Molecular Cloning and Functional Expression of Human Connexin37, an Endothelial Cell Gap Junction Protein

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Abstract

Gap junctions allow direct intercellular coupling between many cells including those in the blood vessel wall. They are formed by a group of related proteins called connexins, containing conserved transmembrane and extracellular domains, but unique cytoplasmic regions that may confer connexin-specific physiological properties. We used polymerase chain reaction amplification and cDNA library screening to clone DNA encoding a human gap junction protein, connexin37 (Cx37). The derived human Cx37 polypeptide contains 333 amino acids, with a predicted molecular mass of 37,238 D. RNA blots demonstrate that Cx37 is expressed in multiple organs and tissues (including heart, uterus, ovary, and blood vessel endothelium) and in primary cultures of vascular endothelial cells. Cx37 mRNA is coexpressed with connexin43 at similar levels in some endothelial cells, but at much lower levels in others. To demonstrate that Cx37 could form functional channels, we stably transfected communication-deficient Neuro2A cells with the Cx37 cDNA. The induced intercellular channels were studied by the double whole cell patch clamp technique. These channels were reversibly inhibited by the uncoupling agent, heptanol (2 mM). The expressed Cx37 channels exhibited multiple conductance levels and showed a pronounced voltage dependence. These electrophysiological characteristics are similar to, but distinct from, those of previously characterized connexins. (*J. Clin. Invest.* 1993. 91:997–1004.) Key words: gap junction • endothelium • intercellular communication • electrophysiology • ion channel

Introduction

Gap junctions are plasma membrane specializations containing intercellular channels that coordinate tissue function by allowing cell-to-cell passage of ions and small molecules. Biochemical and molecular studies have demonstrated that gap junction channels are formed by members of a family of proteins called connexins (1). Topological studies suggest that the extracellular and transmembrane regions of all connexins are highly conserved, whereas the cytoplasmic domains are nearly unique to each protein. These connexin-specific sequences may provide unique physiological channel properties. Thus, the channels derived from cloned connexin DNAs expressed in *Xenopus* oocytes or transfected cells show different biophysical characteristics, including unitary conductance and voltage dependence (2–9).

We have been trying to characterize molecularly the gap junctions between cells of the cardiovascular system, where they play crucial roles in electrical and metabolic function. We have shown that chick heart cells and canine cardiac myocytes express three different gap junction proteins, connexin43 (Cx43),[1] connexin45 (Cx45), and connexin40 (Cx40, dog) or connexin42 (Cx42, chick) (10, 11). These multiple cardiac myocyte connexins likely account for the multiple gap junction channels and voltage-dependent properties observed in these cells (12, 13).

The cells of the blood vessel wall also contain gap junctions and express a multiplicity of connexins. Larson et al. (14) reported that endothelial cells, vascular smooth muscle cells, and pericytes all express the same gap junction protein, Cx43. Lash et al. (15) cloned Cx43 cDNA from bovine aortic smooth muscle cells. However, Moore et al. (16) recently demonstrated that A7r5 rat aortic smooth muscle cells express two physiologically distinct gap junctional channels, and Beyer et al. (17) demonstrated that these cells express two connexins, Cx43 and Cx40.

In the present study, we have identified and molecularly characterized a novel connexin, human connexin37 (Cx37), which is expressed in another component of the blood vessel wall, the endothelial cell. In fact, in some endothelial cells, the steady state mRNA levels for Cx37 are similar to those for Cx43, suggesting that this may be a relatively abundant connexin. In addition, we have demonstrated that the protein encoded by this sequence can form functional cell-to-cell channels. Electrophysiological characterization of those channels demonstrates that they have unique voltage-dependence and unitary conductance properties.

Methods

DNA cloning and sequencing. The initial fragment of mouse Cx37 was amplified from mouse genomic DNA isolated from J774 cells as described in Beyer and Steinberg (18). We used the PCR (19) with a degenerate/consensus primer corresponding to a conserved sequence in the first connexin extracellular loop (AACACTCTGCAGCCTGGC-TGT$^{A}/_{G}$A^{G}/C/$_{A}$AACGTCTGCTA$^{C}/_{T}$GAC) and an antisense primer corresponding to the second connexin extracellular loop (AGCATG-ATGATCATGAAGA$^{T}/_{C}$GGT$^{T}/_{C}$TCNGTGGG). Restriction sites (XhoI and BclI, respectively) were incorporated into the primers to facilitate subcloning into Bluescript (Stratagene, Inc., San Diego, CA) or pGEM (Promega, Corp., Madison, WI) vectors. Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. 30 cycles were conducted.

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A cDNA library in lambda gt11 prepared from human umbilical vein cells (20) was a gift from Dr. J. Evan Sadler (Washington University, St. Louis, MO). The cDNA library was screened by hybridization with the DNA fragment of mouse Cx37 according to Beyer et al. (21) to isolate the full-length human sequence.

DNA sequencing was performed using plasmid templates, Sequenase enzyme (U.S. Biochemical Corp., Cleveland, OH), and oligonucleotide primers as previously described (10). Both strands of the full-length Cx37 cDNA were fully sequenced. DNA sequence acquisition and initial analysis was performed using software (Microgenie; Beckman Instruments, Palo Alto, CA) running on an IBM-compatible microcomputer (22). Protein sequence alignments and comparisons were performed using the CLUSTAL program (23) with the PC Gene software (IntelliGenetics, Mountain View, CA).

DNA and RNA blots. Human genomic DNA was a gift from Dr. Morey Blinder (Washington University, St. Louis, MO) or was purchased from Promega Corp. The genomic DNA was digested with restriction enzymes, electrophoresed in 1% agarose gels, and transferred to nylon membranes as previously described (24).

For RNA analyses, most tissues were obtained from normal adult rats and mice. Uteri were obtained from 19-d pregnant rats; ovarian tissue was obtained from 21-d-old female rats stimulated with pregnant mare's serum gonadotropin as in Beyer et al. (21). Essentially pure endothelial tissue was prepared by razor blade scrapings of bovine (calf) aortas as in Larson et al. (14). These initial scrapings are contaminated only by some blood cells (red blood cells and leukocytes) trapped in adherent fibrin clots. Scrapings prepared in this manner are used to initiate primary cultures of bovine aortic endothelial cells; such cultures contain no morphologically detectable smooth muscle cells. Vital labeling with DiI-Ac-LDL (as in reference 25) shows uniform endothelial identification of such cultures (purity must be > 99.9%; Larson, D. M., unpublished data).

