Connexin 35: A Gap-junctional Protein Expressed Preferentially in the Skate Retina

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Submitted September 28, 1995; Accepted November 30, 1995

Monitoring Editor: Masatoshi Takeichi

We have used low stringency hybridization to clone a novel connexin from a skate retinal cDNA library. A rat connexin 32 clone was used to isolate a single partial clone that was subsequently used to isolate seven more overlapping clones of the same cDNA. Two clones containing the entire open reading frame have a consensus sequence of 1456 bp and predict a protein of 302 amino acids length and molecular mass of 35,044 daltons, referred to as connexin 35 or Cx35. Southern blot analysis suggests that the cloned sequence lies in a single gene with one intron. Polymerase chain reaction amplification from genomic DNA and partial sequencing of this intron showed that it was approximately 950 bp in length, and located within the coding region 71 bp after the translation start site. Hydropathy analysis of the predicted protein and alignments with previously cloned connexins indicate that Cx35 has a long cytoplasmic loop and a relatively short carboxyl terminal tail. Multiple sequence alignments show that Cx35 has similarities to both α and β groups of connexins and suggests that its origins may be near the divergence point for the two groups. Consensus sequences consistent with sites for phosphorylation by protein kinase C and by cAMP- or cGMP-dependent protein kinase were identified. Two transcripts were detected in Northern blot analysis: a 1.95-kb primary transcript and a 4.6-kb minor transcript. In RNA samples from 10 tissues, transcripts were detected only in the retina.

INTRODUCTION

The electrical synapse, mediated by unique intercellular transmembrane channels that constitute the so-called "gap junction," is recognized as one of the fundamental mechanisms of signal transmission in the nervous system of all vertebrate species. The aqueous pores of the gap-junctional channel, formed by hexameric assemblies of proteins (connexins) in the apposed membranes of adjoining cells, permit the passage of ions, metabolites, and other small molecules (<1 kDa), and thereby provide a pathway for both electrical and chemical communication between networks of coupled cells. Interest in the properties of this system has been spurred by growing evidence

that gap junctions are intimately involved in the cellular development and maturation of neuronal tissues (Dermietzel *et al.*, 1989; Guthrie and Gilula, 1989; Peinado *et al.*, 1993; Penn *et al.*, 1994), and by the realization that the plasticity of the junctional synapse is at least as complex as that exhibited by chemical synapses (Obaid *et al.*, 1983; Peracchia, 1991; Bennett *et al.*, 1993; Christ *et al.*, 1994). Indeed, there is abundant evidence that the extent to which cells are electrically coupled can be modulated by a number of physiologically significant variables, e.g., voltage, pH, Ca^{2+} , etc. (Rose and Loewenstein, 1975; Spray *et al.*, 1981; Obaid *et al.*, 1983; Bennett and Spray, 1987).

Similar findings have been obtained in the vertebrate retina, where almost every neuronal cell type appears to be electrically coupled to its neighbors (Raviola and Gilula, 1973; Lamb, 1976; Saito and Kujirakawa, 1988; Cuenca *et al.*, 1993; Hidaka *et al.*, 1993),

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and the modulation of gap-junctional permeability has been well documented (cf. Dowling, 1987; Murakami *et al.*, 1995). However, there are often profound differences in the pharmacology of the gap junctions in related cell types. These differences seem to depend on the species studied, and on the functional properties of the neural network they subserve. For example, electrical coupling between rod-driven horizontal cells of skate (Qian and Ripps, 1992; Qian *et al.*, 1993) and mammals (Pflug and Nelson, 1993) appears to be relatively insensitive to dopaminergic ligands or any of the intermediaries of the dopamine-activated second-messenger system that modulate electrical communication between cone-driven horizontal cells of amphibia and some species of fish (Piccolino *et al.*, 1984; Lasater, 1987; DeVries and Schwartz, 1989; McMahon *et al.*, 1989; Dong and McReynolds, 1991). The pharmacological properties of gap junctions between other classes of retinal neurons have not been studied as extensively, but it seems likely that their relative sensitivities to drugs, transmitters, and other agents will be dependent upon similar factors (cf. Hampson *et al.*, 1992; Cook and Becker, 1995).

