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Cloning, embryonic expression, and functional characterization of two novel connexins from *Xenopus laevis* ☆,☆☆

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Abstract

Vertebrate gap junctions are constituted of connexin (Cx) proteins. In *Xenopus laevis*, only seven different Cxs have been described so far. Here, we identify two new Cxs from *X. laevis*. Cx28.6 displays >60% amino acid identity with human Cx25, Cx29 displays strong homology with mouse Cx26 and Cx30. Cx29 is expressed throughout embryonic development. Cx28.6 mRNA is only transiently found from stage 22 to 26 of development. While no Cx28.6 expression could be detected by whole mount *in situ* hybridization, expression of Cx29 was found in the developing endoderm, lateral mesoderm, liver anlage, pronephros, and proctodeum. Ectopic expression of Cx28.6 failed to produce functional gap-junctions. In contrast, ectopic expression of full-length Cx29 in HEK293 and COS-7 cells resulted in the formation of gap junction-like structures at the cell–cell interfaces. Ectopic expression of Cx29 in communication deficient N2A cell pairs led to functional electrical coupling.
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Gap junctions are aggregates of intercellular communication channels connecting the cytoplasm of adjacent cells. Multicellular animal species have acquired gap-junctions to enable the direct exchange of small signaling molecules, metabolites, and ions between cells. Invertebrates mainly use innexin proteins to constitute their gap-junctions [1]. Chordate species, however, make use of a sequence unrelated class of proteins named connexins (Cxs) as gap-junction building blocks [2]. Recently, a third class of gap-junction proteins was exposed, called pannexins [3]. It became clear that pannexins are expressed in both vertebrates and non-vertebrates. Genome screens demonstrated, however, the presence of only three different pannexins, in contrast to the great numbers of innexins and Cxs. Therefore, although speculative, the different Cxs probably have evolved to encounter the specific requirements of cell–cell communication in more complex organisms, in addition to the pannexins.

To form a gap-junction, a hexamer of innexins or Cxs, the latter is named a connexon, docks at a similar cluster on an adjacent cell thereby forming a functional intercellular channel [4]. On the other hand, connexons by themselves may form functional hemichannels in non-junctional plasmamembranes, which, among other functions, are thought to be involved in osmoregulation [5].

☆ DNA sequences in this report have been deposited at GenBank (Accession Nos.: DQ180493 (Cx28.6) and DQ180492 (Cx29)).
☆☆ *Abbreviations:* Cx, connexin; ORF, open reading frame; EST, expressed sequence tag; WMISH, whole-mount in situ hybridization.
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Currently, at least 21 different Cxs isoforms have been identified and many of them are cloned from mouse, human, and zebrafish, all species of which the genome sequence program has been completed. In contrast, the number of cloned *Xenopus* Cxs lags behind with only 7 (reviewed in [6]). In analogy with mammals and fish, many more may be expected to exist, and we and others already identified several of them in the *Xenopus* EST databases [6,7]. As the *X. laevis* genome sequencing program progresses rapidly, we set out to identify and molecularly clone additional Cxs from this species, in order to enable studying their role in amphibian development and physiology, and to resolve the enigmatic evolution of Cxs.

We now have cloned two new Cxs from *X. laevis*, named by their predicted molecular weight Cx28.6 and Cx29. Cx28.6 resembles human Cx25, and is only weakly and very transiently expressed during development. Upon ectopic expression neither full length protein, nor gap-junction like channels were found. We therefore conclude that Cx28.6 is a non-functional or pseudogene. In contrast, Cx29 displays homology to mouse Cx26 and Cx30, becomes prominently expressed throughout development in endodermal and mesodermal derivatives, e.g., the pronephros, proctodeum, and liver anlage. Furthermore, this Cx forms functional gap junction channels upon ectopic expression in mammalian cell lines.

Materials and methods

In silico and molecular cloning. A *X. laevis* EST database (TIGR) was screened with amino acid sequence 1–228 of *X. laevis* Cx43.

To clone full-length Cx28.6, PCR on cDNA of stage 25 *X. laevis* embryos was performed using the primers 5′-GTCGTGAGATGA GTTGGTCA-3′ (forward) and 5′-CCCTGAGGTATTGAGTTATT-3′ (reverse) encompassing start and stop codon, respectively, at an annealing temperature of 51 °C and 35 amplification cycles. This revealed one product of 780 bp. The product was cloned into pGEM-T (Promega, Madison, WI, USA) and subsequently sequenced.

Full-length Cx29 was cloned from *X. laevis* liver cDNA by PCR using the primers 5′-CGTAACAATGGATTGGGGAA-3′ (forward) and 5′-C TCCATATGAATCATTGGGG-3′ (reverse) encompassing start and stop codon, respectively, at an annealing temperature of 51 °C and 35 cycles. This revealed one product of 820 bp. The product was cloned into pGEM-T-easy (Promega, Madison, WI, USA) and subsequently sequenced.

Alignment and phylogenetic tree construction were performed with MEGA version 3.1 [8] operating with ClustalW and Joint-Neighbor algorithms. Support for each node was determined by interior-branch test.

