Distinct Maturations of N-propeptide Domains in Fibrillar Procollagen Molecules Involved in the Formation of Heterotypic Fibrils in Adult Sea Urchin Collagenous Tissues*

Caroline Cluzel, Claire Lethias, Robert Garrone, and Jean-Yves Exposito‡

From the Institut de Biologie et Chimie des Protéines, CNRS, Unité Mixte de Recherche 5086, Institut Fédératif de Recherche 128 BioSciences Lyon-Gerland, Université Claude Bernard, 7 Passage du Vercors, 69367 Lyon Cedex 07, France

We have characterized the primary structure of a new sea urchin fibrillar collagen, the 5α chain, including nine repeats of the sea urchin fibrillar module in its N-propeptide. By Western blot and immunofluorescence analyses, we have shown that 5α is co-localized in adult collagenous ligaments with the 2α fibrillar collagen chain and fibrosurfin, two other extracellular matrix proteins possessing sea urchin fibrillar modules. At the ultrastructural level, the 5α N-propeptide is detected at the surface of fibrils, suggesting the retention of this domain in mature collagen molecules. Biochemical characterization of pepsinized collagen molecules extracted from the test tissue (the endoskeleton) together with a matrix-assisted laser desorption ionization time-of-flight analysis allowed us to determine that 5α is a quantitatively minor fibrillar collagen chain in comparison with the 1α and 2α chains. Moreover, 5α forms heterotrimeric molecules with two 1α chains. Hence, as in vertebrates, sea urchin collagen fibrils are made up of quantitatively major and minor fibrillar molecules undergoing distinct maturation of their N-propeptide regions and participating in the formation of heterotypic fibrils.

In different sea urchin species, studies have indicated the presence of two fibrillar α chains (1α and 2α) involved in the formation of heterotrimeric molecules [1α]2-2α (6–8). Moreover, traces of a homotrimeric 1α chain have been described in the sea urchin Paracentrotus lividus (9). The complete primary structure of these two fibrillar collagen chains has been characterized in the sea urchin Stronglylocentrotus purpuratus (10, 11), whereas partial sequences have been described in P. lividus (12–14) and Hemicentrotus pulchermissimus (15). The 2α chain presents a large N-propeptide region including 12 repeats of an 140–145 amino acid module that we have named SURF1 for sea urchin fibrillar module (14). During sea urchin embryogenesis, collagen fibrils show a uniform diameter of 25 nm and present at their surface periodically distributed extensions corresponding to the 2α N-propeptide (16). In adults, the collagen fibrils are thicker and have been found in various tissues such as the sutral ligament of the test, the spine ligament, the peristomial membrane that bridges the Aristotle’s lantern or Echinoid jaw to the test, jaw, and tube feet (17–19). Some of these tissues have been defined as mutable collagenous tissues that exhibit a property specific to echinoderms (20). Indeed, mutable collagenous tissues have the ability to modulate their tensile properties in a time scale of seconds under nervous control without the requirement of muscle cells. In sea urchin, we have previously characterized an inter-fibrillar component of the spine collagenous ligament, fibrosurfin (19). This extracellular matrix protein consists of a series of epidermal growth factor-like and SURF modules. It is worth noting that the two sea urchin proteins that include SURF modules (2α and fibrosurfin) are almost exclusively distributed in adult collagenous ligaments. Moreover, we have also partially characterized in the sea urchin S. purpuratus a genomic region that could potentially encode an N-propeptide domain evolutionarily related to the 2α N-propeptide (14). The putative protein encoded by this genomic region includes nine repeats of the SURF module and has been given the name 5α.