Differences in gap-junctional properties suggest that there may be more than one class of electrical synapse on retinal neurons, governed by connexins with different gating characteristics. A knowledge of the structure-function relationships of these connexins is essential to a better understanding of the role of electrical synapses in the processing of information by cells of the vertebrate retina. This study represents a first step toward identifying and characterizing the family of connexins that are expressed in retina. Although there is evidence suggesting the presence of connexins 32 (Finch and Paul, 1989; Vardi *et al.*, 1990; Jones *et al.*, 1992) and 43 (Kadle *et al.*, 1991; Edgerton and McMahon, 1994, 1995) in the retina, we present here the first molecular characterization of a gap-junctional protein, connexin 35, expressed preferentially in a vertebrate retina.

MATERIALS AND METHODS

Preparation of the Skate Retinal cDNA Library

Retinas were isolated from skate (*Raja erinacea* and/or *R. ocellata*) netted in waters near Woods Hole, MA. Approximately 25 μ g of polyA⁺ RNA was isolated from pooled retinas by binding to oligo dT cellulose using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. A cDNA library was prepared commercially (Stratagene, La Jolla, CA) in the λ ZAPII vector.

Cloning of Skate Connexin cDNAs

A low-stringency screening protocol was used to isolate connexin cDNAs from the skate retina library. Approximately 10⁶ plaque-forming units were spread on petri dishes and lifted onto nylon filters (Micron Separations, Westborough, MA) for screening. A rat connexin 32 clone, kindly provided by Dr. David Paul (Harvard

University, Cambridge, MA), was used as the probe. The selection of Cx32 as a probe was based on unpublished observations in our laboratory that demonstrated immunoreactivity to a connexin 32 antibody (monoclonal Ab 72F; provided by Dr. Elliot Hertzberg, Albert Einstein College of Medicine) in the skate retina. The probe sequence was excised from its vector, gel purified, and labeled with [α -³²P]dATP by nick translation (Boehringer Mannheim nick translation kit, Indianapolis, IN) and used to probe the filters. Hybridization was performed at 42°C in a solution containing 30% formamide, 1.5 $\times$ SSPE (1 $\times$ SSPE = 150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA), 0.5% nonfat dry milk, 2% SDS, and 0.5 mg/ml salmon sperm DNA. The filters were washed sequentially at 42°C for 20 min each in solutions containing 2 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl, 15 mM C₆H₅Na₃O₇, pH 7.0), 0.5% SDS, and 0.5 $\times$ SSC, 0.5% SDS. Initial screening of the library under these conditions yielded a 690-bp clone representing the 5' end of a connexin mRNA. The partial connexin clone, named SKCON1, was used to screen the library for other connexin clones under higher stringency conditions. The hybridization was done at 42°C in 40% formamide, 1.5 $\times$ SSPE, 0.5% nonfat dry milk, 2% SDS, 0.5 mg/ml salmon sperm DNA; washes were performed at 42°C as described above. The rescreening yielded an additional 20 connexin clones.

The cDNA clones were restriction mapped and several clones that contained single inserts were further characterized by direct DNA sequencing. Sequencing was performed by the dideoxy chain termination technique (Sequenase kit, United States Biochemical, Cleveland, OH) using a double-strand sequencing protocol involving [α -³²P]dATP incorporation. Sequence data were digitized and analyzed with PC Gene software (IntelliGenetics, Campbell, CA).

Southern Blot Analysis of Skate Genomic DNA

Genomic DNA was isolated from the brain of a single skate (*R. erinacea*) by published protocols (Sambrook *et al.*, 1989). Twenty microgram aliquots were digested with each of the following enzymes: *Pst*I, *Eco*RI, *Hind*III, and *Nco*I. Ten micrograms of each digested DNA sample was run on a 0.8% agarose gel and subsequently transferred to a nylon membrane (Zetaprobe, Bio-Rad Corporation, Richmond, CA) by capillary diffusion in 0.4 M NaOH, 10 $\times$ SSC.

The blot was probed under high stringency conditions with a combination of two skate connexin 35 clones that covered the entire open reading frame as well as 150-bp of 5' and 700-bp of 3' untranslated sequence. The probe sequence covers the region homologous to the position of the single intron described in connexin genes (Miller *et al.*, 1988; Fishman *et al.*, 1990), and contains a single site each for *Pst*I and *Eco*RI. The two skate connexin fragments were amplified by polymerase chain reaction (PCR) from pBluescriptII clones with primers specific to the multiple cloning site of the vector and labeled with [α -³²P]dATP by random primer extension. The blot was probed for 15 h at 42°C in a solution containing 50% formamide, 3 $\times$ SSPE, 0.5% nonfat dry milk, 1% SDS, and 0.5 mg/ml salmon sperm DNA. The blot was washed at 55°C sequentially in buffers containing 0.5% SDS plus 2 $\times$ SSC, 0.5 $\times$ SSC, and 0.1 $\times$ SSC for 20 min each, and exposed to film (Kodak X-Omat AR, Rochester, NY) for 72 h with intensifying screens.

