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Food Chemistry

Food Chemistry 107 (2008) 1467-1472

www.elsevier.com/locate/foodchem

# cDNA cloning and characterization of Type V/XI procollagen $\alpha$ 1 chain in the skate, *Raja kenojei*

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Received 21 June 2006; received in revised form 1 September 2007; accepted 1 October 2007

# Abstract

The full-length cDNA of the Type V/XI procollagen  $\alpha 1$  [pro- $\alpha 1$ (V/XI)] chain was determined by the RACE technique, using a cDNA library constructed from the 4-week embryo of the skate, *Raja kenojei*. The expression property and phylogenetic analysis confirmed that the skate pro- $\alpha 1$ (V/XI) chain was close to other vertebrate pro- $\alpha 1$ (V) chains rather than pro- $\alpha 1$ (XI) chains. The present study is the first evidence for the primary structure of full-length cDNA of the pro- $\alpha 1$ (V/XI) chain in an elasmobranch. © 2007 Elsevier Ltd. All rights reserved.

Keywords: cDNA; Cloning; Collagen; Embryo; RT-PCR; Fish

## 1. Introduction

The fibrillar collagens are the most abundant proteins of extracellular matrices. Among them, Type V and XI collagens are quantitatively minor components, which participate in the formation of the fibrillar collagen network. The current view on the tissue localization of fibrillar collagen is that Type V collagen is associated with Type I and III collagens in non-cartilaginous tissues (Fitch, Gross, Mayne, Johnson-Wint & Linsenmayer, 1984; Linsenmayer et al., 1983). On the other hand, Type XI collagen, associated with Type II collagen, has been discovered in cartilaginous matrices (Mendler, Eich-Bender, Vaughan, Winterhalter & Bruckner, 1989). The tissue localizations of Type V and XI collagens are different, but their structural and biological properties seem to be closely related. Fichard, Kleman, and Ruggiero (1994) demonstrated that several molecules are heterotypic associates of Type V and XI collagen chains, suggesting that these two collagens

are not distinct types but a single type, which can be called Type V/XI collagen. Moreover, minor collagens in the fishes have been recognized as important factors relating to *post-mortem* tenderization under chilled storage (Sato et al., 1997).

We have isolated and characterized molecular species of collagens from the muscle, cartilage and skin of skate (Hwang, Mizuta, Yokoyama & Yoshinaka, 2007; Mizuta, Hwang & Yoshinaka, 2002, 2003). In the course of the work, we found different biochemical characteristics among the minor collagens, in contrast to the fact that the distribution of molecular identity of collagen is fundamentally the same between skin and muscle of minor collagens from some teleosts (Yata, Yoshida, Fujisawa, Mizuta & Yoshinaka, 2001). As for fish minor collagen, there is only one report on the full-length cDNA of Type V/XI procollagen  $\alpha 1$  [pro- $\alpha 1(V/XI)$ ] chain from the red seabream Pagrus major (Touhata, Tanaka, Yokoyama, Sakaguchi & Toyohara, 2001). Its sole examination is still insufficient for understanding characteristics of fish Type V/XI collagen. The present study describes a full-length cDNA of pro- $\alpha 1(V/XI)$  chain from the skate, *Raja kenojei*.

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<sup>0308-8146/\$ -</sup> see front matter  $\odot$  2007 Elsevier Ltd. All rights reserved. doi:10.1016/j.foodchem.2007.10.002

## 2. Materials and methods

### 2.1. Materials

Skate, *Raja kenojei* (body weight, 450–500 g), was obtained alive from local fishermen in Obama, Fukui, Japan, and reared in the running water tank of the Research Center for Marine Bioresources, Fukui Prefectural University in Obama, Fukui, Japan. Newborn embryos in the egg capsules were independently reared in the plastic box, with supply of enough aeration.

# 2.2. RNA isolation

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 dissected out from the adult skate and immediately placed in liquid nitrogen. Frozen tissues were pulverized in a mortar with a pestle and then homogenized with Sepasol (Nacalai tesque, Japan), and total RNAs were isolated according to the manufacturer's instructions. Total RNAs of 4- and 8-week embryos were prepared, as described above, and used for cDNA library construction and rapid amplification of cDNA ends (RACE), respectively.

# 2.3. cDNA cloning of skate pro- $\alpha I(V|XI)$ chain

Total RNA (2 µg) from adult liver was reverse-transcribed by using an oligo(dT)<sub>20</sub> primer and ThermoScript reverse transcription (RT)-PCR system (Invitrogen, USA) according to the manufacturer's instructions. A pair of oligonucleotide mixed primers, 5'-TT(TC)CC-(TCAG)GA (TC)GG(TCAG)GA(AG)TA(TC)TGG-3' and 5'-TC(AG)AA(TCAG)CC(AG)AA(TC)TT(TC)TG-3', was designed, based on highly conserved amino acid sequences in the C-terminal propeptides of vertebrate pro- $\alpha$ 1(V) and pro- $\alpha$ 1(XI) chains, which are conserved in the highest similarity to vertebrate pro- $\alpha$ 1(V) and pro- $\alpha$ 1(XI) chains (Dion & Myers, 1987).

