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cDNA Cloning and Characterization of Type II Procollagen α1 Chain in the Skate *Raja kenojei*

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We determined the partial cDNA of Type II procollagen α1 [pro-α1(II)] chain (1802 bp) of the skate Raja kenojei, which codes 581 amino acid residues. The partial structure of the pro-α1(II) chain consisted of a part of triple helical region (309 residues) and a C-domain (272 residues). Comparing the chain to other vertebrates showed relatively low homology (about 50%) at the amino acid level. However, eight Cys residues in the C-domain of the skate pro-α1(II) chain were conserved in common with those of other vertebrates. The skate pro-α1(II) chain mRNA was detected by RT-PCR of various tissues, but was undetected in tissues containing Type II collagen. The low homology and unexpected expression pattern suggest the presence of another mRNA variant of the skate pro-α1(II) chain. The present study is the first report of the primary structure of pro-α1(II) chain in an elasmobranch.

Key words: cDNA cloning, Collagen, Elasmobranch, RT-PCR, Skate

Introduction

Type II collagen is found predominantly in the cartilage, and consists of three identical α-chains. The Type II collagen α1 chain has the same primary sequence as, or is a very similar genetic variant of, the α3(XI) chain, but they differ in their degree of Lys hydroxylation and glycosylation (Burgeson and Hollister, 1979). Later, both chains were found to be products of the short spliced variant of the COL2A1 gene named α1(IIB). Cartilaginous Type II collagen was shown to be the $[\alpha1(IIB)]_3$ molecule (Fichard et al., 1994).

In addition to the association of α-chains between collagen types, some evidence exists of heterotypic fibrils composed of different fibrillar collagen molecules. Supramolecular interactions between fibrillar collagen types have been demonstrated for Type I and III (Keene et al., 1987), Type I and V (Birk et al., 1988), and Type II and XI (Vaughan et al., 1988). Type II collagen is a main protein component of the cartilage, which occupies a priority space in the skeletal structure of elasmobranches, in contrast to teleosts. However, the primary structure of elasmobranch Type II collagen remains to be characterized. The present study describes a partial cDNA of Type II procollagen α1 [pro-α1(II)] chain from the skate Raja kenojei.

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Materials and Methods

Materials

Live skate *R. kenojei* specimens (body weight, 450-500 g) were obtained from local fishermen in Obama, Fukui, Japan, and reared in a running water tank at the Research Center for Marine Bioresources, Fukui Prefectural University, Obama, Fukui, Japan. Embryos from the egg capsules of adults were independently reared in an aerated plastic box.

RNA isolation

Samples of various tissues (muscle, skin, cartilage, gill, orbit, brain, liver, intestine, heart, stomach, spleen, pancreas, egg, ovary, uterine tube, shell gland, spiral valve, and whole blood) were taken from an adult skate and immediately placed in liquid nitrogen.

Frozen samples were pulverized in a mortar and pestle and then homogenized with Sepasol (Nakalai Tesque, Tokyo, Japan), and total RNA was isolated according to the manufacturer's instructions. Total RNA of four-week-old embryos were also prepared as described above, and used for cDNA library construction and rapid amplification of cDNA ends (RACE).

cDNA cloning of the skate pro-α1(II) chain

Total RNA (2 μg) from adult liver was reverse-transcripted using oligo(dT)$_{20}$ primer and the ThermoScript reverse transcription (RT)-PCR kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. A pair of oligonucleotide mixed primers, 5'-GT(TCAG)TT(TC)TG(TC)AA-(TC)ATGGA-3' and 5'-TT(TC)TT(AG)CA(AG)TG-(AG)TA (TCAG)GT-3', was used for degenerate PCR, based on highly conserved amino acid sequences of VFCNME and GSQNVT in the C-terminal propep-tides of vertebrate pro-α1(II) chains.