PCR Amplification and Cloning of Genomic DNA Fragments

Fragments of skate genomic DNA were amplified by PCR using the Expand PCR kit (Boehringer Mannheim). The reactions were performed following the manufacturer's protocol with 250 ng genomic DNA and 200 ng of each primer in a 50 μ l reaction mixture. The primer sequences used were as follows: 1) 5'-TGAGATGCCAGG-TACAGG-3', 2) 5'-AGGCTTTGTCGTAACAAG-3', 3) 5'-TCCAAA-CAGCGAGAGAGAC-3', and 4) 5'-CGCCAGTTCGTTGGGTCTC-3'. Primer pairs used were 1 and 2, 1 and 4, and 3 and 4. No single clone contained sequence for both primer 1 and any of the other

primers, so positive controls for pairs 1,2 and 1,4 were not done. Amplification of a fragment from clone scxn11 was used as a positive control for pair 3,4. Negative controls (no template) were run with each reaction set.

PCR products containing the Cx35 intron were cloned into a T-vector constructed from pBluescript II KS⁺ (Stratagene). The plasmid was cut at the *EcoRV* site and T's were added to the 3' ends by incubating with *Taq* polymerase for 30 min at 72°C in *Taq* buffer containing only dTTP. PCR products were ligated into this vector at a 10:1 molar ratio of PCR product to vector, and plasmids containing an insert were selected from ampicillin-resistant transformants by disruption of β -galactosidase activity. Clones were restriction mapped and then sequenced using vector-specific primers.

Northern Blot Analysis

Total RNA was isolated from skate retina, brain, heart, stomach, kidney, spleen, liver, skeletal muscle, skin, and lens using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. For the lens RNA preparations, samples from two individual *R. erinacea* were pooled and used for the isolation; for the other tissues, samples were obtained from single individuals. Forty micrograms of each total RNA was run on a 1% agarose gel containing 6.7% formaldehyde in a buffer of 20 mM 3-[N-morpholino]propanesulfonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA. The gel was stained with ethidium bromide and the RNA was transferred to a nitrocellulose (Schliecher & Schuell, Keene, NH) membrane by capillary diffusion in 20× SSC. Transcript sizes were determined by comparison to an RNA molecular weight ladder (Life Technologies).

The blot was probed with a 1-kb skate connexin 35 clone (scxn18) that contains the entire open reading frame. The probe sequence was amplified by PCR, labeled by nick translation, and hybridized to the blot under high stringency conditions (hybridization solution contained 50% formamide, 5× SSC, 1× Denhardt's solution, 0.2% SDS, and 0.25 mg/ml salmon sperm DNA; 43°C). The blot was washed at 55°C as described for the Southern blot. The washed blot was exposed to film for 7 days with intensifying screens.

RESULTS

Isolation of Connexin cDNA Clones

Low stringency screening of a skate retinal cDNA library with a rat Cx32 clone (Paul, 1986) yielded a single 690-bp clone with high sequence homology to previously cloned connexins. This clone, called SKCON1, contained a 530-bp open reading frame that predicted a polypeptide homologous to the amino terminus through the intracellular loop of connexins. Rescreening of the library with this clone at higher stringency yielded 20 additional clones that hybridized to the probe. Of these clones, seven were sequenced and two contained the complete open reading frame coding for a 302-amino acid connexin of predicted molecular mass 35,044 daltons. Figure 1 shows the sequence and translation of one of these clones, scxn11. A number of features identify this as a member of the connexin family of proteins. First, hydropathy analysis (Rao and Argos, 1986) predicts three transmembrane domains, corresponding to the first, second, and fourth transmembrane domains (M1, M2, and M4) of connexins (shaded boxes in Figure 1). The portion of scxn11 analogous to M3 is predicted to

have an extensive region of hydrophobicity interrupted by several charged and polar amino acids, as is the case with other connexins. These amino acids, Arg177, Tyr179, Gln182, Arg186, and Glu190, are homologous in position to the conserved charged and polar amino acids thought to form the lining of the gap-junctional channel (Milks *et al.*, 1988). These features are evident in the alignment of the predicted skate connexin amino acid sequence with several of the previously described connexins (Figure 2). The conserved transmembrane domains are outlined, with conserved residues being denoted by asterisks below the alignment, and conservatively replaced residues indicated by periods. In addition, the scxn11 sequence predicts a proline (Pro89; Figures 1 and 2) in M2 that is conserved and is reported to be important for voltage gating (Suchyna *et al.*, 1993). Thus the typical four-transmembrane domain structure of connexins is conserved in this skate clone.