Degenerate PCR was conducted to obtain cDNA fragment by using the above primer set (0.1  $\mu$ M each), the skate liver RT product as a template and HotStarTag master mix kit (Qiagen, The Netherlands) in a PC-808 (Astec, Japan) with one cycle of 95 °C for 15 min, 30 cycles at 94 °C for 0.5 min, at 50 °C for 0.5 min and at 72 °C for 1 min, and one cycle of 72 °C for 10 min. The resultant products, showing expected size, were subcloned and sequenced. The amplified fragment was subcloned and its nucleotide sequence was determined. Analysis of the nucleotide and deduced amino acid sequences revealed a significant similarity to the C-terminal domains of other vertebrate pro- $\alpha l(V)$  and pro- $\alpha l(XI)$  chains (data not shown). From this result, it was speculated that the clone encodes a part of the skate pro- $\alpha l(V/XI)$  chain. Gene specific primers (GSPs) were designed from the sequence of the degenerate PCR

fragment for extending the skate  $\text{pro-}\alpha 1(V/XI)$  chain cDNA.

The mRNA was purified from 4-week skate embryo by Oligotex<sup>™</sup>-dT30(Super) kit (Roche, Switzerland). Approximately 3 µg of the mRNA was applied for constructing the cDNA library with a Marathon cDNA amplification kit (BD Biosciences Clontech, USA). Using the cDNA library, rapid amplification of cDNA ends (RACE) was conducted by GSPs, based on the sequence obtained from the degenerate PCR, and Marathon adapter primers. The PCR reaction was conducted by using an Adavantage-GC 2 PCR kit (BD Biosciences) with one cycle of 94 °C for 3 min, 30 cycles at 94 °C for 0.5 min, at 68 °C for 3 min, and one cycle of 68 °C for 3 min. The resultant 5' and 3' RACE products were subcloned and sequenced. Moreover, the 5' RACE was further utilized to obtain 5' end of the skate pro- $\alpha 1(V/XI)$  chain cDNA according to the method of Zhi (1996) with some modifications.

### 2.4. Expression analysis by RT-PCR

Total RNA was determined by measuring the absorbance of 260 nm in a spectrophotometer, and the purity of the RNA was confirmed by readings of the 260 nm/ 280 nm ratio in 10 mM Tris–HCl, pH 7.5. Total RNA (5  $\mu$ g) was treated with deoxyribonuclease (Nippon Gene, Japan) to remove contaminant genomic DNA traces, and reverse-transcribed into first strand cDNA with the oligo(dT)<sub>20</sub> primer. PCR amplification was performed by using a pair of the gene specific primer (0.1  $\mu$ M each), and various RT products from skate adult origin as template, and Adavantage-GC 2 PCR kit (BD Biosciences, USA) in a PC-808 (Astec, Japan) with one cycle of 94 °C for 3 min, 30 cycles at 94 °C for 0.5 min, at 60 °C for 0.5 min, and at 72 °C for 1 min, and one cycle of 72 °C for 1 min.

#### 2.5. Sequence analysis

The nucleotide and amino acid sequence analyses were performed by using FASTA (http:www.ddbj.nig.ac.jp/ search/fasta-j.html) and CLUSTAL W (http:www.ddbj.nig.ac.jp/search/clustalw-j.html).

#### 3. Results and discussion

Full-length cDNA of the skate pro- $\alpha 1(V/XI)$  chain (accession no. AB201248) was derived from sequences of six overlapping cDNA clones, and contained a 5580bp open reading frame encoding a protein of 1860 amino acids with a calculated molecular mass of 187,813.2 Da.

Alignment with amino acid sequences of other vertebrate pro- $\alpha 1(V)$ , pro- $\alpha 1(XI)$  and pro- $\alpha 1(V/XI)$  chains demonstrates, more clearly, both the similarity and dissimilarity among them (Chernousov, Rothblum, Tyler, Stahl & Carey, 2000; Gordon et al., 1999; Greenspan, Cheng & Hoffman, 1991; Takahara et al., 1991; Touhata, Tanaka, Yokoyama, Sakaguchi & Toyohara, 2001; Wu, Sumiyoshi, Khaleduzzaman, Ninomiya & Yoshioka, 1998: Yoshioka et al., 1995: Yoshioka & Ramirez, 1990). The difference between  $pro-\alpha I(V)$  and  $pro-\alpha I(XI)$  chains is the number of amino acid residues in the N-domain. The N-domains of  $pro-\alpha 1(V)$  chains (554–560 residues) are longer (over 25 amino acid residues) than those of pro-al(XI) chains (526-528 residues). The N-domain of the skate pro- $\alpha I(V/XI)$  chain was found to consist of 579 amino acid residues. From the result, the skate  $pro-\alpha I(V/$ XI) chain seems to be closer to  $pro-\alpha I(V)$  chains than  $pro-\alpha I(XI)$  chains. Like the N-propertide of other vertebrate  $pro-\alpha I(V)$  and  $pro-\alpha I(XI)$  chains, the N-propertide of the skate pro- $\alpha l(V/XI)$  chain contains a long globular domain, followed by an interrupted collagenous domain. A concentration of mismatches and gaps was seen in the acidic globular subdomains, which lie between the cluster of Cys (234 and 241, Fig. 1) and small collagenous domains (468–559, Fig. 1) in the N-propeptides of the skate pro- $\alpha 1$ (V/XI) chain, as with other vertebrate counterparts.

