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RESEARCH ARTICLE

Cloning and Functional Characterization of a Novel Connexin Expressed in Somites of *Xenopus laevis*

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Connexin-containing gap junctions play an essential role in vertebrate development. More than 20 connexin isoforms have been identified in mammals. However, the number identified in *Xenopus* trails with only six isoforms described. Here, identification of a new connexin isoform from *Xenopus laevis* is described. Connexin40.4 was found by screening expressed sequence tag databases and carrying out polymerase chain reaction on genomic DNA. This new connexin has limited amino acid identity with mammalian (<50%) connexins, but conservation is higher (~62%) with fish. During *Xenopus laevis* development, connexin40.4 was first expressed after the mid-blastula transition. There was prominent expression in the presomitic paraxial mesoderm and later in the developing somites. In adult frogs, expression was detected in kidney and stomach as well as in brain, heart, and skeletal muscle. Ectopic expression of connexin40.4 in HEK293 cells, resulted in formation of gap junction like structures at the cell interfaces. Similar ectopic expression in neural N2A cells resulted in functional electrical coupling, displaying mild, asymmetric voltage dependence. We thus cloned a novel connexin from *Xenopus laevis*, strongly expressed in developing somites, with no apparent orthologue in mammals. *Developmental Dynamics 233:864–871, 2005.*

Key words: connexin; gap junction; *Xenopus*; expression; electrophysiology; somite

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INTRODUCTION

Gap junctions provide the path for direct exchange of small molecules and ions between adjacent cells in all animal species. In general, gap junctions are built of innexins as pore-forming units in invertebrates (Phelan and Starich, 2001) and of connexins (Cxs) in vertebrates and chordates (Goodenough and Paul, 2003). Six Cxs associate to form one hemichannel (connexon), and subsequent docking of two connexons in adjacent cell membranes results in the formation of a gap junction. To date, 20 and 21 different connexin (Cx) isoforms have been described in the mouse and human genome, respectively (Willecke et al., 2002; Söhl and Willecke, 2003). The cumulative expression pattern of the entire set of Cx isoforms covers virtually all organs and tissues within a given species. Furthermore, many structures express more than one Cx isoform. In the amphibian *Xenopus laevis*, only six Cxs have been described, i.e., XlCx30, XlCx31, XlCx38, XlCx41, XlCx43, and XlCx43.4 (Gimlich et al., 1988, 1990; Ebihara, 1989; Yoshizaki and Patiño, 1995; Landesman et al., 2003), some of which display strong homology with mammalian species, i.e., XlCx43 (Van der

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Heyden et al., 2001a, 2004). Others display poor overall homology, i.e., XlCx38 (less than 50% amino acid identity with mammalian Cx37).

At least two observations suggest the existence of additional amphibian Cxs. First, the cumulative expression pattern of all six known *Xenopus* Cxs does not cover every structure in the (developing) frog, leaving "white areas" to be filled in by additional Cxs. Second, from the genome sequencing projects of *Xenopus* (see for example, Klein et al., 2002), it has become evident that additional Cx-like sequences are present in the genomic and expressed sequence tag (EST) databases. We carried out a search for additional *Xenopus* Cxs for two reasons. First, a complete understanding of gap-junction function in *Xenopus* development and adult physiology will require the identification of all Cx isoforms. Second, the identification of all *Xenopus* Cxs will yield important additional information for elucidating Cx evolution. Evolutionary complexity becomes increasingly evident with rapid progression of zebrafish, chordates, and mammalian Cx research (Dermietzel et al., 2000; Willecke et al., 2002; Söhl and Willecke, 2003; Sasakura et al., 2003; Cheng et al., 2003, 2004; White et al., 2004; Zoidl et al., 2004).

To identify new Cxs from *X. laevis*, we screened several EST databases and found additional full-length Cxs in silico. One of these Cxs was subsequently physically cloned, and its characteristics were determined. This XlCx showed limited (<50%) amino acid identity with mammalian Cxs, became expressed after the mid-blastula transition, and was particularly strongly expressed in the paraxial mesoderm and developing somites. In adult frogs, expression was found in kidney, brain, stomach, heart, eye and skeletal muscle. Finally, functional characteristics of this new XlCx were established by electrophysiology.