The two predicted extracellular loops also show the highly conserved features typical of connexins: three cysteines in each loop, and a conserved stretch of amino acids (aa71–77; FPISHIR) near the end of E1. Thus the protein coded for by this sequence constitutes a new member of the connexin family. Based on current nomenclature, the protein will be referred to as connexin 35 or Cx35.

The cytoplasmic loop and carboxyl terminal tail of Cx35 are very poorly conserved, as is the case among most connexins, but contain several interesting features. The cytoplasmic loop is 77 amino acids long, comparable in length to that of Cx45, making it one of the longest among the connexins so far described. It contains two notable stretches of high charge density (aa117–128 and aa162–174), and a consensus sequence for protein kinase C phosphorylation (HQSSK) at Ser101. The carboxyl terminal tail, on the other hand, is rather short (52 aa) and contains a consensus sequence for cAMP- and cGMP-dependent protein kinase phosphorylation (RRKSIY) at Ser273.

Homology of Cx35 to Other Connexins

The skate represents the most primitive vertebrate from which a connexin clone has been reported. Hence it is of interest to determine the relationship of this connexin to other known connexins. The current classification of vertebrate connexins separates the known proteins into two categories termed group I (β group) and group II (α group) (Bennett *et al.*, 1994). The two groups have arisen from a gene duplication relatively early in the process of diversification of the connexins. To better understand the relationship of Cx35 to these groups of connexins, we created an amino acid alignment of the majority of the known connexin types using the Clustal program in the PC-Gen software package. A rodent representative of

each type was chosen, when available, and the carboxyl terminal domain and cytoplasmic loops were eliminated from the comparison to reduce variability (cf. Bennett *et al.*, 1994). The dendrogram of this alignment is shown in Figure 3. This dendrogram does not represent evolutionary relationships of the connexin genes, but rather protein similarity based on an amino acid similarity matrix. From this alignment, it can be seen that the amino acid similarities of skate Cx35 place it near the divergence point between the α and β classes of connexins. A rigorous phylogenetic analysis of the connexins will be required to define its evolutionary position within this gene family.

Analysis of a number of overlapping clones of skate Cx35 revealed that several sequence variants were present in the skate library. Figure 4 shows a nucleotide alignment of a second full-length clone, scxn18, with the clone described in Figure 1 (scxn11). These two clones differ by six single nucleotides within the coding region. None of these changes result in amino

Figure 5A shows a Southern blot of skate genomic DNA digested with a variety of restriction enzymes and probed with a combination of two probes covering 1.5 kb of the Cx35 cDNA sequence. The averaged sum of labeled fragment sizes predicts that the gene is

Figure 2. Multiple sequence alignment of several distinct connexin types with skate connexin 35. The alignment was created with Clustal software using sequences available in the EMBL database. The sequences used are listed along the left edge of the alignment and include one of the two fish sequences currently available, Atlantic croaker Cx32.2 (Yoshizaki *et al.*, 1994). Gaps in the alignment are noted with a dash (-), while residues conserved within the alignment are designated with an asterisk (*) and conservatively replaced residues with a dot (.). The positions of the transmembrane domains are outlined with boxes and the first through fourth transmembrane domains are labeled M1 through M4. A conserved proline residue in M2, as noted in Figure 1, is boldfaced, as are four charged or polar residues within M3 that are thought to line the gap-junctional channel (Milks *et al.*, 1988). The predicted extracellular loops are labeled E1 and E2 and the three conserved cysteines in each loop are boldfaced. Note the long cytoplasmic loop of Cx35 (77 aa) and relatively short carboxyl terminal tail (52 aa).

contained within 14.7 kb. Two enzymes, *Pst*I and *Eco*RI, have recognition sites within the sequence of the cDNA used to probe the blot. Both of these yield three bands in the Southern analysis, while the remaining enzymes yield one or two bands. These data suggest the presence of an intron within the bounds of the cDNA sequence used to probe the blot.