Of note is the presence of two potential Asn-linked carbohydrate attachment sites (Asn-Leu-Ala, 156–158; Asn-Ala-Thr, 393–395, Fig. 1), conserved in the skate pro- $\alpha 1(V/XI)$  chain, in the two globular subdomains of the N-propeptide. Similar potential sites are not found in the N-propeptides of mammalian pro- $\alpha 1(I)$ , pro- $\alpha 1(II)$  and pro- $\alpha 1(III)$  chains. Moreover, the other two sites (Asn-Glu-Thr, 379–381; Asn-Ser-Glu, 400–402, Fig. 1) were located around the second potential Asn-linked site. In the skate pro- $\alpha 1(V/XI)$  chain, at least one of the potential glycosylation sites may be utilized and be of functional significance.

	signal peptide	N-propeptide
skate (V/XI) mouse (V) rat (V) hamster (V) human (V) chicken (V) mouse (XI) human (XI) red seabream (V/XI)	MDDRVRWKWKGWFWDSVLTLQIAILTLTASVS MDVHTRWKAARPGALLLSSPLLLFLLLLWAPPSSR MDVHTRWKAARPGAPLLSSPLLLLLLLWAPPPSR MDVHTRWKDRLPVGPAAVPPLLLLLLLWAPPPSR MDVHTRWKRRSWIRNWQLHVALVLLGAALGR MEPWSSRWKTKR-WLWDFTVT-TLALTFLFQAREVR MEMQRWTTRWKTKR-WLWDFTVT-TLALTFLFQAREVR *** ::::	VEPVDILKILQLDTLPDGIERVTGLCPNRKLTSTPDVAYKVSRLAQLSAQTKQLYPGDTPFPEDFSLVMTVRAQKGKGAFL 114 AQPADLLEMLDFHNLPSGVTKTTGFCATRRSSSEPDVAYRVSKDAQLSMPTKQLYP-ESGFPEDFSILTTVKAKKGSQAFL 116 AQPTDLLEMLDFHNLPSGVTKTTGFCATRRSSRGPDVAYRVSKDAQLSMPTKQLYP-DSDFAEDFSILTTVKAKKGSQAFL 116 AQPTDLLEMLDFHNLPSGVTKTTGFCATRRSSKGPDVAYRVSKDAQLSMPTKQLYP-ASAFPEDFSILTTVKAKKGSQAFL 116 AQPADLLKVLDFHNLPDGITKTTGFCATRRSSKGPDVAYRVTKDAQLSAPTKQLYP-ASAFPEDFSILTTVKAKKGSQAFL 116 APPADLLKVLDFHNLPDGITKTTGFCATRRSSKGPDVAYRVTKDAQLSAPTKQLYP-ASAFPEDFSILTTVKAKKGSQAFL 114 AAPVDIKALDFHNSPVGISKTTGFCTNRKNSKDPDVAYRVTKDAQLSAPTKQLFP-GGTFPQDFSILTTVKAKKGSQFL 113 APVDIKALDFHNSPEGISKTTGFCTNRKNSKGDTAYRVSKQAQLSAPTKQLFP-GGTFPQDFSILFTKKKKGTQFL 114 AAPVDVKALDFHNSPEGISKTTGFCTNRKNSKGSDTAYRVSKQAQLSAPTKQLFP-GGTFPQDFSILFTKKKKGQSFL 115 AEPVDVLKALDFLNSPEGISKTTGFCSVRGSQ-PDTAYRVSKQAQLSAPTKQLFP-GGVFPEDFSILFTKKKKGQSFL 117 . * * * * * * * * * * * * * * * * * * *
skate (V/XI) mouse (V) rat (V) hamster (V) human (V) chicken (V) mouse (XI) human (XI) red seabream (V/XI)	LSIYNEQGIQQLGVSFP-QPHILYEDHAQQPSPDEHPI VSIYNEQGIQQLGLELGRSPVFLYEDHTGKPGPEEYPL VSVYNEQGIQQLGLELGRSPVFLYEDHTGKPGPEEYPL VSVYNEQGIQQIGLELGRSPVFLYEDHTGKPGPEDYPL VSIYNEQGIQQIGLELGRSPVFLYEDHTGKPGPEDYPL LSIYNEQGIQQIGVEVGRSPVFLYEDHTGKPFDPYPL LSIYNEHGIQQIGVEVGRSPVFLFEDHTGKPAPEDYPL LSYNEHGIQQIGVEVGRSPVFLFEDHTGKPAPEDYPL LSYNNQQGIQQLGVEVGRGPVFLYEDQHGKPAPEEYPL :*:**::****:*: *::*:*:	RGINLADGKWHRVAISVHKKTVTMIVDCTKTLTQPLNRSKRPVIDTNGIVVFGTRILDEDVFQGDVQQFLIIADHRAAYDY 233 