Total cellular RNA was prepared from cells or tissues according to Chomczynski and Sacchi (26). RNA was separated on formaldehyde/agarose gels and transferred to nylon membranes as previously described (21). Hybridization was performed using specific ^{32}P-labeled DNA probes prepared using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (10). In one experiment, 25 ng of the rat Cx43 cDNA and 25 ng of the human Cx37 cDNA were separately labelled and then mixed and hybridized with the blot together to compare relative abundances. In some control experiments, blots were also hybridized with probes for rat Cx40 or human fibroblast γ-actin as in previous studies (17, 18).

Cell cultures. Mouse Neuro2A (N2A) neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD). N2A cells were grown in MEM (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% heat inactivated (56°C for 30 min) fetal calf serum (JRH Biosciences, Lenexa, KS), 1× non-essential amino acids (GIBCO/BRL), 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO/BRL). A7r5 and other cell lines were grown in DME (GIBCO) supplemented with 10% fetal calf serum (JRH Biosciences) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO/BRL). Primary cultures of bovine aortic, bovine retinal microvascular, and human umbilical vein endothelial cells, rat aortic smooth muscle cells, and bovine retinal pericytes were isolated and cultured as described previously (14).

Connexin cDNA transfection. The full-length human Cx37 cDNA was cloned into the EcoRI site of pSFFV-neo (27) N2A cells in 60-mm dishes were transfected with 20 μg linearized plasmid using the lipofectin reagent (GIBCO/BRL) according to the manufacturer's directions, and stable, neomycin-resistant colonies were selected in 0.5 mg/ml G418 (GIBCO/BRL). Connexin expression was verified by Northern blotting of total RNA prepared from selected clones.

Electrophysiological studies. Cx37-induced coupling in the transfected N2A cells was studied by double whole-cell recording procedures identical to those described previously (28). Patch electrodes had resistances of 2–4 MΩ when filled with a solution with the following composition (mM): 100 K glutamate, 15 NaCl, 1 KH_2PO_4, 4.6 $MgCl_2$, 0.68 $CaCl_2$, 5 EGTA, 3 Na_2ATP, 3 Na_2Phosphocreatine, 25 Hepes, pH 7.1. The cells were bathed in a solution with the following composition (mM): 142 NaCl, 1.3 KCl, 0.8 $MgSO_4$, 0.9 NaH_2PO_4, 1.8 $CaCl_2$, 5.5 dextrose, 10 Hepes, pH 7.2. All experiments were performed at room temperature (20–22°C). Transjunctional voltages were elicited by stepping the holding potential of cell 1 (V_1) from a common value ($V_1 = V_2 = -40$ mV) to a new value in 10 mV increments between −140 and +60 mV. 6-s duration pulses were applied once every 21 s, except when channel activity was observed and pulse durations were increased to 10 s. Junctional current was taken as the change in holding current of the nonpulsed cell (I_2), since the I_1 trace contains a nonjunctional membrane current associated with the change in V_1 in addition to the negative of the I_2 signal.

Results

Cloning of human Cx37. All connexins studied to date have single copy genes that lack introns in their coding sequences (29, 30) and that contain highly conserved sequences corresponding to four transmembrane and two extracellular domains within the proteins (1). We took advantage of these characteristics and used the PCR to attempt to isolate novel connexin sequences. Using oligonucleotide primers corresponding to the two conserved extracellular regions, we amplified several 350–500-bp sequences from mouse genomic DNA that were subcloned and sequenced. One 456-bp sequence clearly corresponded to a portion of a previously unidentified connexin. (The recently reported sequence of Willecke et al. [31] demonstrates that it corresponds to nucleotides 174–630 of mouse Cx37.) High stringency Northern blots performed using this sequence as probe identified an mRNA of ~ 1.7 kb, which was expressed in many different tissues, but was particularly abundant in endothelial cells (see Fig. 4).

Therefore, to obtain the full-length sequence for this connexin, we used this mouse DNA fragment as probe to screen a cDNA library prepared from human umbilical vein endothelial cells. This cDNA appeared to be relatively abundant in the library, since on rescreening with part of the human sequence we obtained 18 positives from ~ 200,000 plaques. Portions of four different clones were sequenced; all overlapped without discrepancies. The sequence of this human connexin cDNA is shown in Fig. 1. It contains 1,601 nucleotides and ends with a poly(A) tail preceded by the poly-adenylation signal AATAAA (32) at bases 1572–1577. There is a long open reading frame that begins with the first ATG (contained within an appropriate consensus for translational initiation) (33) at base 65. This human cDNA sequence encodes a polypeptide of 333 amino acids with a predicted molecular mass of 37,238 D, which we have therefore termed human Cx37.

Comparison of human Cx37 to the amino acid sequences of other connexins (Fig. 2) demonstrates that this protein is clearly related to other connexins. It is most similar to the Cx37 recently reported from mouse (31) and rat (34); human Cx37 contains 86% identical amino acids to each of these proteins (which are 98% identical to each other). Most of the differ-

1. *Abbreviations used in this paper:* Cx, connexin; g_j, junctional conductance; G_{ss}, normalized steady state junctional conductance; I_1, holding current of cell 1; I_2, holding current of the nonpulsed cell (cell 2); I_j, junctional current; V_1, holding potential of cell 1; V_2, holding potential of cell 2.