PCR was performed using the above primer set (0.1 μM each), the skate liver RT product as a template, and the HotStarTaq master mix kit (Qiagen, Hilden, Germany) on a PC-808 (Astec, Tokyo, Japan). The amplification process was as follows: one cycle of 15 min at 95°C; 30 cycles of 0.5 min at 94°C, 0.5 min at 50°C, and 1 min at 72°C; and one cycle of 10 min at 72°C. An amplified fragment of about 200 bp was excised with ethidium bromide (EtBr)-stained agarose gel (1%), and then purified using GenElute Minus EtBr spin column (Sigma, St. Louis, MO, USA). The fragment was subcloned into pDrive cloning vector (Qiagen) according to the manufacturer's instructions, and transformed into competent Escherichia coli cells, DH5α strain (Inoue et al., 1990). Positive colonies, including the plasmid DNA, were selected from Luria and Bertani (LB)-agar plates using ampicillin (50 mg/mL) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 20 mg/mL). Each clone was further incubated overnight in Terrific Broth (TB) medium with ampicillin, and then plasmid DNA was purified by precipitation with polyethylene glycol (Sambrook et al., 2001). The plasmid DNA sequence was determined with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

The mRNA of four-week-old skate embryo was purified using affinity beads of Oligotex^{TM}-dT30< Super>kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Approximately 3 μg mRNA was used to construct a cDNA library with Marathon cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Using the cDNA library, RACE was performed with gene-specific primers (GSP; SII-1, -2, and -3 in Fig. 1) based on the sequences obtained from the RT-PCR, and Marathon adapter primers. The PCR reaction was performed using an Advantage-GC 2 PCR kit (BD Biosciences Clontech) on a PC-808 (Astec). The amplification process was as follows: one cycle of 3 min at 94°C; 30 cycles of 0.5 min at 94°C and 3 min at 68°C; and one cycle of 3 min at 68°C. The 5'- and 3'-RACE products were subcloned and sequenced as described above.

Expression analysis by RT-PCR

Total RNA (5 μg) was treated with deoxyribonuclease (Nippon Gene, Tokyo, Japan) to remove contaminant genomic DNA traces, and reverse-transcribed into first-strand cDNA with the oligo(dT)$_{20}$ primer. PCR amplification was performed using a pair of the GSP primers (SII-1 and -3 in Fig. 1; 0.1 μM each) and various RT products of adult skate as template, and the Advantage-GC 2 PCR kit (BD Biosciences Clontech) on a PC-808 (Astec). The amplification process was as follows: one cycle of 3 min at 94°C; 30 cycles of 0.5 min at 94°C, 0.5 min at 60°C, and 1 min at 72°C; and one cycle of 1 min at 72°C.

Sequence analysis

Nucleotide and amino acid sequence analyses were performed using FASTA (www.ddbj.nig.ac.jp/search/fasta-j.html) and CLUSTALW (www.ddbj.nig.ac.jp/search/clustalw-j.html).

Results and Discussion

To obtain a cDNA fragment, RT-PCR was performed with degenerate primers corresponding to amino acid sequences of the C-terminal region, which is conserved with the highest similarity in vertebrate pro-α1(II) chains (Dion and Myers, 1987). A major band of about 200 bp was detected by electrophoresis of the amplified fragment. The fragment was subcloned, and its nucleotide sequence was determined. Analysis of the nucleotide and deduced amino acid sequences revealed significant similarity to the C-terminal domains of other vertebrate pro-α1(II) chains (data not shown), suggesting that the clone encodes a part of the skate pro-α1(II) chain (RK2A1-