Among the components of extracellular matrix, collagens are the most abundant of the glycoproteins. In vertebrates, 27 collagen types have been identified (1–4). All of them consist of three identical or different α chains that contain at least one collagenous or triple helical segment, consisting of repeating Gly-Xaa-Xaa triplets. The collagen domains of the three α chains coil around each other into a triple helical structure. The fibrillar collagens, including types I-III, V, and XI, are the best known, and their precursor α chains consist of a main triple helix made up of 338 Gly-Xaa-Xaa triplets flanked by two non-collagenous regions containing the N- and the C-propeptides. During maturation of procollagen into collagen molecules, the two propeptide domains are generally removed by the action of specific proteinases (1). The resulting collagen molecules participate in the formation of supramolecular structures called fibrils. The invertebrate fibrillar α chains so far described present the same overall structure as that of their vertebrate counterparts (5).

‡ To whom correspondence should be addressed. Tel.: 33-4-72-72-26-04; Fax: 33-4-72-72-26-04; E-mail: jy.exposito@ibcp.fr.

1 The abbreviations used are: SURF, sea urchin fibrillar; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; CAPS, 3-cyclohexylamino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
EXPERIMENTAL PROCEDURES

Embryo Culture and Nucleic Acid Purification—P. lividus were purchased from the Arage laboratory (Banyuls-sur-Mer, France). Gamete collection, fertilization, and embryo culture were done according to standard protocols (21). Total RNA from embryonic and adult tissues was purified as described previously (19).

cDNA Synthesis and PCR—For all of the RT-PCR experiments, 500 ng of test RNA were reverse-transcribed using random primers and the reverse transcribe Expand kit (Roche Applied Science) used according to the manufacturer’s recommendations. For RACE experiments, the 5′- and 3′-RACE kit from Invitrogen were used. The oligonucleotides used in this study are listed in Table I and were synthesized by Isoprim (Toulouse, France). For PCR, 35 amplification cycles of target single-stranded cDNA were usually carried out using the Taq Expand polymerase kit (Roche Applied Science). PCR conditions, purification, cloning, and sequencing of PCR fragments were carried out as described previously (19).

Antibody Production—To prepare anti-5α monoclonal antibodies, DNA fragments encoding the SURF module R8 were generated by PCR using the RT-PCR cDNA clone X as template with 5′-TATCTGCAGACCTCTACACTTCT-3′, and washed three times with distilled water.

TABLE I

| cDNA fragment | Forward | Reverse |
|---------------|---------|---------|
| 5-1 | 5′-AAAGCAGAAGTGTAGGAGGCT-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 5-2 | 5′-GGTGGGATCCTGCGACATGTGAG-3′ | 5′-GGTGGGATCCTGCGACATGTGAG-3′ |
| 5-3 | 5′-CAGTATGCGTGGCTGCCAGACCAAC-3′ | 5′-CAGTATGCGTGGCTGCCAGACCAAC-3′ |
| 5-4 | 5′-GGTGGGATCCTGCGACATGTGAG-3′ | 5′-GGTGGGATCCTGCGACATGTGAG-3′ |
| 5-5 | 5′-AAAGCAGAAGTGTAGGAGGCT-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 5-7 | 5′-TACATGCCTGATACATGCCAGAC-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 5′-RACE-1 | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 5′-RACE-2 | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 3′-RACE-1 | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |
| 3′-RACE-2 | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ | 5′-GGGTTAGGTGTGCGAACACCATAG-3′ |

Collagen samples were separated by 6% SDS-PAGE and analyzed by staining with Coomassie Blue or by Western blotting, the latter after electrotransfer onto polyvinylidene difluoride membranes overnight in 10 mM CAPS, pH 11.5, 5% methanol. Blots were incubated with a rabbit polyclonal antibody made against the N-terminal sequence and the available theoretical and experimental masses.

The disaggregated collagen molecules were then solubilized by limited trypsin digestion. The pellet was suspended in a pepstatin solution with a collagen/pepsin ratio of 1000/3 (wt in weight) in 0.5 M acetic acid, and incubated for 16 h at 4 °C. Pepsin was immediately inhibited by the addition of pepstatin A (1 µg/ml, Sigma). The viscous solution was centrifuged at 20,000 × g for 30 min. The supernatant was subjected to gel filtration in acetic acid on a column of ultrafiltration to remove all of the fibrillar collagen molecules present in the initial solution (24). First, the supernatant was dialyzed against 1.5 M NaCl in 0.5 M acetic acid for several days. After centrifugation (20,000 × g, 1 h), the precipitate was suspended in 0.1 M acetic acid and frozen. The supernatant was subjected to a second dialysis with 4 µl NaCl in 50 mM Tris-HCl, pH 8.5, for several days. The small amount of precipitate was centrifuged and suspended in 0.1 M acetic acid and frozen. Bovine type I collagen was purchased from Coléctica (Lyon, France).