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     CTCCGGCCATCGTCCCCACCTCCACCTGGGCCGCCCGCGAGGCAGCGGACGGAGGCCGGG   60

     AGCCATGGGTGACTGGGGCTTCCTGGAGAAGTTGCTGGACCAGGTCCGAGAGCACTCGAC  120
        M  G  D  W  G  F  L  E  K  L  L  D  Q  V  R  E  H  S  T
     CGTGGTGGGTAAGATCTGGCTGACGGTGCTCTTCATCTTCCGCATCCTCATCCTGGGCCT  180
 20  V  V  G  K  I  W  L  T  V  L  F  I  F  R  I  L  I  L  G  L
     GGCCGGCGAGTCAGTGTGGGGTGACGAGCAGTCAGATTTCGAGTGTAACACGGCCCAGCC  240
 40  A  G  E  S  V  W  G  D  E  Q  S  D  F  E  C  N  T  A  Q  P
     AGGCTGCACCAACGTCTGCTATGACCAGGCCTTCCCCATCTCCCACATCCGCTACTGGGT  300
 60  G  C  T  N  V  C  Y  D  Q  A  F  P  I  S  H  I  R  Y  W  V
     GCTGCAGTTCCTCTTCGTCAGCACACCCACCCTGGTCTACCTGGGCCATGTCATTTACCT  360
 80  L  Q  F  L  F  V  S  T  P  T  L  V  Y  L  G  H  V  I  Y  L
     GTCTCGGCGAGAAGAGCGGCTGGCGCAGAAGGAGGGGGAGCTGCGGGCACTGCCGGCCAA  420
100  S  R  R  E  E  R  L  A  Q  K  E  G  E  L  R  A  L  P  A  K
     GGACCCACAGGTGGAGCGGGCGCTGGCCGGCATAGAGCTTCAGATGGCCAAGATCTCGGT  480
120  D  P  Q  V  E  R  A  L  A  G  I  E  L  Q  M  A  K  I  S  V
     GGCAGAAGATGGTCGCCTGCGCATTCCGCGAGCACTGATGGGCACCTATGTCGCCAGTGT  540
140  A  E  D  G  R  L  R  I  P  R  A  L  M  G  T  Y  V  A  S  V
     GCTCTGCAAGAGTGTGCTAGAGGCAGGCTTCCTCTATGGCCAGTGGCGCCTGTACGGCTG  600
160  L  C  K  S  V  L  E  A  G  F  L  Y  G  Q  W  R  L  Y  G  W
     GACCATGGAGCCCGTGTTTGTGTGCCAGCGAGCACCCTGCCCCTACCTCGTGGACTGCTT  660
180  T  M  E  P  V  F  V  C  Q  R  A  P  C  P  Y  L  V  D  C  F
     TGTCTCTCGCCCCACGGAGAAGACCATCTTCATCATCTTCATGTTGGTGGTTGGACTCAT  720
200  V  S  R  P  T  E  K  T  I  F  I  I  F  M  L  V  V  G  L  I
     CTCCCTGGTGCTTAACCTGCTGGAGTTGGTGCACCTGCTGTGTCGCTGCCTCAGCCGGGG  780
220  S  L  V  L  N  L  L  E  L  V  H  L  L  C  R  C  L  S  R  G
     GATGAGGGCACGGCAAGGCCAAGACGCACCCCCGACCCAGGGCACCTCCTCAGACCCTTA  840
240  M  R  A  R  Q  G  Q  D  A  P  P  T  Q  G  T  S  S  D  P  Y
     CACGGACCAGGGTCTTCTTCTACCTCCCCGTGGCCAGGGGCCCTCATCCCCACCATGCCC  900
260  T  D  Q  G  L  L  L  P  P  R  G  Q  G  P  S  S  P  P  C  P
     CACCTACAATGGGCTCTCATCCAGTGAGCAGAACTGGGCCAACCTGACCACAGAGGAGAG  960
280  T  Y  N  G  L  S  S  S  E  Q  N  W  A  N  L  T  T  E  E  R
     GCTGGCGTCTTCCAGGCCCCCTCTCTTCCTGGACCCACCCCCTCAGAATGGCCAAAAACC 1020
300  L  A  S  S  R  P  P  L  F  L  D  P  P  P  Q  N  G  Q  K  P
     CCCAAGTCGTCCCAGCAGCTCTGCTTCTAAGAAGCAGTATGTATAGAGGCCTGTGGCTTA 1080
320  P  S  R  P  S  S  S  A  S  K  K  Q  Y  V  -

     TGTCACCCAACAGAGGGGTCCTGAGAAGTCTGGCTGCCTGGGATGCCCCCTGCCCCCTCC 1140
     TGGAAGGCTCTGCAGAGATGACTGGGCTGGGGAAGCAGATGCTTGCTGGCCATGGAGCCT 1200
     CATTGCAAGTTGTTCTTGAACACCTGAGGCCTTCCTGTGGCCCACCAGGCACTACGGCTT 1260
     CCTCTCCAGATGTGCTTTGCCTGAGCACAGACAGTCAGCATGGAATGCTCTTGGCCAAGG 1320
     GTACTGGGGCCCTCTGGCCTTTTGCAGCTGATCCAGAGGAACCCAGAGCCAACTTACCCC 1380
     AACCTCACCCTATGGAACAGTCACCTGTGCGCAGGTTGTCCTCAAACCCTCTCCTCACAG 1440
     GAAAAGGCGGATTGAGGCTGCTGGGTCAGCCTTGATCGCACAGACAGAGCTTGTGCCGGA 1500
     TTTGGCCCTGTCAAGGGGACTGGTGCCTTGTTTTCATCACTCCTTCCTAGTTCTACTGTT 1560
     CAAGCTTCTGAAATAAACAGGACTTGATCACAAAAAAAAAA                     1601
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Figure 1. Sequence of human Cx37. The nucleotide sequence determined from the human umbilical vein cDNA is shown in light type with residues numbered on the right. The derived amino acid sequence is shown in boldface with residues numbered on the left. The putative polyadenylation signal AATAAA is underlined. These sequence data are available from EMBL/Genbank under accession number M96789.