To test for the presence of an intron, we designed several pairs of PCR primers covering most of the known cDNA sequence and amplified fragments from genomic DNA (Figure 5B). A map of the cDNA sequence with the positions of the primers used and the

known restriction sites is shown in Figure 5C. One primer pair (primers 1 and 2), designed to amplify from position -179 to +199 relative to the translation initiation site, amplifies a fragment approximately 950 bp larger than the predicted 379-bp product. Pair 3,4 amplifies the same product from both genomic DNA and the cDNA clone, while the product amplified from genomic DNA with pair 1,4 is also 950 bp larger than the predicted fragment. The PCR fragment amplified with primers 1 and 2 has restriction sites for *Eco*RI, *Pst*I, and *Hind*III, accounting for all of the fragments labeled in the Southern blot. Portions of the

Figure 3. Dendrogram of a multiple peptide sequence alignment of the majority of known connexin types. The alignment was created with Clustal software and included rodent representatives of most connexin types as well as several connexins cloned from organisms other than rodents. A connexin-like protein from the plant *Arabidopsis thaliana* (Meiners *et al.*, 1991) is included for comparison. Only the relatively conserved transmembrane and extracellular portions of the proteins were included in the alignment. The dendrogram plots the similarity of the sequences to each other based on the Dayhoff amino acid similarity matrix. The alignment resolves the α and β groups of vertebrate connexins as expected, but note the poorly resolved position of Cx35.

sequence of clones of this PCR product are shown in Figure 5D. Comparison of this sequence with the cDNA sequence shows that the intron lies within the coding region, 71 bp after the translation initiation site. The intron donor and acceptor sites follow the consensus sequences for splice junctions (Padgett *et al.*, 1986). The exon sequence in Figure 5D (capital letters) is numbered from the translation initiation site for reference, while the intron sequence (lower case) is not numbered. A notable stretch of G-rich sequence was found in the intron 115 bp upstream of the acceptor splice site (not shown). The labeling pattern of the Southern blot, along with the restriction sites within the intron, suggest that Cx35 is coded for by a single copy gene.

Connexin 35 Is Preferentially Transcribed in the Retina

The tissue distribution of Cx35 expression was examined by Northern blot. Total RNA was isolated from a number of skate tissues and probed with scxn18, covering the entire open reading frame. This probe recognized two transcripts, a major 1.95-kb transcript and a minor 4.6-kb transcript (Figure 6A). Both transcripts are larger than the consensus sequence derived from the overlapping clones, indicating that the cDNA clones containing the entire coding region are not full length. Cx35 transcripts were detected only in the retina; probe hybridization was not evident in RNA from brain, heart, stomach, kidney, liver, spleen, skin, skeletal muscle, or lens, in spite of the relatively long exposure to film (7 days). Ethidium bromide staining of the ribosomal RNA bands (Figure 6B) shows that similar amounts of RNA were loaded in each lane. Additional blots (not shown) also failed to detect Cx35

hybridization in ovary mRNA. These data suggest that Cx35 transcription may be confined to the retina.

DISCUSSION

As noted earlier, gap-junctional coupling among retinal neurons has been well documented, and the physiological and pharmacological properties of these junctions have been studied extensively. However, far less information is available concerning the molecular characterization of retinal connexins mediating gap-junctional communication. Kadle *et al.* (1991) identified Cx43 by Western blot in the rat retina, and other reports describe the reverse transcriptase-PCR amplification of a fragment of a connexin similar to Cx43 from the giant danio retina (Edgerton and McMahon, 1994, 1995). In addition, Cx32 immunoreactivity has been detected in mammalian retinas (Finch and Paul, 1989; Vardi *et al.*, 1990; Jones *et al.*, 1992). However, we were unable to detect Cx32 transcripts in retinal RNAs from several mammals, amphibians, skate, and other fish (our unpublished observations), suggesting that the immunostaining observed with Cx32 antibodies may reflect cross-reactivity with epitopes of other connexins. Nevertheless, the diverse gap-junctional properties of electrically coupled retinal neurons make it likely that several types of connexin are present, and that their distribution may be cell-type specific. The present study was undertaken to begin to characterize the connexins that are expressed in the retina.

By low stringency screening of a skate retinal cDNA library with a Cx32 probe, we have succeeded in cloning a new connexin predicted to have a molecular mass of approximately 35 kDa. We have called this