Xenopus embryo handling and RT-PCR analyses. *Xenopus laevis* embryos were obtained by *in vitro* fertilization and raised in 25% MMR (modified Marc's ringer) at 18 °C. Embryos were staged according to Nieuwkoop and Faber [9]. RNA was isolated from staged embryos using Trizol (Invitrogen, Breda, The Netherlands) and reverse transcribed using oligo(dT) Superscript 3 (Invitrogen). Amplification of Cx28.6 and Cx29 was performed using primers 5′-ACCCTCGCTTCTTGTTGTTC-3′ (Cx28.6 forward), 5′-GTGGTTCTTGCAATCACAGC-3′ (Cx28.6 reverse), 5′-TCACCTGCAACACCTTACAG-3′ (Cx29 forward) and 5′-A AGGCAGCTTCAAAGAGGAC-3′ (Cx29 reverse) at an annealing temperature of 53 °C for 35 amplification cycles. For amplification of histone H4 primers 5′-CGGGATAACATTCAGGGTATCACT-3′ (forward) and 5′-ATCCATGGCGGTAACTGTCTTCCT-3′ (reverse) [10] were used at an annealing temperature of 57 °C and 35 amplification cycles. Products were subsequently analyzed in 1% agarose, ethidium bromide-stained gels.

Whole-mount in situ hybridization. Embryos were fixed in MEMPFA (100 mM Mops (Sigma), pH 7.4; 2 mM EGTA (Sigma); 1 mM $MgSO_4$ (Merck); 4% (w/v) paraformaldehyde (Sigma)) at different developmental stages. For generation of full-length Cx28.6 and Cx29 antisense probes, pGEM-T-Cx28.6 and pGEM-T-Cx29 plasmids were linearized and used as templates for synthesis of DIG-labeled antisense RNA (Roche). RNA probe was purified using RNeasy columns (QIAGEN). Whole mount *in situ* hybridization (WMISH) was performed as described by Harland [11]; hybridization was performed with a probe concentration of 100 ng/mL at 65 °C. For controls, WMISH with similarly produced sense probes was performed. After WMISH the embryos were stained using BM purple (Roche) followed by re-fixation in MEMPFA. To dissipate the embryo's pigmentation, embryos were treated in $K_2Cr_2O_7$ (0.1 M in 5% acetic acid), washed thoroughly in 1× TBS (25 mM Tris, pH 7.4, 136 mM NaCl, and 2.7 mM KCl), and were subsequently bleached in 1× TBS with 5% H_2O_2 under a light source.

Transfection. For fluorescence microscopy, the coding region of Cx28.6 (including the 8 (Cx28.6) and 7 (Cx29) upstream nucleotides from the start codon) was PCR amplified using primers T7 and 5′-TTG GATCCAGCCTGTAACCATCAGGTGCAC-3′ for Cx28.6 and 5′-TTG GATCCTCGGGGTAATGTTCTTTCTGT-3′ for Cx29 (reverse and *Bam*HI restriction site attached) from pGEM-T-Cx28.6 or pGEM-T-easy-Cx29, respectively, thereby deleting the stop codon, and cloned in-frame using *Apa*I/*Bam*HI in front of EGFP in pEGFP-N1 (BD Biosciences, Palo Alto, CA, USA). All constructs were verified by sequencing. The Cx28.6- and Cx29-EGFP fusion constructs were subsequently transfected in HEK293 for localization studies and COS-7 cells for biochemical studies using Lipofectamine2000 (Invitrogen) overnight.

For electrophysiological studies, the Cx29 coding region from pGEM-T-easy-Cx29 was cloned into the *Eco*RI site of pcDNA3 (BD Biosciences) and was transiently co-transfected with pCMV-EGFP for detection of transfected cells, in a 2:1 stoichiometry into communication deficient N2A cells using Lipofectamine2000 for 5 h.

Western blotting. Cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM Na_2HPO_4, 1% (v/v) Triton X-100, 1% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, and 10 μg/ml aprotinin). Lysates were clarified by centrifugation at 14,000*g* for 5′ at 4 °C. Twenty micrograms of protein lysate was mixed with Laemmli sample buffer and proteins were separated by 12.5% SDS–PAGE and subsequently electro-blotted onto nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands). For Western blot detection of the EGFP-tagged Cxs, EGFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a peroxidase labeled secondary antibody (Jackson) were visualized by standard ECL procedures (Amersham Bioscience, Roosendaal, The Netherlands).

Electrophysiology. Double voltage clamp was essentially performed as described before [12]. Measurements of gap-junctional coupling were done in the whole-cell patch clamp configuration using a HEKA EPC 10 double plus amplifier. 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 1.5 s to one cell of a pair. These transjunctional voltage changes (V_j) ranged from −100 to +100 mV, with 10 or 20 mV increments. The junctional conductance ($g_j = I_j/V_j$) at the end of the pulse (g_{ss}) was normalized to the instantaneous conductance (g_{inst}) at the beginning of the pulse. Plots of mean normalized g_{ss} (G_{ss}), as a function of either negative or positive V_j, were fit with a two-state Boltzmann distribution ($G_{ss} = (1 - G_{min})/(1 + \exp(A(V_j - V_0))) + G_{min}$), in which G_{min} denotes the voltage insensitive component of G_j.

Extracellular buffer was a modified Tyrode's solution, containing (in mmol/L) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes 15, $NaHCO_3$ 35, glucose 6, $BaCl_2$ 1, $CsCl_2$ 2, $NiCl_2$ 6, pH 7.20/NaOH. Pipette buffer contained (in mmol/L) $CsCl_2$ 120, TEACl 10, Hepes 5, EGTA 10, $MgCl_2$ 3, $CaCl_2$ 1, Na_2ATP 2, pH 7.20/CsOH. Pipettes were pulled using a Sutter P-2000 puller and had an average resistance of 3.2 ± 0.1 MΩ ($n = 14$). For blocking gap junctional conductance, the saturated supernatant of a

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