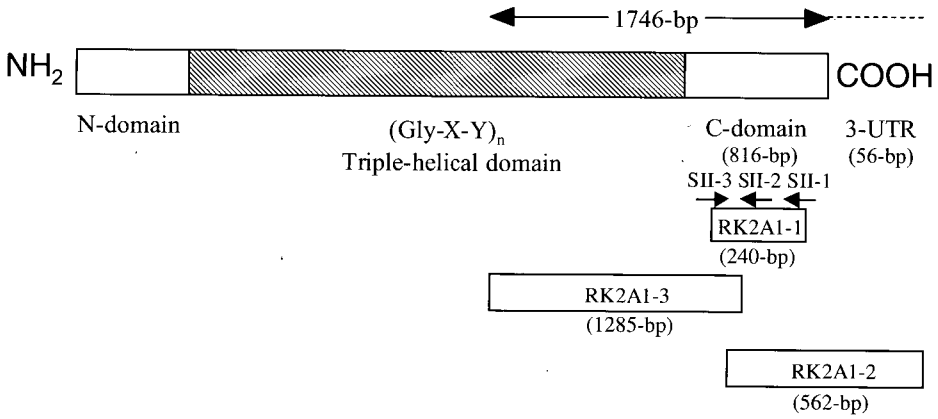

Fig. 1. cDNA cloning strategy for the skate pro-α1(II) chain. The top illustration represents the structure of the skate pro-α1(II) chain; the three rectangles below it represent cDNA clones obtained by degenerate PCR (RK2A1-1) or RACE (RK2A1-2 and 1-3). The primers (SII-1, CCATTCATAGATTCGCCAAACC; SII-2, GTGTGGAA TTTCAGATGGGTCAGC; SII-3, GACACCTGTATACATGCTGACCCATCTG) are indicated by short arrows, which show the 5' to 3' direction.

1; Fig. 1). As shown in Fig. 1, GSPs (SII-1, -2, and -3) were designed from the sequences of the RK2A1-1 fragment to extend the cDNA. The clones RK2A1-2 and RK2A1-3 were obtained by RACE, and the combined three cDNAs from the skate pro-α1(II) chain contained 1746 bp encoding 581 amino acids with a calculated molecular mass of 58,838.58 Da.

Figure 2 shows the nucleotide sequence of partial skate pro-α1(II) chain cDNA and its deduced amino acid sequence. The 3'-untranslated region (56 bp) contained a polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976). The partial cDNA of the chain encodes the triple-helical domain of $(Gly\text{-}X\text{-}Y)_{103}$ and a C-domain of 272 amino acids. The remaining portion of the chain, including the N-domain and possibly $(Gly\text{-}X\text{-}Y)_{235}$, was not determined.

The results were compared to published nucleotide and deduced amino acid sequences of pro-α1(II) chains of the human (Strausberg et al., 2002), horse (Richard and Dodge, 1997), dog (Du et al., 2000), rat (Krebsbach et al., 1996), mouse (Metsaranta et al., 1991), chicken (Deak et al., 1985), newt (Asahina et al., 1999), frog (Su et al., 1991), and zebrafish (Yan et al., 1995). As shown in Fig. 3, the C-domain of the skate pro-α1(II) chain was less conserved than other vertebrate counterparts, whereas the amino acid identity with human, mouse, bovine, and chicken (Metsaranta et al., 1991) was more than 85%.

To determine the most conserved regions, which may perform structural or functional roles in common with other vertebrate counterparts, the amino acid sequences were also analyzed by alignment (Fig. 4). In Type II procollagen, the site cleaved by C-proteinase is most likely between Ala and Asp, due to a CNBr dipeptide at the C-terminus of Type II collagen (Miller, 1972). This position is also conserved in the C-proteinase cleavage site Ala-Asp (335-336, Figs. 2 and 4) of the skate pro-α1(II) chain. Heterogeneity was the highest in the telopeptide regions and the N-terminal side of the C-propeptide. Both N- and C-telopeptides may participate in linear assembly of fibrils from triple-helical molecules to produce fibers characteristic of collagen types, and C-telopeptide may be involved in the lateral assembly that gives rise to the periodic arrangement of the collagen fibril (Capaldi and Chapman, 1982; Sandell et al., 1984). However, in this study, it was difficult to postulate any function of the skate pro-α1(II) chain from the alignment results alone (Fig. 4).