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Human Cx37    MGDWGFLEKLLDQVREHSTVVGKIWLTVLFIFRILILGLAGESVWGDEQS    50
Mouse Cx37    ::::::::::::::Q::::::::::::::::::::::::::::::::::    50
Xenopus Cx38  :AG:EL:KL:::D:Q::::LI::V:::::::::F::SV::::::T::::    50
Rat Cx43      ::::SA:G::::K:QAY::AG::V::S::::::::L::T:V::A::::::    50

Human Cx37    DFECNTAQPGCTNVCYDQAFPISHIRYWVLQFLFVSTPTLVYLGHVIYLS   100
Mouse Cx37    ::::::::::::::::::::::::::::::::::::::I:::::::::    100
Xenopus Cx38  ::I:::Q::::::::::::::::V::::::::::::::::T:::MV:::   100
Rat Cx43      A:R:::Q::::E:::::KS:::::V:F::::II:::V:::L::A::F:VM   100

Human Cx37    RREERLAQKEGELRALPAKDPQVERALAGIELQMAKISVAEDGRLRIPRA   150
Mouse Cx37    ::::::R:::::::::::S::LH::::::A::H:::::::::::::::RG:   150
Xenopus Cx38  KK::KER:::N:S:I:V:NEA:T:------------VYSSATKKI::QGP   138
Rat Cx43      :K::::NK::E::KVAQTDGVN::MH:KQ::IKKF:YGIE:H:KVKMRGG   150

Human Cx37    LMGTYVASVLCKSVLEAGFLYGQWRLYGWTMEPVFVCQRAPCPYLVDCFV   200
Mouse Cx37    ::::::V::::::::::::::::::::::::::::::::::::HI:::Y:   200
Xenopus Cx38  ::C::TT::VF::IF::::L:::YI::FV:S:I:::E:I::KHK:E:::   188
Rat Cx43      :LR:::II:I:F:::F:VA::LI::YI::FSLSA:YT:K:D:::HQ:::L   200

Human Cx37    SRPTEKTIFIIFMLVVGLISLVLNLLELVHLLCRCLSRGMRARQGQDAPP   250
Mouse Cx37    ::::::::::::::::V:::::::::::::::::V::EIK:::DH::R:   250
Xenopus Cx38  :::M:::::::::::::S::::L:::M::I::SFK:FQH:IK---EGATC:   235
Rat Cx43      ::::::::::::::::S:V::A::II::FYVFFKGVKDRVKG:SDPYHAT   250

Human Cx37    TQGTSSDPYTDQGLLL-PPRGQGPSSPPC------PTYNGLSS-------   286
Mouse Cx37    A::SA::::PE:-VFFYL:M:E:::::::------:::::::--------   286
Xenopus Cx38  PT:I---:FNGA:NRM--:PQEYTNP:SSNQDIDL:A::KM:G-------   273
Rat Cx43      :GPL:PSKDCGSPKYAYFNGCSS:TA:LSP--MSP:G:KLVTGDRNNSSC   298

Human Cx37    -------SEQNWANLTTEE-RLASSRPP----------------------   306
Mouse Cx37    -------T:::::::::::-::T:::::----------------------   306
Xenopus Cx38  -------G-H::SSIQN:Q-QVNGLVKPKCQC---------DCWSQSAIS   305
Rat Cx43      RNYNKQA:::::::YSA:QN:MGQAGSTISNSHAQPFDFPDDNQNAKKVA   349

Human Cx37    LFLDPPPQ--NGQKPPSRPSSSASKKQY-----V    333
Mouse Cx37    P:VNTA::--G:R:S:::N:::::::-----:    333
Xenopus Cx38  VVVSGA:GIISNMDAVK:NHQTS::Q::------:    334
Rat Cx43      AGHELQ:LAIVD:R:S::A::R::SRPRPDDLEI    382
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Figure 2. Comparison of human Cx37 to several closely related connexins. The derived amino acid sequences of human Cx37 and those of mouse Cx37 (31), *Xenopus* Cx38 (2), and rat Cx43 (21) are shown as optimally aligned by CLUSTAL (23). Residues that are identical to their counterparts in human Cx37 are represented by (:); nonidentical residues are listed in single letter code. Dashes represent spaces added to optimize alignment.

ences from the rodent sequences fall in regions predicted to be in the cytoplasmic carboxyl-terminal tail. The homology of human Cx37 to mouse Cx37 is confirmed by comparison of the nucleotide sequences; 79% of residues in our sequence are identical to those in the mouse sequence reported by Willecke et al. (31). Many identical residues are found in both the coding region and the 3′-untranslated region. The 5′-untranslated regions differ; however, the reported mouse sequence was derived from genomic DNA and may contain nontranscribed regions. The sequences of other connexins are somewhat less homologous; they all contain many identical residues in predicted transmembrane and extracellular regions, but few matches in cytoplasmic domains. The next closest connexins are *Xenopus* Cx38 (50% identity) and rat Cx43 (48% identity) (Fig. 2). The predicted cytoplasmic regions of human Cx37 contain a serine-rich region (residues 301–333) and the sequence SEQNWAN (amino acids 287–293); although the functions of these regions are unknown, they have also been identified in a number of other connexins, which Bennett et al. (35) called class II connexins.

Southern blots. Southern blots of restriction enzyme–digested human genomic DNA were hybridized with human Cx37 probes. As shown in Fig. 3, the Cx37 cDNA probe hybridized only to single bands in multiple enzyme digests, suggesting that human Cx37 is a single copy gene. The uniqueness of the Cx37 gene is confirmed by the difference between this blot and published Southern blots probed with other human connexins (30, 36). The blots also suggest that, like other connexins, the Cx37 coding sequence contains no introns. PCR amplification using primers derived from the coding or 3′-untranslated regions of Cx37 gave identical products from genomic DNA or from the cDNA, further supporting the absence of introns in these regions (data not shown).

Expression of Cx37 mRNA. To determine the pattern of expression of Cx37, we performed Northern blot analysis of total RNA isolated from many rodent or bovine organs and tissues. Fig. 4 shows filters probed at high stringency with the probe for mouse Cx37. Cx37 hybridization was most prominent in heart, uterus, and ovary, but low levels were also detected in many other tissues.