RESULTS

In Silico and Molecular Cloning of *Xenopus laevis* Cx40.4: Sequence Analysis and Comparison

A *X. laevis* EST database (TIGR) was screened with part of the *X. laevis* Cx43 amino acid sequence encompassing all four transmembrane regions. This search resulted in four hits, two of which appeared to be full-length Cxs. One had strongest homology with mammalian Cx26 (63.5% at amino acid level; TIGR TC72011; GenBank accession no. BC043797), which might in fact be the amphibian Cx26 orthologue. The other had strongest homology with mammalian Cx46 (~49% at amino acid level; TIGR TC111754; GenBank accession no. BC043801) but no obvious mammalian orthologue. To clone this Cx, we designed primers based on the EST sequence that would give an amplification product covering the complete protein coding region. These were used to amplify the Cx coding region from genomic DNA of *X. laevis*. PCR amplification revealed two products: one of ~550 bp and the other of the expected size of ~1,140 bp. The largest product was subsequently cloned in pGEM-T-easy and sequenced.

The deduced amino acid sequence displays the predicted features of a genuine Cx protein (Hua et al., 2003): four predicted transmembrane regions with two extracellular loops each containing three conserved cysteine residues, an intracellular N- and C-terminus and one intracellular loop (Fig. 1). The predicted relative molecular mass of this Cx protein is 40.4 kDa. It was, therefore, designated XlCx40.4.

We next compared the amino acid sequence of XlCx40.4 with other Cxs. As shown in Table 1 and Figure 1, XlCx40.4 is not identical to the original EST clone. Furthermore, overall identity with other *X. laevis* Cxs varies between 29.7% and 41.4%, indicating that a new Cx isoform had been cloned from *X. laevis*. Based on amino acid homology, XlCx40.4 had a 63.3% identity to an unknown protein of the fish *Tetraodon nigroviridis*, which is most likely a Cx protein, and 62.3% identity with zebrafish Cx39.9. Amino acid identity with mammalian Cxs was fairly low, 49% to mouse Cx46 and 49.6% to bovine Cx44, suggesting that there are no obvious mammalian orthologues based on sequence characteristics only.

Nucleotide based phylogenetic analyses of all full-length XlCxs revealed three subgroups, comparable to the three defined subgroups (α, β, and unnamed) in humans (White et al., 2002; Fig. 2). XlCx40.4 falls within the α-subgroup, of which XlCx43 is one of the determining members. XlCx26 falls within the β-subgroup.

Embryonic and Adult Expression of XlCx40.4

To determine the developmental expression pattern of XlCx40.4, we isolated whole embryo RNA at different developmental stages and then carried out reverse transcriptase-polymerase chain reaction (RT-PCR) using primers encompassing the entire XlCx40.4 coding region. No expression was detected before the mid-blastula transition. Expression was first evident in stage 10 of development and increased at later stages (Fig. 3A). After stage 30, relative expression decreased slightly.

RT-PCR was also carried out on several isolated adult tissues and organs. Strong XlCx40.4 expression was detected in stomach and kidney, whereas lower expression levels were detected in brain, eye, heart, and leg muscle. No expression was found in ileum, skin, colon, or lung (Fig. 3B). In addition, NCBI UniGene database information for MGC53082 provides identical ESTs derived from stage 15, stage 19–26 pooled embryos, stage 25 embryos, stage 31/32 embryos and the kidney, in agreement with our RT-PCR expression data.

To determine the distribution of XlCx40.4 during development, whole-mount in situ hybridization was carried out. In stage 19/20 embryos, specific expression was found in the paraxial mesoderm regions (Fig. 4). At later stages, XlCx40.4 expression was detected in the developing somites in a segmented pattern. This is one of the "white areas" of *Xenopus* Cx expression. Strongest expression was detected within the somites, lower expression levels were apparent in the intersomitic regions. At these stages the presomitic paraxial mesoderm also showed expression of XlCx40.4 as a continuous sheet.