The C-propeptide must have an important role in the assembly of procollagen α-chains because the process starts at the C-propeptide. As shown in Figs. 2 and 4, the homologous regions in both skate and other vertebrates are located mainly between the C-propeptide at the Asn-Ile-Thr sequence (482-484) and eight Cys residues (376, 382, 399, 408, 416, 487, 532, 579). Biochemical studies of chicken procollagen α chains have revealed an oligosaccharide side chain at the Asn-Ile-Thr position (Pesciotta et al., 1981). Similarly, the Asn portion of the Asn-Ile-Thr sequence conserved in the skate chain may participate in glycosylation (Fig. 4). Cys plays an important role in the assembly of triple-helical molecules during pro-α1(II) collagen synthesis (Rosenbloom et al., 1976), and may serve a similar purpose in the skate pro-α1(II) chain.

In the triple-helical domain, the Arg-Gly-Asp se-

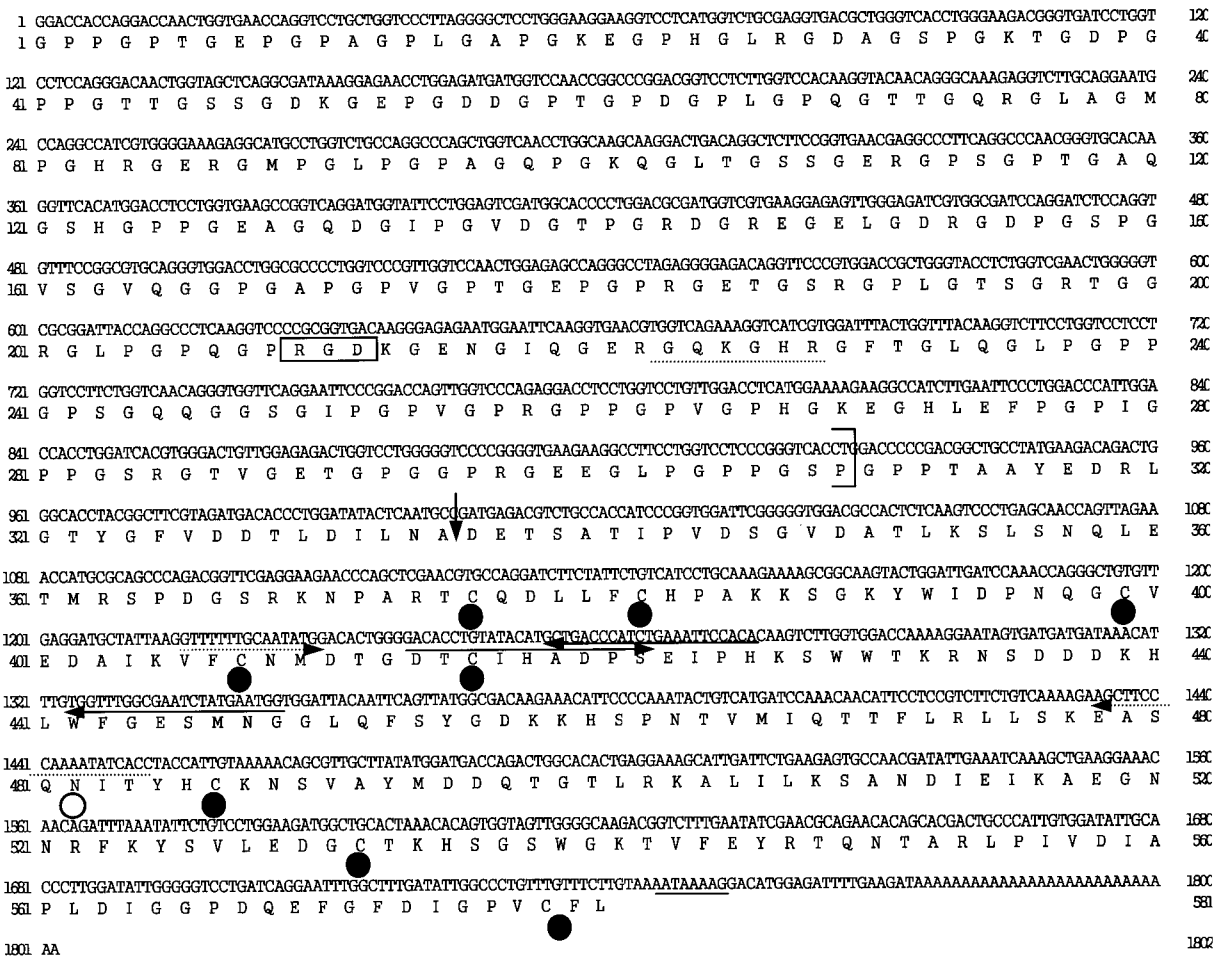

Fig. 2. Nucleotide sequence of partial cDNA of the skate pro-α1(II) chain and its deduced amino acid sequence. Three overlapping cDNA clones were combined to construct the cDNA. The potential cleavage site is shown by an arrow: Ala-Asp is the cleavage site for C-proteinase. The C-terminus of the triple-helical domain is bracketed. Potential cell-binding sites are boxed, and the dotted bar indicates the location of the putative cross-linking site. Open and closed circles indicate the glycosylation site and Cys residues, respectively. Broken and solid arrows indicate degenerate and gene-specific primers, respectively. The potential polyadenylation signal is underlined. The DDBJ accession number of the nucleotide sequence is AB201248.