Since Cx37 mRNA was expressed in so many locations, we examined its expression by established cell lines and primary

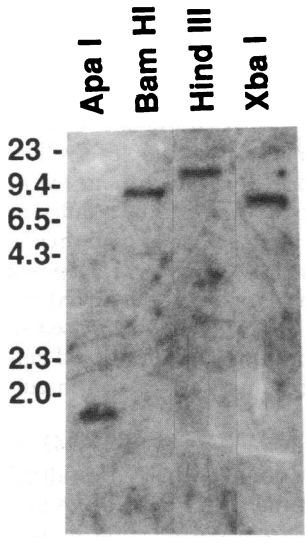

Figure 3. Southern blot analysis of human genomic DNA. DNA was digested with several restriction endonucleases as indicated, separated by agarose electrophoresis, and transferred to nylon membranes. The blot was hybridized with a cDNA probe corresponding to nucleotides 68–603 of the human Cx37 sequence. None of the enzymes used has predicted sites within this probe. Lambda phage DNA digested with HindIII was used as molecular mass standards with size in kilobase indicated to the left of the blot. For all enzymes shown, hybridization produced only one band.

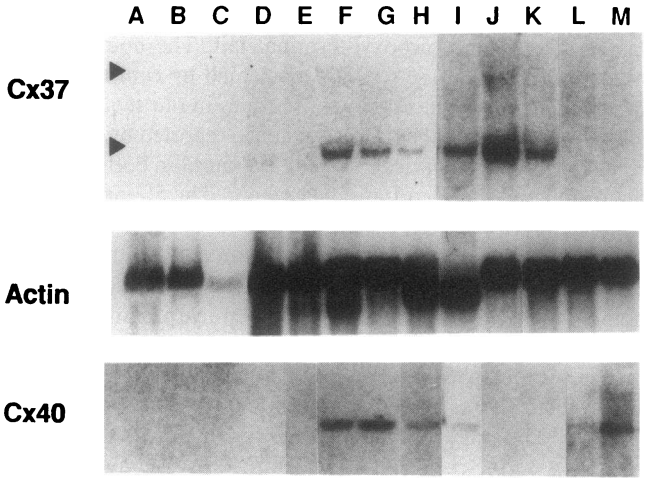

Figure 4. Northern blots demonstrating tissue distribution of expression of Cx37 mRNA. Total cellular RNA was prepared from cells or tissues, separated on formaldehyde/agarose gels (10 μg per lane) and prepared for Northern blotting as described in Methods. Blots were then incubated with specific ^{32}P-labeled DNA probes corresponding to nucleotides 174–630 of mouse Cx37. RNAs were prepared from mouse kidney (*A*), mouse brain (*B*), mouse liver (*C*), mouse spleen (*D*), mouse stomach (*E*), rat uterus (*F*), rat ovary (*G*), rat heart (*H*), mouse heart (*I*), human umbilical vein endothelial cells (*J*), bovine aortic endothelial cells (*K*), rat aortic smooth muscle cells (*L*), and A7r5 rat aortic smooth muscle cells (*M*). Arrowheads indicate the migration of 18S and 28S rRNAs. The identical blots were subsequently hybridized with a cDNA probe for human fibroblast γ-actin (*Actin*) to examine equivalence of loading and RNA integrity. Identical samples were also hybridized with DNA probes for rat Cx40 (indicated as *Cx40*).

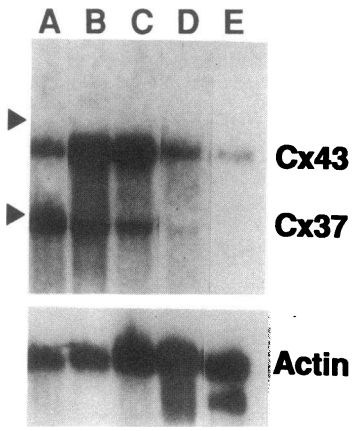

Figure 5. Northern blots comparing expression of Cx37 and Cx43 by cultured cells. Total RNA was prepared from fresh scrapings of bovine aortic endothelium (*A*) or cultured bovine aortic endothelial cells (*B, C*), bovine retinal microvascular endothelial cells (*D*), or bovine retinal pericytes (*E*). 10 μg of total RNA was applied in each lane. The blot was hybridized with an equal mixture of identically prepared specific cDNA probes for rat Cx43 (nucleotides 1–1,394) (21), which hybridizes to an mRNA of ~ 3 kb and human Cx37 (nucleotides 68–1,601), which hybridizes to an mRNA of 1.7 kb. The positions of the bands corresponding to Cx43 and Cx37 are indicated to the right of the blot. Arrowheads indicate the migration of 18S and 28S rRNAs. The blot was subsequently rehybridized with a cDNA probe for human fibroblast γ-actin (*Actin*) to confirm equivalence of loading and RNA integrity.

cultures of various cells. Cx37 mRNA was most abundant in RNA derived from primary cultures of endothelial cells from human umbilical vein and bovine aorta (Fig. 4). Other vascular cells (which express Cx40 or Cx43), including smooth muscle cells and pericytes, did not contain detectable Cx37 mRNA. We examined RNA derived from multiple cell lines of different sources, including vascular smooth muscle cells (A7r5, A10), skeletal myoblasts (BC_3H1), fibroblasts (NRK), glioma cells (C_6), macrophages (J774), hepatoma cells (SK Hep1, MH_1C_1), and neuroblastoma cells (Neuro2A), but did not detect Cx37 hybridization (data not shown).

Rehybridization of the blots from Fig. 4 with a control probe for human fibroblast γ-actin (which hybridizes to the different mammalian actin isoforms under the conditions used) confirmed the integrity and relative equivalence of loading of the samples, except for the mouse liver sample for which less RNA apparently had been loaded. Because of our recent observation that Cx40 appears to be expressed by a different component of the blood vessel wall (vascular smooth muscle cells), we also hybridized the same RNA samples with a probe for rat Cx40. These results, also shown in Fig. 4, confirmed our previous findings that Cx40 mRNA, while also expressed in several different organs, was abundant in A7r5 cells and cultured vascular smooth muscle cells, but not detected in cultured endothelial cells.