Collagen samples were separated by 6% SDS-PAGE and analyzed by staining with Coomassie Blue or by Western blotting, the latter after electrotransfer onto polyvinylidene difluoride membranes overnight in 10 mM CAPS, pH 11.5, 5% methanol. Blots were incubated with a rabbit polyclonal antibody made against the N-terminal sequence and the available theoretical and experimental masses.

To obtain fibrillar samples, gastric mucosa of P. lividus was isolated from the body region, minced and dispersed in distilled water. The samples were incubated for 30 min at 4 °C, centrifuged, and washed three times with distilled water.

The disaggregated collagen molecules were then solubilized by limited trypsin digestion. The pellet was suspended in a pepstatin solution with a collagen/pepsin ratio of 1000/3 (wt in weight) in 0.5 M acetic acid, and incubated for 16 h at 4 °C. Pepsin was immediately inhibited by the addition of pepstatin A (1 µg/ml, Sigma). The viscous solution was centrifuged at 20,000 × g for 30 min. The supernatant was subjected to gel filtration in acetic acid on a column of ultrafiltration to remove all of the fibrillar collagen molecules present in the initial solution (24). First, the supernatant was dialyzed against 1.5 M NaCl in 0.5 M acetic acid for several days. After centrifugation (20,000 × g, 1 h), the precipitate was suspended in 0.1 M acetic acid and frozen. The supernatant was subjected to a second dialysis with 4 µl NaCl in 50 mM Tris-HCl, pH 8.5, for several days. The small amount of precipitate was centrifuged and suspended in 0.1 M acetic acid and frozen. Bovine type I collagen was purchased from Coléctica (Lyon, France).
fixed for 4 h at 4 °C in 2.5% paraformaldehyde in artificial sea water. Tissues were demineralized with 0.5 M EDTA at 4 °C and then rinsed and frozen in liquid nitrogen. Thin sections were cut on a Cryostat (Leitz) and immunolabeled with 27-7F10 and 11-4E11 monoclonal antibodies (1/30, British Biocell International, Cardiff, United Kingdom), respectively. Sections were incubated with fluorescein-conjugated goat anti-mouse IgG (1/400, Jackson ImmunoResearch, West Grove, PA) and observed on a Zeiss AxioPlan microscope.

For electron microscopy, tests were dissected from individual P. lividus, rinsed with artificial sea water, and fixed at room temperature for 12 h in 3% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.8). Samples were rinsed in the same buffer and post-fixed for 1 h in 1% osmium tetroxide in PIPES buffer (0.1 M, pH 7.4). After rapid washing in water, sections were dehydrated in a graded ethanol series and embedded in London Resin White at 60 °C. Blocks were cut to the surface of the tissue and demineralized in 0.5 M EDTA for several days. After reembedding in London Resin White, ultrathin sections were cut on a Reichert-Jung Ultracut ultramicrotome and then immunolabeled with 27-7F10 and 11-4E11 (anti-SURF modules) antibodies used were a goat anti-mouse IgG conjugated to 5-nm gold particles and a goat anti-rabbit IgG conjugated to 10-nm gold particles and a goat anti-rabbit IgG conjugated to 10-nm gold particles (1/100). Negative controls were carried out by omitting the primary antibody. The secondary antibodies used were a goat anti-mouse IgG conjugated to 5-nm gold particles and a goat anti-rabbit IgG conjugated to 10-nm gold particles (1/100). Negative controls were carried out by omitting the primary antibody. The secondary antibodies used were a goat anti-mouse IgG conjugated to 5-nm gold particles and a goat anti-rabbit IgG conjugated to 10-nm gold particles (1/100). Negative controls were carried out by omitting the primary antibody. The secondary antibodies used were a goat anti-mouse IgG conjugated to 5-nm gold particles and a goat anti-rabbit IgG conjugated to 10-nm gold particles (1/100).