quence (210-212; Fig. 2) is a potential cell-binding site (Ruoslahti and Pierschbacher, 1986), and the Gly-Gln-Lys-Gly-His-Arg sequence (223-228; Fig. 2) may be an important attachment site for lysyl oxidase (Kuhn, 1987). The highly conserved sequences in skate suggests that the skate pro-α1(II) chain may serve similar functions.

A phylogenetic analysis was performed to clarify the relationships among the C-domains of vertebrate pro-α1(II) chains (Fig. 5). As expected, the resulting tree placed the skate on the same branch as the zebrafish, and far from all other vertebrates.

Figure 6 shows the expression of pro-α1(II) chain mRNA in various skate tissues. Bands amplified by RT-PCR were detected in the muscle, skin, orbit, brain, liver, intestine, stomach, spleen, pancreas, egg, ovary, uterine tube, shell gland, and spiral valve, and not in cartilage, gill, heart, or whole blood. PCR products were further defined by cloning and sequencing. As expected, all products were identical to the respective regions of the skate pro-α1(II) chain cDNA. Although Type II collagen is predominant in cartilaginous tissues (Bornstein and Sage, 1980), the RT-PCR products of the skate pro-α1(II) chain were not detected in the cartilage or gill. Miosge et al. (1998) reported that the relatively low amount of Type II collagen mRNA in the chondrocytes of adult human articular cartilage might be explained by the slow turnover of Type II collagen in cartilaginous tissues. In addition, von der Mark and von der Mark

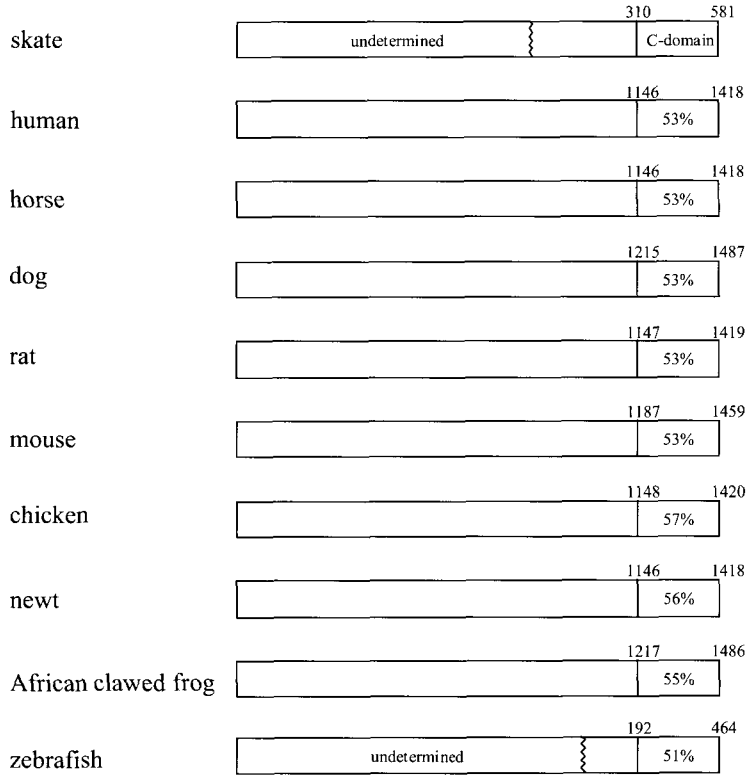

Fig. 3. Domain structure of the skate pro-α1(II) chain and homology with other vertebrate counterparts. The figures in boxes indicate the homology of the C-domain corresponding to the skate pro-α1(II) chain. The numbers above each box refer to the position of amino acids in each domain.