Functional Characterization of XlCx40.4

To test the capacity of the cloned XlCx40.4 to form gap junctions, the Cx

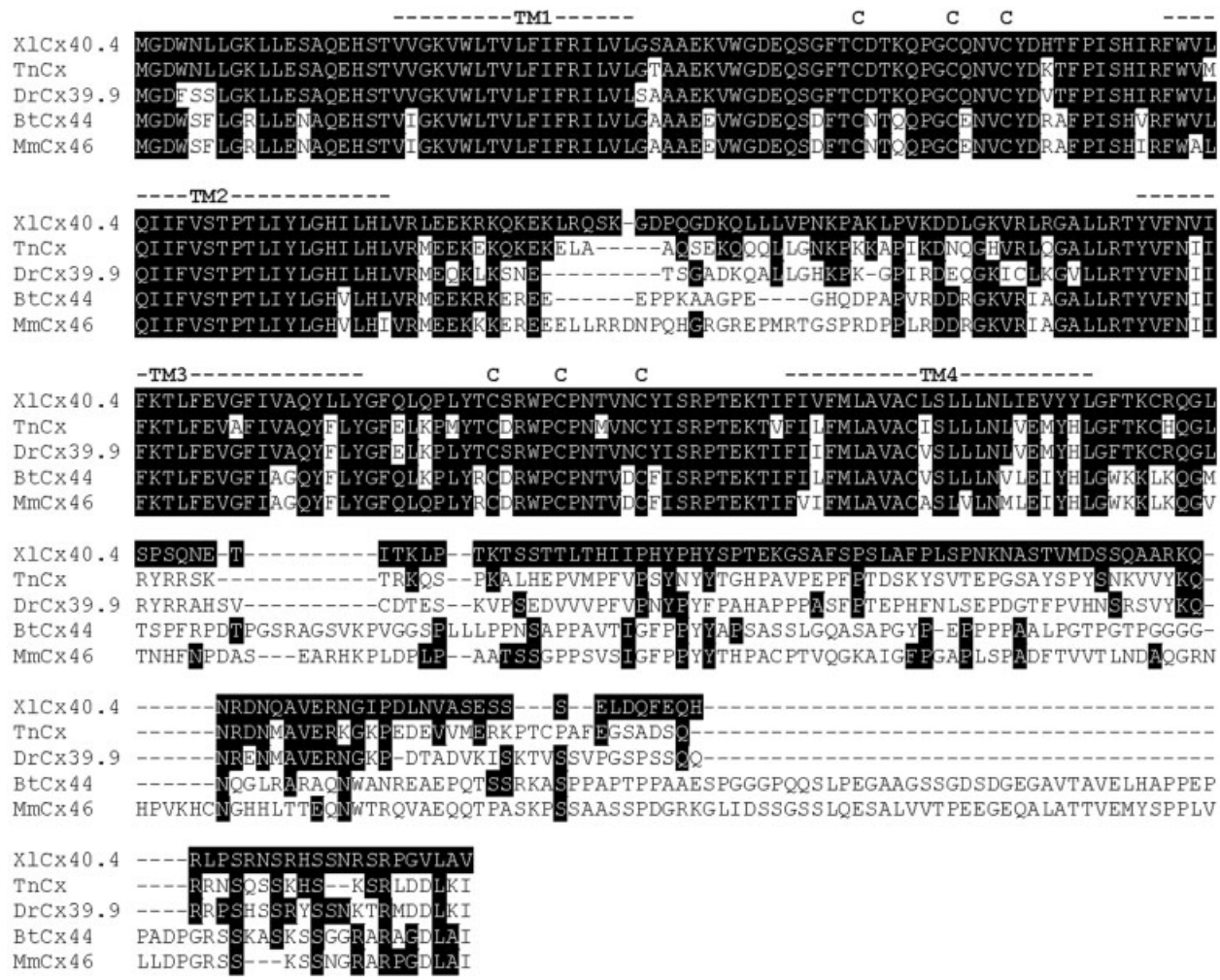

Fig. 1. Comparison of the deduced amino acid sequence of XlCx40.4 with that of *Tetraodon nigroviridis* presumed Cx protein, *Danio rerio* Cx39.9, *Bos taurus* Cx44, and *Mus musculus* Cx46. Putative transmembrane regions (TM1 to TM4) were predicted with the Web-based program TMHMM2.0 (Krogh et al., 2001) and indicated with dashed lines above the sequence. Conserved cysteine residues within the presumed extracellular loops are indicated by a "C" above the sequence. Identical amino acids between the sequences are shaded black. The GenBank accession number of *X. laevis* Cx40.4 is AY792358.

coding region was fused with the fluorescent protein HcRed and the expression construct was transfected into HEK293 cells. A proportion of the XlCx40.4-HcRed was expressed at sites of cell–cell interaction in typical gap junctional plaque-like structures (Fig. 5A,B). Furthermore, the fusion protein was found intracellularly, as often observed with Cx overexpression experiments.