SCXN11	-	ATGCACAGAGATGGGAGATCG	22
SCXN18	-	TGAAATCTACCAGCACGGCCGTGCTTGTAGTCGCGTTCGCTCGCCGGATGCACAGAGATGGGAGATCG	70
SCXN11	-	ACAATACTAGAGAGGTTGCTGGAGGCGGCTGTTCAACAGCATTTCGACAATGATAGGAAGGATCTTGCTGA	92
SCXN18	-	ACAATACTAGAGAGGTTGCTGGAGGCGGCTGTTCAACAGCATTTCGACAATGATAGGAAGGATCTTGCTGA	140
SCXN11	-	CAGTGGTAGTGATTTTCCGAATCTTGGTGGTGGCCATCGTGGGCGAGACAGTATATGATGACGAGCAGAC	162
SCXN18	-	CAGTGGTAGTGATTTTCCGAATCTTGGTGGTGGCCATCGTGGGCGAGACAGTATATGATGACGAGCAGAC	210
SCXN11	-	CATGTTTGTATGCAACACCCTCCAGCCCGGCTGTAAACAGGCTTGTTCAGACAAAGCCTTCCCATCTCA	232
SCXN18	-	CATGTTTGTATGCAACACCCTCCAGCCCGGCTGTAAACAGGCTTGTTCAGACAAAGCCTTCCCATCTCA	280
SCXN11	-	CATATACGCTACTGGGTCTTCCAGATCATCATGGTATGCACTCCAGCCTCTGCTTTATAACGTA	302
SCXN18	-	CATATACGCTACTGGGTCTTCCAGATCATCATGGTATGCACTCCAGCCTCTGCTTTATAACGTA	350
SCXN11	-	TGCACCAGTCTCCAAACAGCGAGAGAGACAGTACTCCACCGTCTTCATCACCTTGGATAAAGACAAGAA	372
SCXN18	-	TGCACCAGTCTCCAAACAGCGAGAGAGACAGTACTCCACCGTCTTCATCACCTTGGATAAAGACAAGAA	420
SCXN11	-	GCGAGAAGACAACAAGATCAAGAACACGACAGTGAACGGGGTGCTGCAGAACTCGGAATTTTTCACCAAG	442
SCXN18	-	GCGAGAAGACAACAAGATCAAGAACACGACAGTGAACGGGGTGCTGCAGAACTCGGAATTTTTCACCAAG	490
SCXN11	-	GAGATGCAGTCGGACTTTTGAAGTCAAAGAAATGCAAACTCGGCTGCGAGGAACAGCAAAATGTCAA	512
SCXN18	-	GAGATGCAGTCGGACTTTTGAAGTCAAAGAAATGCAAACTCGGCTGCGAGGAACAGCAAAATGTCAA	560
SCXN11	-	AGATTCGACGCGCAGGAAGGGATCTCGCGTTTCTACATCATCCAGGTTGTTTCAGGAATGCTTTGGAGAT	582
SCXN18	-	AGATTCGACGCGCAGGAAGGGATCTCGCGTTTCTACATCATCCAGGTTGTTTCAGGAATGCTTTGGAGAT	630
SCXN11	-	TGTTTCTTGATGGGACAGTACTTCTCTACGGGTTCAAGGTGCCGTCCATGTACGAGTGAACCGGTAC	652
SCXN18	-	TGTTTCTTGATGGGACAGTACTTCTCTACGGGTTCAAGGTGCCGTCCATGTACGAGTGAACCGGTAC	700
SCXN11	-	CCGTGCGTGAAGATGGTTCGAGTGCTACGTGTCCAGACCCACGGAGAAGACTGTCTTCTGCTTTCATGT	722
SCXN18	-	CCGTGCGTGAAGATGGTTCGAGTGCTACGTGTCCAGACCCACGGAGAAGACTGTCTTCTGCTTTCATGT	770
SCXN11	-	TCGCCGTGAGTGGTCTTTGCGTGATCCTCAATCTGGCAGAGTTGAACCACTTGGGTTGGAGGAAGATCAA	792
SCXN18	-	TCGCCGTGAGTGGTCTTTGCGTGATCCTCAATCTGGCAGAGTTGAACCACTTGGGTTGGAGGAAGATCAA	840
SCXN11	-	AACGGCGGTGAGGGGGCGCAGGAGAGGGCGCAATCCATATATAGAGATCAGGAACAAGGACTCGCCGCAT	862
SCXN18	-	AACGGCGGTGAGGGGGCGCAGGAGAGGGCGCAATCCATATACGAGATCAGGAACAAGGACTCGCCGCAT	910
SCXN11	-	AGAATCGGGTGCCCAACTTCGGGCGAACTCAGTCCAGCGACTCTGCTTATGTGATGGGAGGGAGG	932
SCXN18	-	AGAATCGGGTGCCCAACTTCGGGCGAACTCAGTCCAGCGACTCTGCTTATGTGATGGGAGGGAGG	980
SCXN11	-	A-----CGCTATTCTCTCTTGTGCTCGGCTCGAAGGTGAAGCTGCCGAGGCGCAGCCCAAGCACGCCA	996
SCXN18	-	GCAGGCTCGCTATTCTCTCTTGTGCTCGGCTCGAA	1016
SCXN11	-	GGGATGGTCCCGTGGACACGGGGTCCCCGCTACCCGCTTCGTTTCGGAATCTGTCTGGTGGAGTTTAC	1066
SCXN11	-	AACGTACCGAAATCAGAATGAGCCACGCGTCCGTTACACATTGTGCAATCATTTTACACATTGCATGC	1136
SCXN11	-	TGTTTACCTCGCCAAATCTGTGTGCATTTTAAATGTTCACCGAGCTCCTTCGAGACTTTGAGACCC	1206
SCXN11	-	AACGAATGCGGAAATCCACGCGAAACCGTTCGTAAGTTCTAGGAGCAGAATTCAGATTTCGGCCCAT	1276
SCXN11	-	CGAGTCTACTCCAAATGAATCATGGTGTCTATCTGTCCTGCCAACCTCGTTCTGATGCCTTCTCG	1346
SCXN11	-	CCATAACCCCTGACAAATCAAGAATTAAGTTGCTTGTTCGGATTGTGCGTGAA	-1402