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                telopetide                                  C-propetide
skate     --GPPTAAYEDRLGTYGFVDDTLDILNADETSATIPVDSGVDATLKSLSNQLETMRSPDGSRKNPARTCQDLLFCHPAKKSGKYWIDPNQGCVEDAIKVFCNMDTGDTCIHADPSEIPHK  427
frog      GPGIDMSAFAGLSQPEKGPDPMRYMRADQASN---SLPVDVEATLKSLNNQIENIRSPDGTKKNPARTCRDLKLCHPEWKSGDYWIDPNQGCTVDAIKVFCDMETGETCVYPNPSKIPKK 1333
newt      GPGIDMSAFAGLSQPEKGPDPLRYMRADQASSTLRQHDAEVDATLKSLNNQIESIRSPEGNRKNPARTCRDLKLCHPEWKSGDYWIDPNQGCTLDAIKAFCNMETGETCVYPNPSKIPSK 1265
horse     GPGIDMSAFAGLGPREKGPDPLQYMRADFAAGGLRPHDEEVEATLKSLNNQIESIRSPEGSRKNPARTCRDLKLCHPEWKSGDYWIDPNQGCTLDAMKVFCNMETGETCVYPNPANVPKK 1265
human     GPGIDMSAFAGLGPREKGPDPLQYMRADQAAGGLRQHDAEVDATLKSLNNQIESIRSPEGSRKNPARTCRDLKLCHPEWKSGDYWIDPNQGCTLDAMKVFCNMETGETCVYPNPANVPKK 1265
dog       GPGIDMSAFAGLGQREKGPDPLQYMRADQAAGDLRQHDAEVDATLKSLNNQIESIRSPEGSRKNPARTCRDLKLCHPEWKSGDYWIDPNQGCTLDAMKVFCNMETGETCVYPNPASVPKK 1334
mouse     GPGIDMSAFAGLGQREKGPDPMQYMRADEADSTLRQHDVEVDATLKSLNNQIESIRSPDGSRKNPARTCQDLKLCHPEWKSGDYWIDPNQGCTLDAMKVFCNMETGETCVYPNPATVPRK 1306
rat       GPGIDMSAFAGLGQREKGPDPLQYMRADEADSTLRQHDVEVDATLKSLNNQIESIRSPDGSRKNPARTCQDLKLCHPEWKSGDYWIDPNQGCTLDAMKVFCNMETGESCVYPNPATVPRK 1266
chicken   GTGIDMSAFAGLGQTEKGPDPIRYMRADEAAGGLRQHDVEVDATLKSLNNQIESIRSPEGSKKNPARTCRDIKLCHPEWKSGDYWIDPNQGCTLDAIKVFCNMETGETCVYPTPSSIPRK 1267
zebrafish GPGIDMSAFAGLSQPEKGPDPLRYMRADEASSSLRQHDVEVDATLKSTNGQIEDIRSPDGSRKNPARSCRDLKLCHPEWKSGDYWVDPNLGSAADAIKVFCNMETGETCVKPSTPKIPRK  311
          *   :*: .       *    :   : .        *:*****:.,*:* :***:*.:*****:*:*: :***  ***.**:*** *., **:*.**:*:**::*: . .. :* *