Because the HcRed tag might influence the electrophysiological properties of the XlCx40.4 containing gap junctions, we cotransfected both XlCx40.4 and HcRed1 expression constructs in a 10:1 stoichiometry into communication deficient N2A cells to record coupling characteristics quantitatively. Transfected cell pairs displayed functional electrical coupling, which was sensitive to the gap junctional uncoupler halothane (Fig. 6A). Mean gap junctional conductance was 3.5 ±0.9 nS. Junctional currents displayed voltage and time-dependent inactivation in response to transjunctional potentials (Fig. 6A). The relationship between mean normalized g_{ss} and V_j followed a two-state Boltzmann distribution (Fig. 6B). For negative values of V_j, the voltage-sensitive component of g_j was half-maximal ($V_0 \pm$ SEM) at -54 ± 4 mV. The normalized voltage-insensitive component of g_j ($G_{min} \pm$ SEM) was 0.37 ± 0.03, the steepness of voltage sensitivity ($A \pm$ SEM) was -0.067 ± 0.004. The fit parameters for positive values of V_j were $V_0 = 57 \pm 4$ mV, $G_{min} = 0.44 \pm 0.03$ and $A = 0.10 \pm 0.04$. These measurements demonstrate that XlCx40.4 forms functional gap junctions.

DISCUSSION

Cx proteins are characterized by several relatively conserved regions, such as four transmembrane domains and three conserved cysteine residues in each of the two extracellular loops. Ongoing genome sequencing projects have revealed many genomic and EST sequences, which can be screened using the specific features of Cx proteins. This approach has resulted in the identification of 20 and 21 Cx isoforms in the fully sequenced mouse and human genomes, respectively (Willecke et al., 2002; Söhl and Wil-

TABLE 1. Percentage of Amino Acid Identity of XlCx40.4 With Other Connexins

Cx	Identity	GenBank accession no.
EST	99.7%	BC043801
XlCx30	30.4%	Y00791
XlCx31	29.7%	AY057997
XlCx38	39.4%	J03091
XlCx41	41.3%	U26256
XlCx43	41.4%	X17243
XlCx43.4	34.6%	AY057998
TnCx	63.3%	CAG02972
DrCx39.9	62.3%	AAN08576
BtCx44	49.6%	I46053
MmCx46	49.0%	NM_016975

EST, expressed sequence tag; Xl, *Xenopus laevis;* Tn, *Tetraodon nigroviridis;* Dr, *Danio rerio;* Bt, *Bos taurus;* Mm, *Mus musculus.*

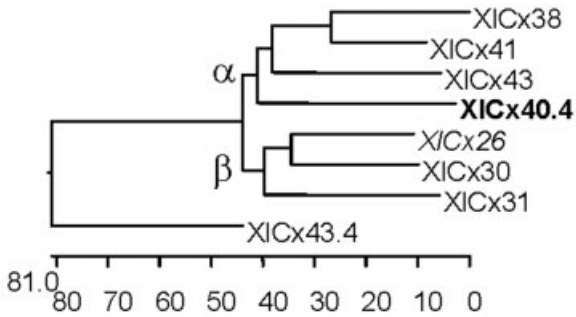

Fig. 2. Cladogram of *Xenopus laevis* Cx sequences. The nucleotide sequences of the protein coding region of 8 Cxs were analyzed using the ClustalW method of the Megalign program of the Lasergene software package DNASTAR. The scale beneath the tree measures the evolutionary distance between the sequences, and units indicate the number of substitution events.

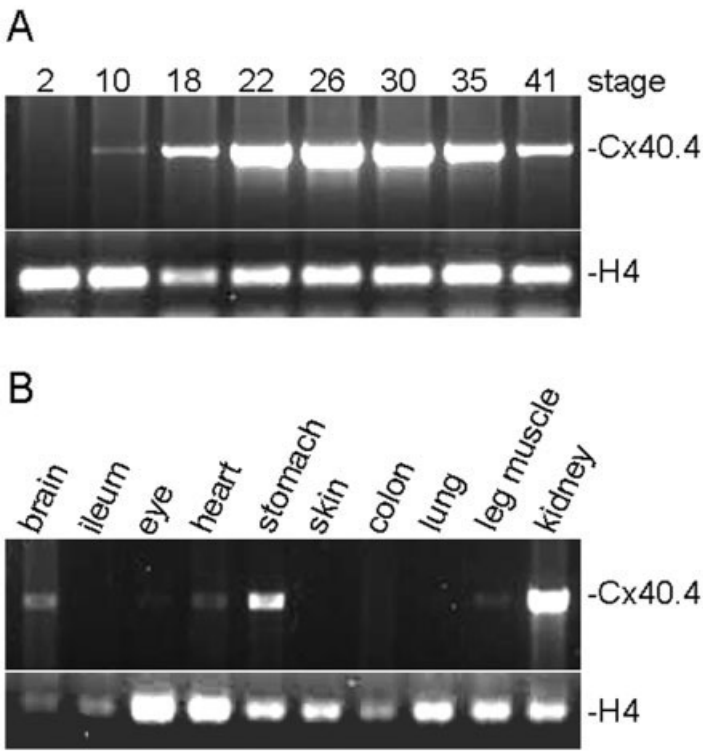

Fig. 3. Developmental and organ expression of XlCx40.4. **A:** Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *X. laevis* total RNA at different stages of development. Nieuwkoop and Faber stages are indicated at the top. **B:** RT-PCR analysis of adult *X. laevis* organ and tissue RNA. PCR with histone H4 was used as loading control.