PGINLSDGKWHRIALSVYKKNVTLILDCKKKITKFLSRSDHPIIDTNGIVMFGSRILDDEIFEGDIQQLLFVSDNRAAYDY 236 PGINLSDGKWHRIAISVYKKNVTLILDCKKKITKFLNRGDHPIIDVNGIIMFGSRILDDEIFEGDIQQLLFVSDHRAAYDY 236 RGINLSDGKWHRIALSVYKKNVTLILDCKKKVVKFLNRSDHPIIDVNGIIMFGSRILDDEIFEGDIQQLLFVSDHRAAYDY 236 RGINLSDGKWHRVAISVCKKNVTLILDCKKKITKFLDRSDHPIIDVNGIIVFGTRILDEEVFEGDIQQLLFVSDHRAAYDY 236 RGINLADGKWHRVAISVCKKNVTLILDCKKKITKFLDRSDHPIIDVNGIIVFGTRILDEEVFEGDIQQLLFVSDHRAAYDY 236 RGINLADGKWHRVAISVCKKNVTLILDCKKKITKFLDRSDHPIIDVNGIIVFGTRILDEVFEGDIQQLLFVSDHRAAYDY 237 STVNIADGKWHRVAISVCKKNTVTIVDCKKKITKFLDRSERAIVDTNGITVFGTRILDEEVFEGDIQQLLIVADPRAAVDY 235 RSINLADGKWHRVAISVEKKAVTIIVDCKKKITKFLDRSERAIVDTNGITVFGTRILDEEVFEGDIQQLLIVADPRAAVDY 237 ::::******:**:**:********************
skate (V/XI) mouse (V) rat (V) hamster (V) human (V) chicken (V) mouse (XI) human (XI) red seabream (V/XI)	CEHYSPDCDSPLPETPQNQDPDTLDYDPAADSYY CEHYSPDCDTAVPDTPQSQDPNPDEYYPEGEGETYY CEHYSPDCDTAVPDTPQSQDPNPDEYYPEGEGETYY CEHYSPDCDTAVPDTPQSQDPNPDEYYPEGDGETYY CEHYSPDCDTAVPDTPQSQDPNPDEYYTDGGEGEGTYY CCHYSPDCDTAVPDAPQSQDPNQDEYYTDGEGEGDTYY CDHYSPDCDTAVPDAPQSQDPNQDEYYTDGEGEGDTYY CEHYSPDCDTAVPDAPQSQDPNQDEYYTDGEGEGDTYY CEHYSPDCDTAVPDAPQSQPNQDEYTDGEGEGDTYY CEHYSPDCDTAVPDAPQSQPNQDEYTDGEGEGDTYY CEHYSPDCDTSAPKAAQAQEPQIDEYAPEDIIEYDYEY CEHYSPDCTPNHHDTPQAQEPEGEYTNYDY *:******: : : * : *	EYPYYEDINDDKTKESEDGAEGAETDFSAQITGPITAVPDQQETYPTEGPEEVITVTIPGTTAEAAIDEYPTVETGYYDNY 349 EYPYYEDPEDPGKEPAPTQKPVEAARETTEVPEEQTQPLPEAPTVPETSDTAGKEDNPGIGDYDYVPP 341 EYPYYEDPEDPGKEPAPTQKPVEAARETTEVPEEQTQPPSEAPTVPETSDTAGKEDDPGIGDYDYVPT 341 EYPYYEDPEDLGKEPAPTQKPVEAARETTEVPEEQTQPPPEAPTVPETSDPAGKEDDPGGDYDVVPT 341 EYPYYEDPEDLGKEPTPSKKPVEAAKETTEVPEEQTQPPTEAAPMPETSEGAGKEEDVGIGDYDVVPT 343 EYPYYEDVEAEKPTPSKKPVEAAKETTEVPEELTPTPTEAAPMPETSEGAGKEEDVGIGDYDVVPT 343 ETDYKEAESVTEMPTFTEETVAQTEANIVDDFQDYNGTMEPYQTETPRRVSGSNEPNP 333 EAEYKEAESVTEGPTVTEETIAQTEANIVDDFQEYNGTMESYQTEAPRHVSGTNEPNP 333 ETYDYIEGELTTDSPPTEGAKTETNVGGDYYTGEYDYSIVDGNQPETPTDTAGQGQSYDGEI 331 *
skate (V/XI) mouse (V) rat (V) hamster (V) human (V) chicken (V) mouse (XI) human (XI) red seabream (V/XI)	DDDYYFPELTLTEETPEKEDNPSSGQEFENETSITFER -DDYYTPPPYEDFGYGEGVENPDQPTNPDSGAEVPTST -DDYYTTSPYEDFGYGEGVENPDQPTNPDSGAEVPTST -DDYYTPAPYEDLGYGEGVENPDQPTNPDSGAEVPTST -EDYYTPAPYEDLTYGEGEENPDQPTDPGAGAEIPTST NEEYFTPLPYEDINYNEEVDPQGGLTENAVEAELPTST VEEGFTEEYLTGEDYDVQRNTSEDILYGNKGVDGROSD VEEIFTEEYLTGEDYDSQRKNSEDTLYENKEIDGRDSD VDDYITGVEQVGVDPTVAVETAAPVDPNEKVDPATDVY ::	VFSHNATTFEDNSEHTIEIVEEAIPGQGSRNEDKFNVTTIGEEDPNTYFDEFEVYYDYDDVTVGPGAPVLTDTDYVRGP 467 