                                                                                                                                    
skate     SWWTKRNSDDDKHLWFGESMNGGLQFSYGDKKHISPNTVMIQTTFLRLLSKEASQNITYHCKNSVAYMDDQTGTLRKALILKSANDIEIKAEGNNRFKYSVLEDGCTKHSGSWGKTVFEYR  547
frog      NWWSAK-GKEKKHIWFGETINGGFQFSYGDDSSAPNTANIQMTFLRLLSTDASQNITYHCKNSIAFMDEASGNLKKAVLLQGSNDVEIRAEGNSRFTYNALEDGCKKHTGKWSKTVIEYR 1452
newt      NWWTSK-SKDKKHIWFGETINGGLQFSYGDDSSAPNTASIQMTFLRLLSTDASQNITYHCKNSIAFMDEASGNLKKALLLQGSNDVEIRAEGNSRFTYHALEDGCKKHTGKWGKTVIEYR 1384
horse     NWWSSK-SKDKKHIWFGETINGGFHFSYGDDNLAPNTANVQMTFLRLLSTEGSQNITYHCKNSIAYLDEAAGNLKKALLIQGSNDVEIRAEGNSRFTYTVLKDGCTKHTGKWGKTTIEYR 1384
human     NWWSSK-SKEKKHIWFGETINGGFHFSYGDDNLAPNTANVQMTFLRLLSTEGSQNITYHCKNSIAYLDEAAGNLKKALLIQGSNDVEIRAEGNSRFTYTALKDGCTKHTGKWGKTVIEYR 1384
dog       NWWSSK-SKDKKHIWFGETINGGFHFSYGDDNLAPNTANVQMTFLRLLSTEGSQNITYHCKNSIAYLDEAAGNLKKALLIQGSNDVEIRAEGNSRFTYTVLKDGCTKHTGKWGKTMIEYR 1453
mouse     NWWSSK-SKEKKHIWFGETMNGGFHFSYGDGNLAPNTANVQMTFLRLLSTEGSQNITYHCKNSIAYLDEAAGNLKKALLIQGSNDVEMRAEGNSRFTYTALKDGCTKHTGKWGKTVIEYR 1425
rat       NWWSSK-SKEKKHIWFGETMNGGFHFSYGDGNLAPNTANVQMTFLRLLSTEGSQNITYHCKNSIAVLDEAAGNLKKALLIQGSNDVEMRAEGNSRFTYTALKDGCTKHTGKWGKTTIEYR 1385
chicken   NWWTSK-TKDKKHVWFAETINGGFHFSYGDENLSPNTASIQMTFLRLLSTEGSQNVTYHCKNSIAYMDEETGNLKKAILIQGSNDVEIRAEGNSRFTYSVLEDGCTKHTGKWGKTVIEYR 1386
zebrafish NWWTSK-SKAQKHVWFGESMNGGFHFSYADGSQTPSTTTIQLNFLRLLSTEATQTITYHCKNSVAYMDQATGNLKKAILLQGSNDVEIRAEGNSRFTYGVLEDGCKKHTGQWAKTVIEYK  430
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skate     TQNTARLPIVDIAPLDIGGPDQEFGFDIGPVCFL  581
frog      TQKTSRLPIVDIAPMDIGGADQEFGVDIGPVCFL 1486
newt      TQKTSRLPIVDIAPMDIGGADQEFGVDIGPVCFL 1418
horse     SQKTSRLPIIDIAPMDIGGPEQEFGVDIGPVCFL 1418
human     SQKTSRLPIIDIAPMDIGGPEQEFGVDIGPVCFL 1418
dog       SQKTSRLPIIDIAPMDIGGPEQEFGVDIGPVCFL 1487
mouse     SQKTSRLPIIDIAPMDIGGAEQEFGVDIGPVCFL 1459
rat       SQKTSRLPIVDIAPMDIGGPDQEFGVDIGPVCFL 1419
chicken   LQKTSRLSIVDTAPMDIGGADQEFGVDIGPVCFL 1420
zebrafish TQKTSRLPIMDIAPMDIGGADQEFGVDIGAVCFL  464
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Fig. 4. Alignment of amino acid sequences of the C-domain of vertebrate pro-α1(II) chains. The dashed lines indicate gaps inserted to give the best alignment. Asterisks, colons, and dots represent fully conserved, conserved, and similar amino acids, respectively.