lecke, 2003). Almost all of them have been characterized experimentally to some extent. We exploited the rapid progress in the *Xenopus* genome sequencing projects to search for new putative Cx genes in the frog. We thus found two putative full-length Cx sequences: XlCx26 and XlCx40.4. We then cloned XlCx40.4 to establish whether the putative Cx gene encoded a functionally gap junction protein.

Most abundant XlCx40.4 expression was found in the developing somites. Strongest expression was found within the somite, whereas lower expression was detected in the intersomitic regions. Unsegmented presomitic mesoderm of the developing embryo also showed expression. It was established previously that cells in the presomitic mesoderm of *Xenopus* are electrically coupled (Blackshaw and Warner, 1976). Furthermore, both intra- and intersomite coupling was observed. This finding is thought to play a role in coordinating muscle contraction initiated in neurally innervated head somites and spreading along the somites through gap junctions (Blackshaw and Warner, 1976). During maturation of the trunk muscle, in which each somite becomes independently neurally innervated, both gap junctions and electrical coupling are lost (stage 36–37; Armstrong et al., 1983). In addition, gap junctional communication is also implicated in (terminal) differentiation of skeletal muscle cells (Proulx et al., 1997; Constantin and Cronier, 2000; Araya et al., 2005).

XlCx40.4 gap junctions displayed a mild voltage dependence of macroscopic gap junctional conductance (V_0 ~55 mV) and a steady state conductance (G_{min}) of approximately 0.4. Preliminary data provided a single channel conductance between 90 and 120 pS. Gap junctional electrophysiological properties have been studied extensively in isolated somite-derived muscle cells (Chow and Poo, 1984; Chow and Young, 1987). This study has revealed single channel conductance of around 100 pS and mild voltage-dependent gating (Chow and Young, 1987; Young, 1991), values similar to our data. Thereby, XlCx40.4 could in part provide a molecular basis for the observations made in somite muscle cell studies.

Cx expression in somites has been reported previously for mammals (Cx40, Cx43, Cx45; Ruangvoravat and

Lo, 1992; Yancey et al., 1992; Dahl et al., 1995; Krüger et al., 2000), chicken (Cx36, Cx42, Cx43, and Cx45; Berthoud et al., 2004), and zebrafish (Cx45.6; Christie et al., 2004). Even though *Xenopus* Cx43 is highly homologous to mammalian and chicken Cx43 (Van der Heyden et al., 2001a, 2004) and it is expressed at many identical sites, it is absent in the developing somites of *Xenopus* (Van der Heyden et al., 2001b). XlCx40.4, therefore, is the only reported somitic Cx in the frog to date. However, given the presence of multiple somitic Cxs in mouse and chicken, additional Cxs are likely to be present in these structures in amphibians.

Cxs evolved relatively late during evolution (Stout et al., 2004; White et al., 2004). It was generally accepted until recently that connexin molecules are restricted to vertebrates. Recent evidence, however, shows the presence of functional Cxs in tunicates, invertebrate chordates (Sasakura et al., 2003; White et al., 2004). Nevertheless, several Cxs appear to have limited sequence homology, even between the different vertebrate classes, whereas others have high levels of amino acid identity. When comparing *Xenopus* Cxs already described, with those of mice, three of them display amino acid identity above 60% (XlCx30 is 67.5% identical with MmCx32; XlCx31 68.9% with MmCx31; XlCx43 85.6% with MmCx43), whereas the other four exhibit lower maximal amino acid identities (XlCx38 48.4% with MmCx37; XlCx40.4 49% with MmCx46; XlCx41 57.8% with MmCx37; XlCx43.4 51.6% with MmCx45). Similar observations are made in the studies of zebrafish Cxs (i.e., Dermietzel et al., 2000). There too, some of the zebrafish connexins display a high degree of amino