Figure 4. Alignment of two sequence variants of Cx35 cloned from the skate retina library. The two clones, scxn11 and scxn18, differ by six nucleotides in the coding region as well as a deletion in the 3'-untranslated region; these differences are labeled with asterisks and boldfaced. The translation initiation and termination codons are outlined for reference. None of the six nucleotide changes within the coding region result in amino acid differences in the predicted polypeptides.

connexin 35, in keeping with the current nomenclature for the connexin family. Several sequence variants of Cx35 were represented in the group of cDNA clones derived from the library (e.g. Figure 4). Although this led initially to the notion that several copies of the Cx35 gene may be present in the skate, Southern blot analysis indicated that there is only one copy of the gene. The presence of sequence variants in the cDNA library is due, perhaps, to the fact that the library was made from RNA prepared from several different individual skate captured from a wild population; this population may have several allelic variants of the gene. It is interesting to note, however, that these variants do not code for differences in the amino acid sequence of the protein, and thus are silent as far as the protein is concerned.

It is apparent that Cx35 is a member of the same family of gap-junctional proteins identified in other

tissues. The protein predicted by the cDNA clones displays the characteristic features of a connexin: four transmembrane domains with several charged and polar residues in conserved positions in M3, as well as the highly conserved extracellular loop structure with three stereotypically spaced cysteines in each loop that are required for gap-junctional channel formation (Dahl *et al.*, 1992). However, Cx35 differs from other connexins in having an unusually long cytoplasmic loop (77 amino acids) coupled with a short carboxyl terminal tail (52 amino acids). It is also unique in having an intron located within the coding region of the gene. All other connexins for which elements of the gene structure have been determined have a single intron in the 5'-untranslated portion of the transcribed sequence (cf. Miller *et al.*, 1988; Fishman *et al.*, 1990; Hennemann *et al.*, 1992; Beyer, 1993; Church *et al.*, 1995).

Figure 5. Southern blot analysis of skate genomic DNA and identification of an intron. (A) Ten microgram aliquots of skate genomic DNA digested with *Pst*I, *Eco*RI, *Hind*III, or *Nco*I were run through an agarose gel, transferred to a nylon membrane, and probed with a pair of clones covering the entire Cx35 cDNA sequence. Internal sites for *Eco*RI and *Pst*I are present in the cDNA sequence (see 5C). (B) PCR amplification of DNA fragments from skate genomic DNA reveals the presence of an intron in the 5' end of the sequence. Primer pair 1,2 (see Figure 5C) is predicted to amplify a 379-bp fragment, but yields an approximately 1.32-kb fragment (lane 1). Primer pair 1,4 is predicted to amplify a 1378-bp fragment but amplifies an approximately 2.3-kb fragment from genomic DNA (lane 2), while primer pair 3,4 amplifies a 906-bp fragment from both genomic DNA (lane 3) and the cDNA clone scxn11 (lane 4). PCR product 1,3 containing the intron was cloned, restriction mapped, and partially sequenced. Restriction sites for *Eco*RI, *Hind*III, and *Pst*I were identified within the intron and its location was found to be in the 5' end of the coding region. (C) Map showing the positions of the PCR primers and restriction sites in the Cx35 cDNA. The open reading frame is denoted by the shaded box. The triangle represents the intron identified by genomic PCR. (D) Sequence of the junctions of the intron and the two exons of the coding sequence. The exon sequence (capital letters) is numbered from the translation start site; the intron sequence (lower case) is not numbered. The accession number for the connexin 35 intron acceptor site is U43288. The accession number for the connexin 35 intron donor site is U43289.