VTSNTSNPAPGEGKDDLGGEFTEETIKNLEENYYDPYFDPDSDSNVSPSEIGPGMPANQDTIFEGIG 446 VTSNTSNPAPSPEGKODLGGEFTEETIKNLEENYYDPYFDPDSDSNVSPSEIGPGMPANQDTIFEGIG 448 ITSNTSNPAPSPGEGKADLGGEFTEETIKNLEENYYDPYFDPDSDSNVSPSEIGPGMPANQDTIFEGIG 446 ITSNTSNPAPSPGEGADDLGGEFTEETIKNDURYYDPYTDFSSPSEIGPGMPANQDTIFEGIG 446 ITYNETDAAQGGDDLDKDFTEETIKEYDGNYYYYDTSDIGPGMPANQDTIFEGIG 442 LVDGDLGEYDFYEYKEYEERTTSPNEEFGPGVPAETDFTETSINGH 417 LVDGDLGEYDFYEYKEYEDKPTSPNEEFGPGVPAETDIFETSINGH 419 FKEYDLKEYEHKEFVEKPYEYGDYGDYGTADTKPTSATTEEFGPGVPAETDIFETSINGH 4129 .*** *. *
skate (V/XI) mouse (V) rat (V) hamster (V) human (V) chicken (V) mouse (XI) human (XI) red seabream (V/XI)	GIRGEKGEKGEPAIIEPGMLIEGPPGSEDPPGVSGHPG GPRGEKGQKGEPAIIEPGMLIEGPPGPEGPAGLPGPPG GPRGEKGQKGEPAIIEPGMLIEGPPGPEGPAGLPGPPG GPRGEKGQKGEPAIIEPGMLIEGPPGPEGPAGLPGPPG GPRGEKGQKGEPAIIEPGMLVEGPPGPEGPAGLPGPPG GAYGEKGQKGEPAVVEPGMLVEGPPGPAGPAGLMGPPG GAYGEKGQKGEPAVVEPGMLVEGPPGPAGPAGLMGPPG SYLGEKGEKGEPAVVEPGMLVEGPQGAPGQAGVMGPPG	telopeptide PGPPGPVGDPGSRGLLGRSGLPGADGLPGPPGTMLMLPFRFTGGGGAGEKGPMVSAQEAQAQAILQQARLALR 579 TGPTGQMGDPGERGPPGRPGLPGADGLPGPPGTMLMLPFRFGGGGDAGSKGPMVSAQESQAQAILQQARLALR 558 TGPTGQVGDPGERGPPGRPGLPGADGLPGPPGTMLMLPFRFGGGGDAGSKGPMVSAQESQAQAILQQARLALR 560 MGPTGQVGDPGERGPPGRPGLPGADGLPGPPGTMLMLPFRFGGGGDAGSKGPMVSAQESQAQAILQQARLALR 558 TGPVGLMGDPGERGPPGRPGLPGADGLPGPPGTMLMLPFRFSGGGDAGSKGPMVSAQESQAQAILQQARLALR 554 QGPSGLPGDPGDRGPGPGPGLPGADGLPGPPGTMLMLPFRFSGGGDAGSKGPMVSAQEAQQAILQQARLALR 554 QGPSGLPGDPGDRGPGPRGLPGADGLPGPPGTMLMLPFRFSGGDAGSKGPMVSAQEAQQAILQQARLALR 554 QGPSGLPGDPGDRGPPGRPGLPGADGLPGPPGTMLMLPFRFSGGDAGSSKGPTISAQEAQQAILQQARLALR 528 QGPPGPPGDPGDRGPPGRPGLPGADGLPGPPGTMLMLPFRYGGDCSKGPTISAQEAQAQAILQQARLALR 538 TGPFGQVGDAGRGPGRPGLPGADGLPGPPGTMLMLPFRYGGDSSKGPTISAQEAQAQAILQQARLALR 538 TGPFGQPGDHGERGPPGRPGLPGADGLPGPPGTMLMLPFRYGGDSSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGPGDHGERGPPGRPGLPGADGLPGPFGTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGPGDHGERGPPGRPGLPGADGLPGPGTTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGPGDHGERGPPGRPGLPGADGLPGPGTTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGDHGERGPPGRPGLPGADGLPGPGTTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGDHGERGPPGRPGFFGRFGLPGADGLPGPGTTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR 538 TGPFGPGDHGERGPPGRPGFFGRFGLPGADGLPGPGTTMLMLPFRYGGDSHKGPVSAQEAQAQAILQQARLALR 538 TGPFGPGDHGERGPPGRPGFFGRFGLPGADGLPGFFGTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLALR TGPFGPGDHGERGPPGRPGFFGFFGFFGFFGTMLMLPFRYGGDSKGPTISAQEAQAQAILQQARLQARAF TGPFGFFGFFGFFGFFGFFGFFGFFGFFGFFGFFGFFGFFFFGFFGFFGFFGFFGFFGFFGFFFF