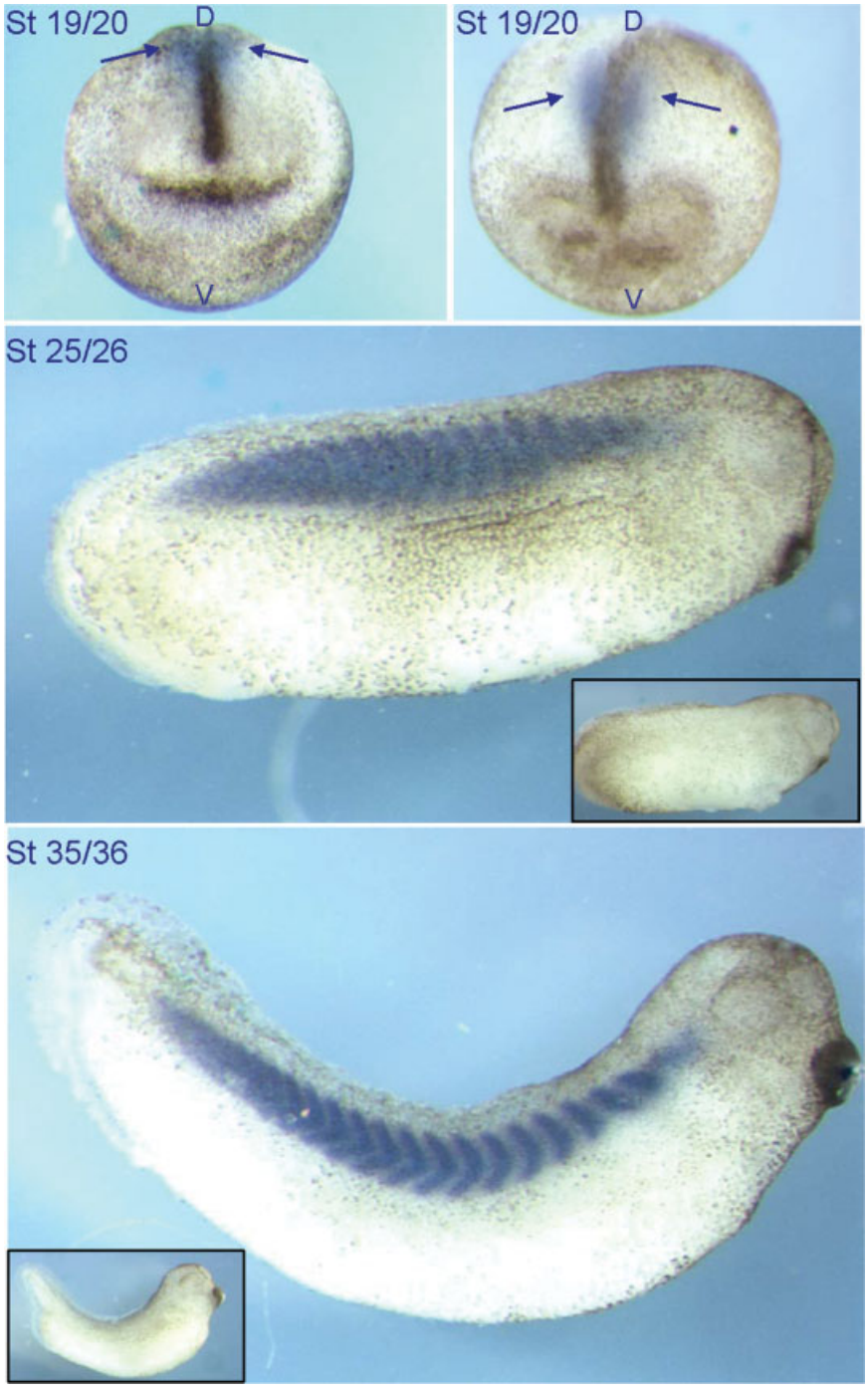

Fig. 4.

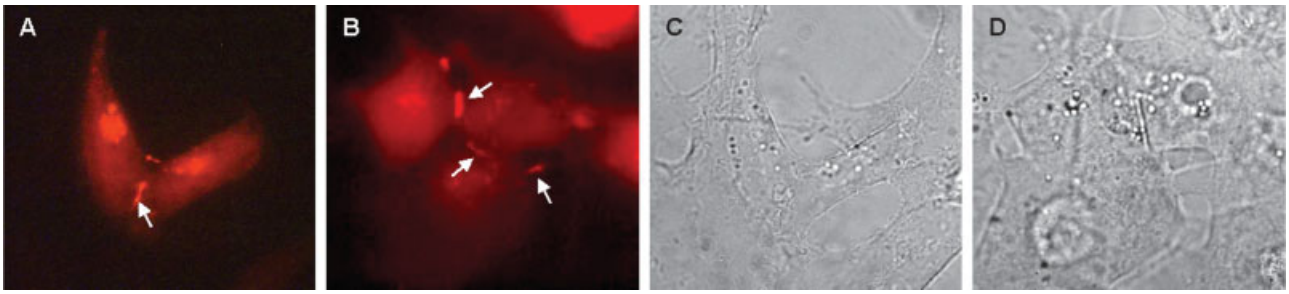

Fig. 5.