The connexins cloned and sequenced thus far belong to a superfamily of gap-junctional genes (Beyer *et al.*, 1988; Bennett *et al.*, 1991, 1994). Within the vertebrates, the members of this superfamily fall into two groups, α (group II) and β (group I), which probably arose by a gene duplication early in the course of vertebrate evolution or before the appearance of vertebrates (Bennett *et al.*, 1994). The relationship of Cx35 to either the α or β group of

connexins is not entirely clear. The amino acid similarity comparison shown in Figure 3 suggests that Cx35 falls near the divergence point between the α and β groups. This is, perhaps, not surprising considering that this clone is derived from the most primitive vertebrate from which a connexin clone has been reported to date. The presence of an intron within the coding region of Cx35 also sets it apart from other connexins. Based on this information, it

Figure 6. Northern blot analysis of skate total RNA. Forty micrograms of total RNA were run on a formaldehyde agarose gel and transferred to nitrocellulose. (A) The blot was probed with Cx35 clone scxn18 and exposed to film for 7 days. Hybridization was detected only in the retinal RNA sample. (B) Ethidium bromide staining of the gel reveals approximately equal loading of RNA samples in all lanes. The 18S rRNA band is shown.

is difficult to determine if Cx35 is a primitive member of either the α or β group of connexins, a precursor to these groups, or a member of yet a third group. Further resolution of this question will require cloning of other related connexins.

It is noteworthy that Cx35 transcripts have been detected thus far only in the retina, and in this respect, it is unique among the connexins already characterized. The RNAs examined were derived from excitable tissues such as brain and heart, as well as a variety of tissues in which the presence of gap junctions has been demonstrated. Its retinal specificity makes Cx35 an interesting candidate for functional studies. The cloning and expression of connexin genes in other tissues has provided valuable information as to their unusual physiological properties. The divergent portions of the gap junction polypeptide, e.g. differences in amino acid sequence, the length of the carboxyl terminus and intracellular loop, or the location of phosphorylation sites, probably adapt channel activity to special tissue requirements. A number of functions have already been attributed to specific res-

idues or regions of the connexin proteins. For example, important determinants of junctional compatibility between adjacent cells have been localized to the second extracellular loop by deletion and domain swapping analyses (White *et al.*, 1994). Similarly, domain swapping and point mutation analyses with connexins 26 and 32 have established the N-terminus as well as residues near the border of the first transmembrane and first extracellular loops as functionally important in voltage gating (Suchyna *et al.*, 1993; Verselis *et al.*, 1994).

Of particular interest is the possible modulation of gap-junctional conductance by phosphorylation of connexin 35. Two consensus sequences consistent with sites for protein kinase phosphorylation are present in Cx35 (Figure 1). A protein kinase C consensus site is located on the cytoplasmic loop at position 101, shortly after the second transmembrane domain. In addition, there is a consensus site for cyclic AMP- and cyclic GMP-dependent protein kinase phosphorylation at serine 273 in the carboxyl terminal tail. Sequence analysis with PCGene software locates similar potential phosphorylation sites for protein kinase C in a broad range of connexins, and for cyclic AMP- or cyclic GMP-dependent protein kinase in Cx32, Cx40, and Cx42. Moreover, regulation of gap-junctional conductance by protein phosphorylation has been extensively documented (Bennett *et al.*, 1991; Beyer, 1993). With regard to retinal neurons, phosphorylation of a gap-junctional protein by a cAMP-dependent protein kinase represents a fundamental mechanism for the regulation of intercellular communication (Piccolino *et al.*, 1984; Lasater, 1987; McMahon *et al.*, 1989), a process that is known to play an important role in receptive field organization and visual adaptation (Baldridge and Ball, 1991; Dong and McReynolds, 1991; Weiler and Akopian, 1992). Thus, the presence of these phosphorylation sites, and the seemingly unique retinal disposition of Cx35, suggest that this connexin may be involved in the adaptive process. However, at what level within the neural network of the skate retina this may occur is unknown. Functional expression and in situ hybridization studies will be useful in identifying the pharmacological properties and cellular localization of Cx35.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants EY-06516 (H.R.) and AR-08254 (J.O.), by the Lions of Illinois, and by an award from the Foundation Fighting Blindness (M.R.A.).

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