Fig. 1. Alignment of amino acid sequences of N-domains of vertebrate  $pro-\alpha I(V)$ ,  $pro-\alpha I(XI)$  and  $pro-\alpha I(V/XI)$  chains. The dashed lines indicate gaps inserted to give the best alignment. Asterisks, colons and dots symbolize fully conserved, conserved and similar amino acids, respectively.

Lys residues are important in the formation of intermolecular covalent cross-links, responsible for much of the mechanical stability of collagen fibrils, and have previously been found in either one or both telopeptides of the major fibrillar collagen chains (Bernard et al., 1983, 1988; de Wet et al., 1987; Greenspan, Cheng & Hoffman, 1991; Kimura et al., 1989; Loidl et al., 1984; Myers, Loidl, Seyer & Dion, 1985; Sandell, Prentice, Kravis & Upholt, 1984). The skate  $pro-\alpha l(V/XI)$  chain lacks a Lys residue in the N-telopeptide, as like other vertebrate  $pro-\alpha 1(V)$  and  $pro-\alpha 1(XI)$ chains (Fig. 1). On the other hand, one Lys residue (1606, Fig. 2) in the C-telopeptide was observed in the non-identical position with other counterparts (Fig. 2). It was, therefore, suggested that a relative paucity of crosslinks in fibrils containing the Type V and XI collagen may utilize forms of cross-linking different from those employed by other fibrillar collagens.

As shown in Fig. 2, amino acid sequences, including those sequences in the C-telopeptide and amino-terminal side of the C-propeptide, are relatively divergent when Type I, II and III procollagen  $\alpha$  chains are compared (Dion & Myers, 1987). This localized variability is highlighted in the present comparison between pro- $\alpha 1(V)$  and pro- $\alpha 1(XI)$ chains and suggests that this region may be important in providing the specificity for combining with other procollagen chains of the appropriate type (Dion & Myers, 1987). Most parts of C-propeptides, which are highly conserved between pro- $\alpha 1(V)$  and pro- $\alpha 1(XI)$  chains, may reflect the ability of the two chains, under certain conditions, to substitute for each other in the formation of cross-type heterotrimers (Mayne & Burgeson, 1987a; Niyibizi & Eyre, 1989). A similar function could be retained in the skate pro- $\alpha 1(V/XI)$  chain, because of high similarity to other vertebrates (Fig. 2). A putative C-proteinase cleavage site (Glu-Glu, 1616–1617) of the skate pro- $\alpha 1(V/XI)$  chain is assigned in Fig. 2, based on comparisons of the demonstrated and postulated cleavage sites of the other vertebrate procollagen chains (Bernard et al., 1988; Greenspan et al., 1991). In contrast to the C-proteinase cleavage site of pro- $\alpha 1(XI)$  chain shown to be in a different position, the skate pro- $\alpha 1(V/XI)$  chain seems to be close to the pro- $\alpha 1(V)$  chain group, however, the Asp residue of the cleavage site in others was substituted for the same acidic amino acid, Glu (Fig. 2).

The locations of the eight Cys residues (1661, 1667, 1684, 1693, 1702, 1768, 1811, 1857) found within the Cpropeptide of the skate pro- $\alpha$ 1(V/XI) chain were identical to the locations of the Cys residues found within the C-propeptides of other vertebrate pro- $\alpha$ 1(V) chains (Fig. 2). The presence of eight conserved Cys residues in the skate pro- $\alpha$ 1(V/XI) chain assures that it could be close to the pro- $\alpha$ 1(V) chains, in contrast to seven Cys residues in the pro- $\alpha$ 1(XI) chains, including the red seabream counterpart (Fichard et al., 1994; Touhata et al., 2001; Vuorio & de Crombrugghe, 1990).

In the skate pro- $\alpha l(V/XI)$  chain, Lys residues of two sites (661–666, 1519–1524) were conserved toward the Nand C-ends of the main triple helical domain. These two Lys residues, as well as those of other vertebrate pro- $\alpha l(V/XI)$  chains, are thought to be important as an attach-

