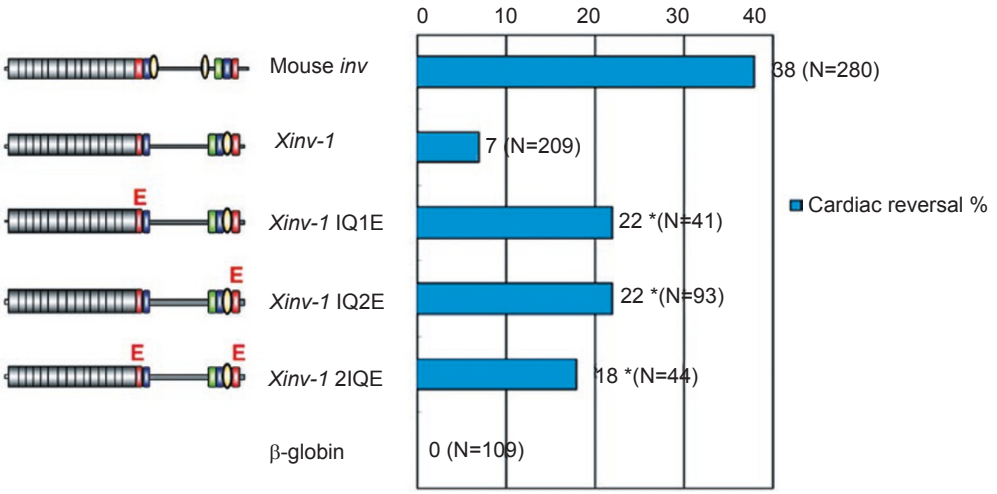

Figure 6 IQ-mutated but not WT Xinv mRNA injection reverses cardiac orientation. Cardiac reversal in *Xenopus* embryos injected with the mRNAs coding WT ORF or mutated construct of Xinv-1. Schematic description of the constructs indicated in the left lane. The red letters 'E' over the IQ motifs (depicted as red blocks) represent the amino-acid substitution. In the right graph, the percentages of embryos with cardiac reversal are shown. All three mutant *Xinv* mRNAs affected L–R asymmetry through the right-sided microinjection of mRNA, while the wild-type Xinv mRNAs did not. *Statistically different from that of the group injected with the Xinv-1WT RNA into a right blastomere.

present study identified two *Xenopus inv* genes (*Xinv-1* and *-2*), *Xinv-2* mRNA was not detected by RT-PCR, suggesting that *Xinv-1* is transcribed mainly during *Xenopus* development, and that *Xinv* expression patterns shown by whole-mount *in situ* hybridization (Figure 3) are likely to reflect the distribution of *Xinv-1* mRNA.

A previous study showed that the injection of mouse *inv* mRNA into *Xenopus* two-cell-stage embryos reverses cardiac orientation [14], and suggested that calmodulin signalling was involved in the *inv* pathway for the establishment of L–R asymmetry. Unexpectedly, mRNAs encoding wild-type *Xenopus* inv proteins did not affect cardiac orientation when injected into *Xenopus* two-cell stage embryos. As the N-terminal ankyrin repeats are highly homologous, the difference between mouse and *Xenopus inv*s on cardiac inversion is likely to lie in the C-terminal half of inv proteins, probably by the interaction between calmodulin and IQ motifs. IQ-mutated *Xinv-1* mRNA randomized cardiac orientation and lost interaction with calmodulin (Figure 6), supporting this idea. The results suggest that the interaction with calmodulin also regulates the *Xenopus* inv function. The mutation in only one of the two IQ motifs eliminated the overall CaM binding activity of Xinv protein (Figure 6), suggesting an intramolecular mechanism controlling the interaction between calmodulin and inv protein.

Randomization of the L–R asymmetry was induced only when these *inv* mRNAs were injected into the right blastomere, whereas injection into the left blastomere was ineffective. Our results suggest a factor(s) that activates inv protein differently between two blastomeres. The present study suggests that calmodulin is an important factor in the control of inv function. Calmodulin is a conserved Ca^{2+}-binding protein involved in a variety of cellular calcium-dependent signalling pathways [23-25]. Ca^{2+} regulates the interaction between calmodulin and inv protein [14]. As recent studies suggest that there is a difference in Ca^{2+} concentration between the right and left sides in mice [26] and chickens [27], Ca^{2+} might be a candidate as a factor in making the difference in cardiac reversal induced by *inv* mRNA injection between right and left *Xenopus* blastomeres.

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