To compare the relative expression of Cx37 and Cx43 by the vascular endothelial cells, we simultaneously hybridized RNA blots with probes for both connexins (Fig. 5). RNA prepared from endothelium scraped from bovine aorta showed hybridization to both the 1.7-kb Cx37 mRNA and the 3-kb Cx43 mRNA. Cx37 and Cx43 both showed strong hybridization to RNA from cultured bovine aortic endothelial cells, although the relative abundance of Cx37 appeared somewhat less than in the tissue. The relative amounts of Cx37 and Cx43 hybridization appeared nearly identical in two different preparations of RNA from first passage cultures of bovine aortic endothelial cells. In RNA from cultured bovine retinal microvascular endothelial cells, Cx37 hybridization was much less than Cx43, and in RNA from the nonendothelial bovine pericytes, only Cx43 was detected. These Northern blots suggest that both Cx37 and Cx43 are endothelial connexins, since the mRNAs are so abundant in the freshly isolated tissue and in such homogenous primary cell cultures. We have no data suggesting that Cx37 in these preparations could derive from other contaminating cell types. Cx37 mRNA was not detectable in RNA prepared from peripheral blood monocytes, monocyte-derived macrophages, lymphocytes, aortic media, or cultured vascular smooth muscle cells (data not shown). The integrity and relative equivalence of loading of these RNA samples was confirmed by hybridization with the actin probe (Fig. 5). The Cx40 probe did not show significant hybridization with these RNA samples (data not shown).

Functional expression of Cx37. To verify that Cx37 was indeed capable of forming functional cell-to-cell channels and to study the biophysical properties of those channels, we stably transfected the communication-deficient cell line N2A with the human Cx37 cDNA. We have recently used this system to examine the macroscopic and junctional currents induced by three chick embryo cardiac connexins (9). The parent N2A cell line shows no detectable gap junctional channels when screened by double whole cell patch clamp recordings and shows no detectable connexin expression as assayed by Northern blotting with probes for Cx26, Cx31, Cx32, Cx37, Cx40, Cx42, Cx43, Cx45, Cx46, and Cx56 (data not shown). Connexin-transfected N2A clones were selected in G418. RNA blot analysis of total RNA prepared from the transfected cells demonstrated that they expressed Cx37 mRNA, whereas no hybrid-

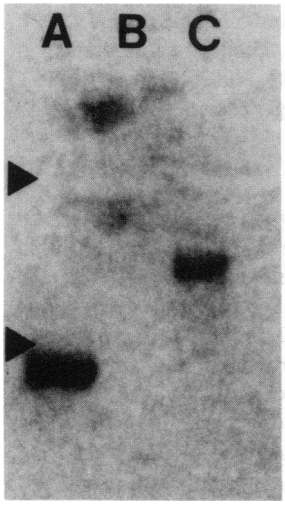

Figure 6. Northern blots demonstrating expression of Cx37 in transfected cells. Total RNA was prepared from bovine aortic endothelial cells (*A*), N2A cells transfected with pSFFV-neo vector alone (*B*), or from N2A cells transfected with pSFFV-neo constructs containing the human Cx37 cDNA sequence (*C*). Blots containing 10 μg per lane were hybridized with the human Cx37 probe. Arrowheads indicate the migration of 18S and 28S rRNAs. The Cx37 hybridizing band in lane *C* has the expected slower mobility for the vector/cDNA construct than the authentic mRNA alone in lane *A*.

ization was detected in cells transfected with vector alone (Fig. 6).

Clones testing positive for connexin expression were examined for functional coupling using the double whole cell recording technique. Electrical communication was evident in 60% of the pairs examined. Junctional conductance (g_j) averaged 4.45±1.03 nS (±SEM, n = 7) for Cx37-transfected cell pairs. The external application of pharmacological uncouplers, 1 mM 1-octanol or 2 mM 1-heptanol, reversibly inhibited g_j by > 90% (n = 5 pairs) (see Fig. 7).

In many gap junctions, conductance can be modulated by transjunctional potential (V_j) gradients (for review see reference 37). To examine the V_j sensitivity of g_j for Cx37, V_j pulses were applied to paired Cx37-transfected N2A cells. Instantaneous and steady state junctional currents were measured from the first and last 10 ms of each V_j pulse and junctional current–voltage (I_j–V_j) relationships were determined for each experiment. In all experiments, the instantaneous I_j–V_j relationships were linear, whereas the steady state I_j–V_j relationships were nonlinear. To illustrate these differences, the steady state g_j was normalized to the instantaneous g_j of each pulse and resulting normalized steady state junctional conductance (G_{ss}) was plotted as a function of V_j for each experiment. The average instantaneous and normalized steady state g_j–V_j relationships for Cx37 are illustrated in Fig. 8. Cx37 conductance underwent a significant reduction (> 20%) in G_{ss} even at low V_j values (≤ ±20 mV). The experimental minimum steady state junctional conductance (G_{min}) at ±100 mV was 0.23–0.30.

The G_{ss} – V_j curve for Cx37 was mathematically represented by the two-state Boltzmann relationship used to de-

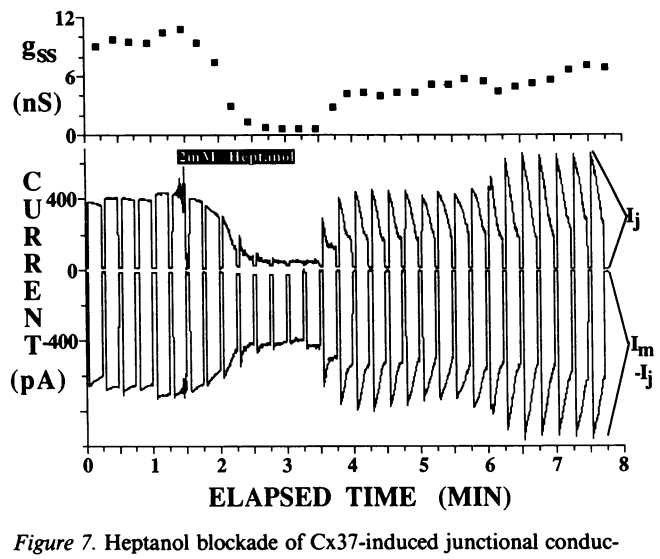

Figure 7. Heptanol blockade of Cx37-induced junctional conductance. Steady state g_j (G_{ss}) was determined from the end of 5-s pulses to V_j = ±40 mV applied at the rate of 4/min to a Cx37-transfected cell pair. The top current trace corresponds to the junctional current (I_j) measured in the nonpulsed cell whereas the currents from the pulsed cell are shown below (I_m – I_j). 9 s of each recovery interval (V_j = 0 mV) between successive pulses were deleted from the current traces for clarity of presentation. G_{ss} dropped from a control value of 9.87 to 0.82 nS during rapid exposure to 2 mM heptanol added to the bath by bolus injection. Heptanol blockade was slowly reversible by superfusion with normal saline (2 ml/min, 2 ml bath vol).