	Type V J	<sup>⊤</sup> <sup>Type XI</sup>	
	telopeptide	C-propeptide	
skate (V/XI)	IOSLPFHSSR-RAKRSIDASOFLEEG	T NPENYLDYADGMEEIFGSLNSLKLEIEOMKHPMGTONNPARTCKDLYLCHPDFPDGEYWIDPNOGCSRDSFKVLCNVTSGGETCIHPDKKSEGA 17	712
hamster (V)	IQPLPIQASRTRRNIDASQLLDDG	AGESYLDYADGMEEIFGSLNSLKLEIEOMKRPLGTOONPARTCKDLOLCHPDFPDGEYWVDPNOGCSRDSFKVYCNFTAGGSTCVFPDKKSEGA 16	<u> </u>
rat (V)	IQPLPIQASRTRRNIDASQLLDDG	AGESYVDYADGMEEIFGSLNSLKLEIEQMKRPLGTQQNPARTCKDLQLCHPDFPDGEYWVDPNQGCSRDSFKVYCNFTAGGSTCVFPDKKSEGA 16	592
mouse (V)	IQPLPIQASRTRRNIDASQLLDDG	AGESYVDYADGMEEIFGSLNSLKLEIEQMKRPLGTQONPARTCKDLQLCHPDFPDGEYWVDPNQGCSRDSFKVYCNFTAGGSTCVFPDKKSEGA 16	590
human (V)	IQPLPIQASRTRRNIDASQLLDDG	NGENYVDYADGMEEIFGSLNSLKLEIEQMKRPLGTQQNPARTCKDLQLCHPDFPDGEYWVDPNQGCSRDSFKVYCNFTAGGSTCVFPDKKSEGA 16	590
chicken (V)	IQPLPIQSSK-RTRRNIDASQLVDDG	VADNYMDYADGMEEIFGSLNSLKLEIEQMKHPLGTQHNPARTCKDLQLCHPDFPDGEYWVDPNQGCSRDSFKVYCNFTAGGETCIFPDKKSEGA 16	587
mouse (XI)	IQPLPILSPK-KTRRHTESIQGDA	AGDNILDYSDGMEEIFGSLNSLKQDIEHMKFPMGTQTNPARTCKDLQLSHPDFPDGEYWIDPNQGCSGDSFKVYCNFTAGGETCIYPDKKSEGV 16	356
human (XI)	IQPLPILSSK-KTRRHTEGMQADA	ADDNILDYSDGMEEIFGSLNSLKQDIEHMKFPMGTQTNPARTCKDLQLSHPDFPDGEYWIDPNQGCSGDSFKVYCNFTSGGETCIYPDKKSEGV 16	358
red seabream (V/XI)	IQPLPMQQSARKTRRQAEMQGDEAMAL	DYGVDGVGMEGMEDVFGSLNNLKQDIERMKFPMGTQDNPARTCNDLHLSQPDYPDGEYWIDPNQGCIGDAIKVFCNFTAGGETCIFPDKKSTGV 16	572
	**.**:* :	.***:.*****.** .**.** ***** ***********	
skate (V/XI)	RVTAWPKEIPGSWFSEFKRGKLLSYA	DAEGNPIGVVQMTFLRLLSASAHQNLTYNCHQTVAWYDSTSDSYDKAIRFLGSNDEEMSYDNNPYIQALVDKCAEKKGYQKTILEINTPKVEQL 18	332
hamster (V)	RITSWPKENPGSWFSEFKRGKLLSYV	DAEGNPVGVVQMTFLRLLSASAHQNITYNCYQSVAWQDAATGSYDKAIRFLGSNDEEMSYDNNPYIRALVDGCATKKGYQKTVLEIDTPKVEQV 18	312
rat (V)	RITSWPKENPGSWFSEFKRGKLLSYV	DAEGNPVGVVQMTFLRLLSASAQQNITYNCYQSVAWQDAATGSYDKAIRFLGSNDEEMSYDNNPYIRALVDGCATKKGYQKTVLEIDTPKVEQV 18	312
mouse (V)	RITSWPKENPGSWFSEFKRGKLLSYV	DAEGNPVGVVQMTFLRLLSASAHQNVTYNCYQSVAWQDAATGSYDKAIRFLGSNDEEMSYDNNPYIRALVDGCATKKGYQKTVLEIDTPKVEQV 18	310
human (V)	RITSWPKENPGSWFSEFKRGKLLSYV	DAEGNPVGVVQMTFLRLLSASAHQNVTYHCYQSVAWQDAATGSYDKALRFLGSNDEEMSYDNNPYIRALVDGCATKKGYQKTVLEIDTPKVEQV 18	310
chicken (V)	RITSWPKENPGSWFSEFKRGKLLSYV	DSDGNPIGVVQMTFLRLLSASAHQNITYNCYQSVAWHDATTDSYDKAIRFLGSNDEEMSYDNNPYIRAAFDGCAAKKGYQKTVLEINTPKVEQV 18	307
mouse (XI)	RISSWPKEKPGSWYSEFKRGKLLSYL	DVEGNSINMVQMTFLKLLTASARQNFTYNCHQSAAWYDVLSGSYDKALRFLGSNDEEMSYENNPHIKALYDGCASRKGYEKTVIEINTPKIDQV 17	776
human (XI)	RISSWPKEKPGSWFSEFKRGKLLSYL	DVEGNSINMVQMTFLKLLTASARQNFTYHCHQSAAWYDVSSGSYDKALRFLGSNDEEMSYDNNPFIKTLYDGCTSRKGYEKTVIEINTPKIDQV 17	778
red seabream (V/XI)	RISNWPKEAPGSWFSEFKRGKILNYV	DMGESTINTVQMTFLKLLSSSARQNFTYICHQSVAWYDAKADNYDKALRFLGSNDEEMSYDNNPFIKPLMDGCSLKQGYSKTVMEINTPRIDQL 17	792
	* . **** **** *************************	* *******_***_***_**_**_**_**_**_*	
skate (V/XI)	PEIDIMENDEGESGOKEGEEIGPVCE		
hamster (V)	PIVDIMENDEGEASOKEGEEVGPACEL	LG 1840	
rat (V)	PIVDIMFTDFGEASOKFGFEVGPACFL	LG 1840	
mouse (V)	PTVDTMENDEGEASOKEGEEVGPACEL	G 1838	
human (V)	PIVDIMENDEGEASOKEGEEVGPACE	MG 1838	
chicken (V)	PIVDIMFNDFGEASOKFGFEVGPACF	MG 1835	
mouse (XI)	PIIDVMINDFGDQNQKFGFEVGPACFL	LG 1804	
human (XI)	PIVDVMISDFGDQNQKFGFEVGPVCFL	LG 1806	
red seabream (V/XI)	PIIDVMLNDFGESNORFGFEVGPVCFL	LG 1820	
( · ····)	* * . * * * . * . * * * * *	•*	