RESULTS

Using a probe coding for SURF module R8 of the 2α chain, we had previously isolated from a P. lividus genomic DNA library 54 positive clones exhibiting variable labeling intensities.

Using the available coding sequences, RT-PCR and 5′-RACE were carried out using RNA extracted from test tissue (Fig. 1B; RT-PCR fragments 5-1 to 5-5 and 5′-RACE clone). This permitted us to characterize the first 1,788 residues of the 5α chain. At this point, the sequence revealed that 5α probably represented a new sea urchin fibrillar collagen chain evolutionarily related to the 2α chain. The sequence included 1 putative signal peptide (residues 1–31), 1 large N-propeptide made up of a Tsp-2 module, 9 SURF modules and 1 minor interrupted triple helix, 1 N-telopeptide region, and 90 Gly-Xaa-Xaa triplets. Comparable domains of the 2α and the 5α chains presented an average of 69% identity (Fig. 1B). To determine the C-terminal sequence of the 5α chain, two properties of the 5α chain were used to generate a 3′ primer set, i.e. the strong similarity between the 2α and 5α chains, and the presence of 54 positive clones exhibiting variable labeling intensities. Hence, shotgun analysis of two weakly positive overlapping genomic clones coupled to RT-PCR and RACE approaches led us to characterize fibrosurfin, an extracellular matrix protein that contains 13 SURF modules (19). Using the same strategy, we analyzed two moderately positive overlapping genomic clones (Fig. 1A). Shotgun sequence analysis revealed that we had identified part of a gene that corresponded to the P. lividus ortholog of the S. purpuratus COL5α gene (14). Hence, as for the 2α and the 2α chains, comparable domains of the 5α chain characterized in these two sea urchin species presented 86–95% identity (Fig. 1A).
highly conserved stretches of amino acids in the C-propeptides of all known fibrillar α chains. This led to the synthesis of two RT-PCR products encoding the major triple helix and the N-terminal region of the C-propeptide (RT-PCR products 5-6 and 5-7 in Fig. 1B). This RT-PCR approach, coupled to a 3’-RACE, led us to complete the primary structure of the 5α chain, confirming that we had identified a new sea urchin fibrillar collagen chain (Fig. 1B). It is worth indicating that the N-telopeptide region represents the least conserved region between these two fibrillar collagen chains (Fig. 1C).

To investigate the nature of the 5α chain, we first prepared monoclonal antibodies against a recombinant protein consisting of SURF module R8 of this chain. These antibodies were tested for their cross-reactivity (see “Experimental Procedures”) with the SURF module R8 of the 2α chain because these comparable domains presented 89% identity, whereas the identity with other SURF modules of 2α, 5α, and fibrosurfin was <35%. The specificity of these antibodies was also confirmed by the absence of cross-reactivity with recombinant proteins built of SURF modules of 2α and fibrosurfin (data not shown). The presence of the 5α chain was analyzed by Western blot experiments during embryogenesis and in different adult tissues. Similar to fibrosurfin (19), the 5α chain is not detectable during the embryogenesis (data not shown). In adults, immunoreactive bands for the 5α chain were detected in the test, peristomial membrane, Aristotle’s lantern, the base of the spine, and the tube feet (Fig. 2). The same positive adult tissue pattern has previously been defined for both 2α and fibrosurfin (19). Two clusters of immunoreactive bands for the 5α chain were detected in these adult tissues. Cluster A included bands of molecular mass higher than 200 kDa, whereas cluster B comprised bands of 100–180 kDa (Fig. 2). The significance of the two clusters will be discussed later in the light of the next results. The identification of multiple bands in the cluster A and of the lowest apparent molecular mass bands in cluster B might result from proteolytic events during urea extraction from the mineralized tissues. A similar degradation pattern has previously been shown for proteins that include SURF modules, i.e. fibrosurfin and the 2α chain (19). Fig. 3 shows immunostaining of the catch apparatus using monoclonal antibodies specific for the N-propeptide of both the 2α and 5α chains. These two α chains were co-localized to the spine collagenous ligament of the catch apparatus (Fig. 3, A and B) and were also present under the epithelium surrounding the muscle layer at the base of the spines (Fig. 3, D and E). A background of autofluorescence was detected within cells in these regions, resulting from a brown pigmentation (Fig. 3, C and F). Co-localization of these two α chains was also demonstrated in the peristome and in the suture ligaments of the test (data not shown).