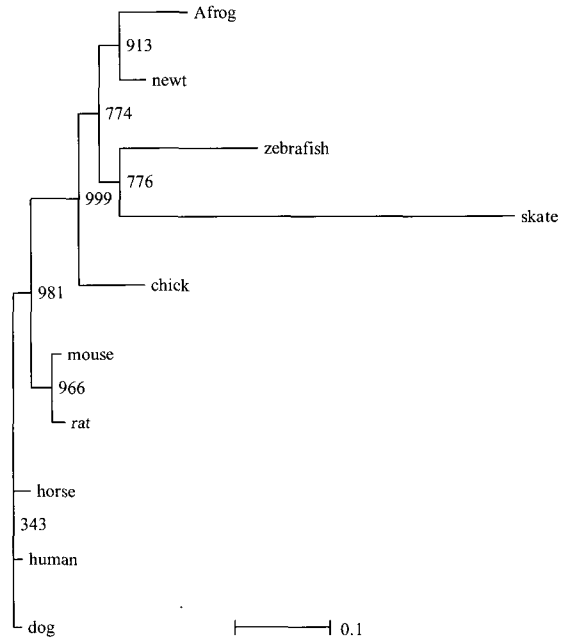

Fig. 5. Phylogenetic tree based on the C-domain of the vertebrate pro-α1(II) chains. The scale bar shows substitution of one amino acid residue per ten residues. The phylogenetic tree was constructed by joining the amino acid sequences of various species. The numbers at the nodes indicate bootstrap values for 1000 runs.

(1977) demonstrated that a gradual and continuous transition occurs from Type I to Type II collagen during chondrogenic differentiation in the limb mesodermal cells of chicks. However, the most plausible explanation may be that Type II procollagen can be expressed in two forms by differential splicing of the primary gene transcript. For example, Type II procollagen mRNA showed a distinct tissue distribution during chondrogenesis, that is, Type IIB procollagen mRNA was expressed in chondrocytes and the Type IIA counterpart was expressed in cells surrounding cartilage in prechondrocytes in the human fetal vertebral column (Sandell et al., 1991). Similarly, small amounts of Type IIA collagen have been detected at the epitheliomesenchymal interfaces during morphogenesis of the cartilaginous neurocranium and in other noncartilaginous tissues of developing chicken embryos (Newsome et al., 1976; Linsenmayer and Little, 1978). Therefore, it is possible that at least one other mRNA for Type II collagen, expressed mainly in the cartilage, may exist in the skate, and that the expression pattern of mRNA for the skate pro-α1(II) chain may vary according to the developmental stage of the animal.

This study presents the partial structure of the skate pro-α1(II) chain, derived from sequences of three overlapping cDNA clones. Further studies are currently under way to determine the full structure of the skate chain, in addition to another Type II procollagen variant, and to elucidate the expression properties of skate pro-α1(II) chain mRNA during embryonic development of the animal.

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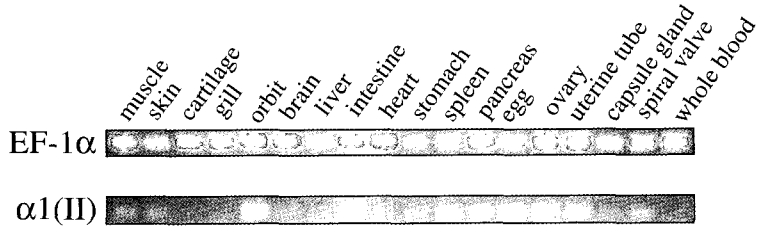

Fig. 6. Expression of skate pro-α1(II) chain mRNA. Total RNA from muscle, skin, cartilage, gill, orbit, brain, liver, intestine, heart, stomach, spleen, pancreas, egg, ovary, uterine tube, capsule gland, spiral valve, and whole blood in adult skates were employed to generate cDNA for PCR amplification using the skate pro-α1(II) chain-specific primers together with EF-1α (DDBJ accession no. AB214933) as a positive control.

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