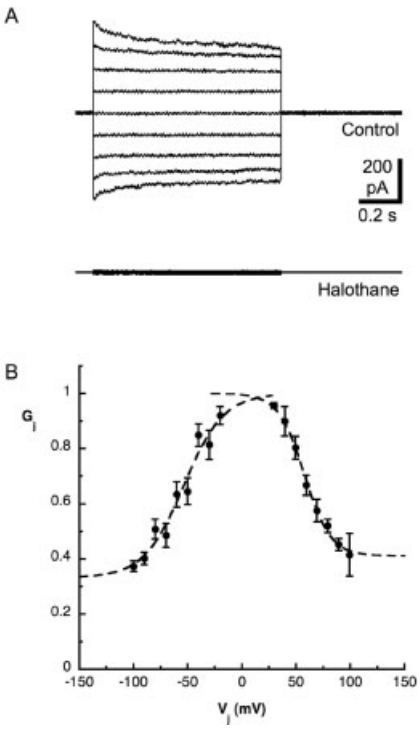

Fig. 6. Electrophysiological evaluation of XlCx40.4 gap junction channels in N2A cells. **A:** Time-dependent inactivation of junctional coupling in response to transjunctional potentials (control). Inhibition of junctional coupling by halothane (halothane). **B:** Relationship between gap junction steady-state conductance and V_j. Mean normalized steady state conductance ± SEM (g_{ss}/g_{inst}) of eight independent recordings plotted against V_j matches a two-state Boltzmann distribution.

acid identity, whereas several others show only limited amounts of amino acid identity with mammals, or higher vertebrates. As stated by others earlier for zebrafish "this observation raises the question why evolution does conserve some connexins . . . but not others?" (Zoidl et al., 2004). It seems this issue can now be extended into the *Xenopus* field. A definitive answer awaits further research. Based on sequence characteristics only, we were unable to define a genuine mammalian orthologue for XlCx40.4. On the other hand, overall amino acid identity of XlCx40.4 with a presumed connexin in the fish *Tetraodon nigroviridis* is 63.3% and with zebrafish Cx39.9, identity is 62.3%. It could be argued, therefore, that XlCx40.4 finds its orthologue in fish rather than mammals. Unfortunately, characterization of these connexins in fish is not available currently.

In addition to sequence homology, "functional orthologues" or "distinct connexins fulfilling similar functional needs" could be defined on basis of sites of expression and functional properties. XlCx40.4 displays the highest sequence homology to mammalian Cx46, which is strongly expressed in lens fiber cells (Paul et al., 1991). In the adult frog eye, we were able to detect only small amounts of XlCx40.4 by RT-PCR, whereas no expression was found in developing eyes. Based on its developmental expression pattern, XlCx40.4 could play the same role in frogs as murine Cx43 does during mouse somitogenesis. However, because Cx40 and Cx45 also display somite expression in the mouse (Dahl et al., 1995; Krüger et al., 2000), these Cxs may be considered as having a similar role as XlCx40.4. Electrophysiological characterization revealed Boltzmann parameters that are most comparable to mouse Cx40 (V_0 ~55 mV, G_{min} 0.35; Beblo et al., 1995).

We have cloned a new connexin from *Xenopus laevis* and assessed its expression pattern during development and in adult organs and tissues and determined functional properties. Our expression data suggest that XlCx40.4 plays a role in embryonic trunk muscle contraction and/or development.

Fig. 4. XlCx40.4 expression in the developing *Xenopus* embryos is present in the presomitic paraxial mesoderm and somites. Whole-mount in situ hybridization was performed on stage 19/20, stage 25/26, and stage 35/36 embryos. Upper left panel: anterior view; signal is visible in the first somites (arrows); dorsal (D) and ventral (V) sides of the embryo are indicated. Upper right panel: dorsoposterior view; signal is visible in the first somites/presomitic paraxial mesoderm (arrows); dorsal (D) and ventral (V) sides of the embryo are indicated. Middle and lower panels: right lateral view; signal is visible in the somites. No signals were found in sense negative controls (inserts).

Fig. 5. A,B: Localization of ectopically expressed XlCx40.4-HcRed fusion protein in HEK293 cells in two independent transient transfections. Arrows indicate typical gap junctional distribution of HcRed-tagged fusion protein. **C,D:** Corresponding transmission images are shown.