Fig. 2. Alignment of amino acid sequences of C-domains of vertebrate  $pro-\alpha I(V)$ ,  $pro-\alpha I(XI)$  and  $pro-\alpha I(V/XI)$  chains. The dashed lines, asterisks, colons and dots are the same as in Fig. 1.

ment site for lysyl oxidase (Mayne & Burgeson, 1987c). Moreover, two Arg-Gly-Asp sequences (666–668, 684– 686), known to be a potential cell-binding site (Ruoslahti & Pierschbacher, 1986), are conserved in the central triple-helical domain (data not shown). In addition, one characteristic of pro- $\alpha$ 1(XI), pro- $\alpha$ 2(XI) and pro- $\alpha$ 1(V) chains is the presence of a heparin binding site, which is absent in the pro- $\alpha$ 2(V) chain (Fichard et al., 1994; Yaoi, Hashimoto, Koitabashi, Takahara, Ito & Kato, 1990). In the skate pro- $\alpha$ 1(V/XI) chain, sequence 918–950 of the triple helical region corresponding to the heparin binding site location, is extremely well conserved, together with other vertebrate pro- $\alpha$ 1(V) and pro- $\alpha$ 1(XI) chains (data not shown).

Comparison of the skate pro- $\alpha 1(V/XI)$  chain with other vertebrate pro- $\alpha 1(V)$  and pro- $\alpha 1(XI)$  chains confirmed the high homology of the amino acid sequence among them (data not shown). The skate pro- $\alpha 1(V/XI)$  chain exhibited a high level of sequence homology, in the order of triplehelical, C-terminal and N-terminal domains, as with the results obtained between human pro- $\alpha 1(V)$  and pro- $\alpha 1(XI)$  chains (Greenspan et al., 1991).

The phylogenetic analysis is performed in order to clarify whether the skate pro- $\alpha l(V/XI)$  chain is close to the pro- $\alpha l(V)$  or the pro- $\alpha l(XI)$  chain. The phylogenetic analyses of, not only N- and C-terminal domains, but also fulllength, suggest that the skate pro- $\alpha l(V/XI)$  chain could be an intermediate form of pro- $\alpha l(V)$  and pro- $\alpha l(XI)$  chains (data not shown). However, the skate pro- $\alpha l(V/XI)$  chain was included in the same cluster as the pro- $\alpha l(V)$  chains, whereas the red seabream pro- $\alpha l(V/XI)$  chain was included in the same cluster with pro- $\alpha l(XI)$  chains when the phylo-



Fig. 3. Phylogenetic tree based on full-length of the skate and red seabream pro- $\alpha l(V/XI)$  chains, together with human and mouse fibrillar procollagen  $\alpha l$  chains. The scale bar shows substitution of one amino acid residue per 10 residues. The phylogenetic tree was constructed by joining amino acid sequences of various species. Numbers at nodes indicate bootstrap values for 1000 runs.



Fig. 4. Expression of the skate  $\text{pro}-\alpha 1(\text{V/XI})$  chain mRNA. Total RNA from various tissues from the adult skate was used to generate cDNA for PCR amplification, using the skate  $\text{pro}-\alpha 1(\text{V/XI})$  chain-specific primers, together with EF-1 $\alpha$  (accession no. AB214933), as a positive control.

genetic tree was constructed by limiting to fibrillar procollagen  $\alpha 1$  chains of human and mouse (Fig. 3).

Fig. 4 shows the expressions of the pro- $\alpha l(V/XI)$  chain mRNA in various tissues of the skate. Amplified bands by RT-PCR were clearly detected in the muscle, skin, orbit, brain, liver, intestine, stomach, spleen, pancreas, egg, ovary, uterine tube, shell gland and spiral valve, except for cartilage, gill, heart and whole blood. PCR products were further defined by cloning and sequencing. As expected, all products were identical to a part of the skate pro- $\alpha l(V/XI)$  chain. This result indicates that the skate pro- $\alpha l(V/XI)$  chain is expressed widely in various tissues, and is consistent with biochemical and immunocytochemical studies which have indicated a wide tissue distribution of Type V collagen, in contrast to Type XI collagen, which has been found mainly in cartilaginous tissues (Mayne & Burgeson, 1987a, 1987b).

The skate pro- $\alpha 1(V/XI)$  chain, comprised of 1860 amino acids, is the longest fibrillar procollagen chain characterized to date. Moreover, the skate pro- $\alpha 1(V/XI)$  chain can be classified as pro- $\alpha 1(V)$  chains rather than pro- $\alpha 1(XI)$ chains, by conserved structural properties, as well as broad expression pattern. Further studies are now in progress to elucidate the expression properties of the skate pro- $\alpha 1(V/XI)$ chain during embryonic development.

#### Acknowledgement

We are grateful to Dr. David Barnes, Mount Desert Island Biological Laboratory, USA, for his invaluable advice in this work.

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