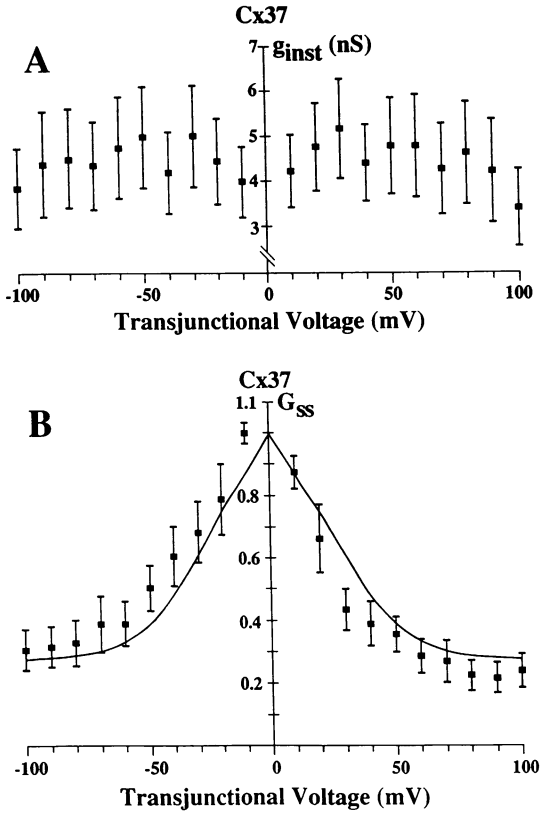

Figure 8. Junctional conductance-voltage relationships for human Cx37. (*A*) The filled squares and error bars represent the mean instantaneous g_j±SEM for all seven Cx37 cDNA-transfected N2A cell pairs where a complete I_j – V_j relationship was obtained. (*B*) The filled squares and error bars represent the mean G_{ss} (= steady state g_j/instantaneous g_j) SEM. The G_{ss} – V_j relationship for Cx37 was fitted with a theoretical line determined by the Boltzmann relation $G_{ss} = (1 - G_{min})/\{1 + \exp[A(V_j - V_o)]\} + G_{min}$, where G_{min} = the voltage-insensitive component of G_{ss}, V_o = the half-inactivation voltage for the voltage-sensitive component of G_{ss}, and A = slope factor expressing the charge sensitivity of the transition between the high and low conductance states. A is determined by the expression zq/kT, where z is the number of equivalent electrons q, k is Boltzmann's constant, and T is absolute temperature. The values for the relevant parameters are listed in the text.

scribe other V_j-dependent gap junctions (Fig. 8 *b*). According to this analysis, G_{ss} achieves a G_{min} of 0.27 at ±100 mV with a half inactivation voltage (V_o) of ±28 mV for the voltage-sensitive component of G_{ss} ($G_{ss} - G_{min}$). The slope of the curve (A = 0.08) is proportional to the number of equivalent electrons that serve as the voltage-sensing mechanism for the voltage-dependent gate, which has a valence of 2.0 for human Cx37. The two-state Boltzmann equation implies that the time-dependent decay of I_j follows first-order kinetics. Exponential fits of the time-dependent decay of I_j revealed two time constants of 80–300 ms and 1–3 s between V_j values of ±50 and ±100 mV. Thus, a two-state Boltzmann model is not truly sufficient to describe the kinetics of this time-dependent process, but it does provide a useful indicator for comparing the relative V_j dependence of connexin-specific gap junctions.

Some cell pairs exhibited low levels (< 1 nS) of g_j without the addition of pharmacological uncoupling agents, allowing the resolution of single Cx37 channels. Examples of the junctional current activity obtained under these conditions are illustrated in Fig. 9. In the representative cell pair shown, the expressed Cx37 channels exhibited a unitary conductance of 200–250 pS at V_j = 30 mV; however, larger voltages (80 mV) produced channel conductances of 35–50 and 60–80 pS and a complete absence of 200 pS channels. Gap junction channel activity was observed in six Cx37-transfected cell pairs over a cumulative time of 2 min with similar results. The combined data from all cell pairs showed that channel conductances averaged 219±22 pS (±SD, n = 139 channel openings), 165±6 pS (n = 102), 123±13 pS (n = 172), and 53±1 pS (n = 73) at V_j = 30–40 mV. At V_j = 80–90 mV, channel conductances of 94±4 pS (n = 39), 69±5 pS (n = 138), and 39±10 pS (n = 163) were observed in the absence of larger channel activity.