EXPERIMENTAL PROCEDURES

In Silico and Molecular Cloning

A *X. laevis* EST database (TIGR) was screened with amino acid sequence 1 to 228 of *X. laevis* Cx43. To clone XlCx40.4, genomic DNA was isolated from *X. laevis* heart and XlCx40.4 was amplified using the primers 5′-TGCCACTGGCTGAGATGTGA-3′ (forward) and 5′-GGTGCACCCAG-TATTTTGGC-3′ (reverse) at an annealing temperature of 52°C and 35 cycles. This revealed two products, of 1,140 and ~550 bp. The 1,140-bp product was excised from gel, purified and cloned into pGEM-T-Easy (Promega, Madison, WI), and subsequently sequenced. Alignment and phylogenetic tree construction was performed with Lasergene software (DNASTAR, Madison, WI), operating with ClustalW and Joint Neighbor algorithms.

RT-PCR Analyses

RNA was isolated from staged embryos and adult organs using Trizol (Invitrogen, Breda, The Netherlands) and reverse transcribed using oligo-dT Superscript 3 (Invitrogen). Amplification of XlCx40.4 was performed with the same primers as for the molecular cloning. For amplification of histone H4 primers 5′-CGGGATAACATT-CAGGGTATCACT-3′ (forward) and 5′-ATCCATGGCGGTAACTGTCT-TCCT-3′ (reverse; Niehrs et al., 1994) were used. Products were analyzed in 1% agarose, ethidium bromide stained gels.

Whole-Mount In Situ Hybridization

Methods of egg collection, fertilization, and embryo culture were as described by Gao et al. (1994). In situ hybridization was performed as described by Molenaar et al. (1998), using the entire *X. laevis* Cx40.4 coding region to generate riboprobes for whole-mount in situ hybridization on albino *X. laevis* embryos.

Transfection

For fluorescence microscopy, the coding region of XlCx40.4 (including 26 upstream nucleotides from the start codon) was cloned in frame in front of HcRed in pHcRed1-N1 (BD Biosciences, Palo Alto, CA). The XlCx40.4-HcRed fusion construct was subsequently transfected in HEK-293 cells

using Lipofectamin (Invitrogen) overnight. For electrophysiology, XlCx40.4 was cloned into pcDNA3 (BD Biosciences) and was transiently cotransfected with pHcRed1-N1 for detection of transfected cells, in a 10:1 stochiometry into N2A cells using Lipofectamin for 5 hr.

Electrophysiology

A symmetrical setup of two Axopatch 200B amplifiers (Axon Instruments) was used to measure gap junctional conductance between cell pairs with the double voltage clamp technique (Van Rijen et al., 2001). In voltage clamp mode, both cells were clamped at a holding potential of 0 mV. Macroscopic junctional currents (I_j) were elicited by applying square voltage pulses of varying amplitude lasting 1 second to one cell of a pair. These transjunctional voltage changes (V_j) ranged from −100 to +100 mV, with 10 or 20 mV increments. Data was on-line filtered at 0.5 or 1.0 kHz using custom-made filters and digitized at 10 kHz using custom data acquisition (Scope) and data analysis (MacDaq) programs. Plots were made using KaleidaGraph (Synergy Software).

The junctional conductance ($g_j = I_j/V_j$) at the end of the pulses (g_{ss}) was normalized to the instantaneous conductance (g_{inst}) at the beginning of the pulse. Plots of mean normalized g_{ss} as a function of negative and positive values of V_j were fit separately with a two-state Boltzmann distribution ($G_{ss} = (1 - G_{min})/(1 + \exp(A(V_j - V_0))) + G_{min}$). Differences between mean fit parameters (G_{min}, V_0, A) were tested with the paired-sample Student's *t*-test, significance was assumed if $P < 0.05$.

Composition of solutions is in millimoles per liter. Patch pipette solution: potassium gluconate 125, KCl 10, HEPES 5, EGTA 5, $MgCl_2$ 2, $CaCl_2$ 0.6, Na_2ATP 4, adjusted to pH 7.2 with KOH. Extracellular solution: NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 6, HEPES 15, $NaHCO_3$ 17.5, adjusted to pH 7.2 with NaOH. Patch pipettes were firepolished and had a resistance of 2–5 MΩ when filled with the pipette solution. All experiments were done at 20°C. For blocking gap junctional conductance, the saturated supernatant of a halothane/extracellular solution mixture was added 1:3 to the extracellular solution and subsequently infused into the recording chamber.

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