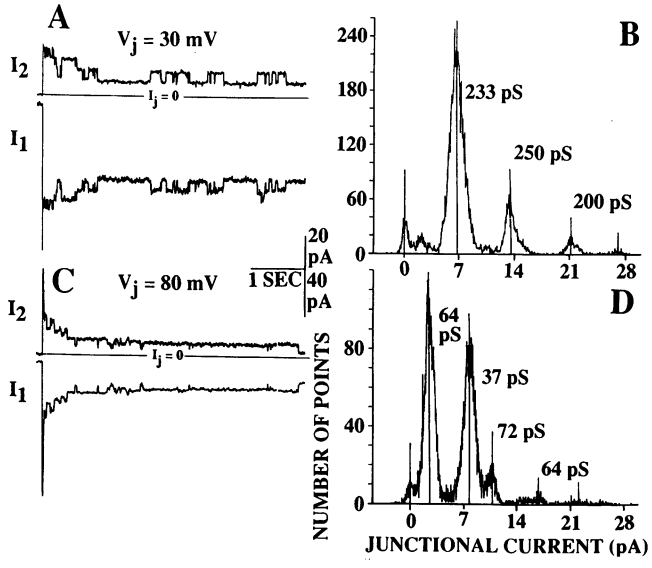

Figure 9. Junctional channel currents obtained from a Cx37 cDNA-transfected N2A cell pair. The left panels (*A* and *C*) illustrate the paired whole cell currents (I_1 and I_2) obtained during a V_j pulse to the indicated value (30 mV in *A*, 80 mV in *C*). The I_2 trace represents the junctional current (I_j) signal. Quantal fluctuations in the upward direction of the I_2 trace (which are mirrored in the I_1 trace) represent channel openings. Paired whole-cell currents were recorded from the onset of a 10-s V_j pulse applied to a pair of Cx37-transfected cells. All current traces were low-pass filtered at 125 Hz and digitized at 1 kHz. The right panels (*B* and *D*) show the channel amplitude histograms compiled from the 10-s junctional current record. The area under each peak is proportional to the time spent in each state. The unitary channel amplitudes shown were calculated from the differences in current between the means of adjacent peaks.

Discussion

We have reported the molecular cloning and functional expression of a novel gap junction protein, human Cx37. This sequence likely corresponds to the gene that Willecke et al. (38) localized to chromosome 1pter-q12 using a mouse Cx37 probe. Two other human connexins (Cx32 and Cx43) and a pseudogene (Cx43) have been previously reported (5, 36).

RNA blots demonstrate that Cx37 mRNA is expressed in many different organs and tissues but is particularly abundant in endothelial cells. In richly vascularized tissues, total RNA samples would be expected to contain a significant proportion of endothelial RNA. Hence, it is likely that endothelial cells in the lung were the source of the mouse Cx37 reported by Willecke et al. (31), and endothelium may also explain our finding Cx37 mRNA in heart, uterus, and ovary. However, the marginally detectable Cx37 mRNA in spleen, brain, and cultured microvascular endothelial cells suggests a differential expression in different vascular beds or at different levels of the vasculature. Indeed, electron microscopic studies have demonstrated that the distribution of endothelial gap junctions varies with different vascular beds (for review see reference 39). Pepper et al. (40) recently reported differences in Cx43 expression and responses to wounding of cultured microvascular and large vessel endothelial cells.

Gap junctions in the blood vessel wall may perform a number of metabolic and electrical functions (39), including the coordination of signal transduction and growth control. Experiments have suggested that vascular gap junctions may coordinate vasomotor responses to humoral agents by mediating cell-to-cell spread of a dilating or constricting wave (41, 42). The expression of both Cx37 and Cx43 by these cells might provide a multiplicity of pathways for such processes. In addition, gap junctions have been implicated in interactions between endothelial cells and other cell types, including smooth muscle cells (43), monocytes (44), and metastatic tumor cells (45). Expression of multiple connexins by endothelial cells might facilitate such interactions.

The expression of both Cx37 and Cx43 by endothelial cells is an example of what may be a general cellular strategy: expression of multiple connexins in a single cell. Hepatocytes express Cx26 and Cx32 (46). Cardiac myocytes express Cx40, Cx43, and Cx45 (11). A7r5 smooth muscle cells express Cx40 and Cx43 (17). Keratinocytes and leptomeningeal cells both express Cx26 and Cx43 (47, 48). The significance of the expression of multiple connexins and their disparate, but overlapping distributions is unclear. Multiple connexins within a single cell should allow it to form different channels and might coordinate interactions of diverse cell types. It is of interest that our data suggest that two cells of the blood vessel wall both express Cx43, but one (the endothelial cell) expresses Cx37, whereas the other (the smooth muscle cell) makes Cx40.

Our data on the functional expression of human Cx37 by the stable transfection of N2A cells shows that Cx37 channels are quite voltage dependent. However, our data differ somewhat from those obtained by Willecke et al. (31) when they expressed mouse Cx37 in *Xenopus* oocytes. We can approxi-

mate the $G_{ss} - V_j$ relation for Cx37 by a simple Boltzmann function whereas they proposed a model with two superimposed gates described by different Boltzmann parameters. The human Cx37 expressed in transfected N2A cells demonstrated a linear instantaneous $G_j - V_j$ relationship; in contrast, Willecke et al. (31) reported a nonlinear instantaneous $G_j - V_j$ relationship for mouse Cx37, apparently because of the inability of the oocyte voltage clamp to obtain G_j measurements during the first 20 ms of each V_j step. We did not observe any fast time constants (< 20 ms) within the ±100 mV range that could account for these differences between mouse and human Cx37.

It may be that these differences are functional consequences of the differences between the mouse and human sequences. However, the differences may also simply reflect differences of properties observed by voltage clamp in *Xenopus* oocytes and by whole cell patch clamp in transfected cells; Cx43, for instance, shows differing voltage-dependence properties in the two systems (3–5). Resolution will require expression of the mouse and human sequences in both systems. However, if the differences are confirmed, mutagenesis experiments to locate residues responsible for voltage dependence should be very straightforward; there are ≥ 10 amino acid differences (all in predicted cytoplasmic locations) that represent charged amino acid substitutions.

This study provides the first observations of single Cx37 channels. The Cx37 channels exhibited multiple conductance levels of ~ 219, 165, 123, and 53 pS at low voltages, but appear to be modulated by V_j since unitary conductances of only ~ 94, 69, and 39 pS were present at V_j values ≥ 80 mV. Similar V_j-dependent shifts in channel conductances have been observed in embryonic chick heart gap junctions (49).

Finally, these electrophysiological observations demonstrate that Cx37 channels have different biophysical properties than other molecularly characterized connexins (5–7). The significance of voltage dependence or unitary conductance of gap junction channels is not known, but these have been the best-studied and quantitated properties. These differences raise the possibility that Cx37 channels might also differ from other connexins in other biologically important properties, such as ion and metabolite permeability or response to intracellular modulators.

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