The co-localization of the 2α and 5α chains in adult tissues and the Western blots realized both in this study and in a previous work (19) is difficult to correlate with the data, indicating that pepsinized sea urchin fibrillar collagen molecules are made of two α chains, the 1α and 2α (6, 7, 9). Moreover, Edman degradation analysis of pepsinized fibrillar collagens extracted from test after SDS-PAGE separation reveals only sequences specific to the 1α and 2α chains (8). To clarify the discrepancy between our immunological and biochemical studies, we first used immuno-electron microscopy using monoclonal antibodies specific for the N-propeptide of the 2α or 5α chain or polyclonal antibodies made against the N-telopeptide and the N-terminus of the triple helix of the 2α chain. For 5α, 5-nm gold particles were detected on the surface of all of the collagen fibrils in suture ligaments (Fig. 4, A–C), indicating that its N-propeptide was either unprocessed or closely associated with these fibrils. For the 2α chain (Fig. 4D), its N-propeptide was not present on the surface of those fibrils that contained the 2α chain as shown in Fig. 4, E and F, where 10-nm gold particles coupled to antibodies specific for the 2α N-telopeptide were used. This agrees with a previous study (19) indicating the processing of the 2α N-propeptide in adult tissues and its localization at the periphery of the bundles made of collagenous fibrils aligned in parallel. The low number of gold particles in Fig. 4, E and F, results from the availability of the 2α N-telopeptide at the surface of the fibrils. Hence, we have previously used this polyclonal antibody specific of the 2α telopeptide to localize the 2α chain during the embryogenesis (16). Only a treatment of embryos by 8 M urea at 40 °C prior the immunolabeling has permitted us to detect the 2α chain in the tissues (16). A similar treatment of tissues analyzed by electron microscopy could not give accurate results because of the damage of the tissue structure.

The second analysis was carried out to identify the 5α chain at the protein level. Pepsinized fibrillar collagen purified from test was sequentially precipitated with 1.5 and 4 M NaCl (see “Experimental Procedures”). These salt precipitates were analyzed by SDS-PAGE (Fig. 5A). The amount loaded on the gel corresponded to 0.05 and 50% of the total precipitate, respectively. Most of the collagen molecules were recovered after the 1.5 M NaCl precipitation (Fig. 5A, lane 1.5M). The same pattern of bands was obtained for the 4 M NaCl precipitate (Fig. 5A, lane 4M), although the fastest migrating band seemed to have a slightly lower apparent molecular mass than the comparable band in the 1.5 M NaCl precipitate. In the 4 M NaCl lane, the higher and lower molecular mass bands were called 1 and 2,
two spots were present, a strongly stained spot and a lightly stained spot (called 3), which was more acidic and seemed to have an apparent molecular mass slightly less than the more basic spot. Bands 1 and 2 of the 4 M lane (Fig. 5A) and spot 3 after two-dimensional gel electrophoresis were analyzed by MALDI-TOF. Similar tryptic peptide profiles were obtained for band 2 of the 4 M lane and spot 3 after two-dimensional gel electrophoresis, whereas band 1 of the 4 M lane generated a distinct pattern. Experimental data were compared with the theoretical molecular masses of tryptic fragments generated from the 5α chain and the available sequences of the P. lividus 1α and 2α chains (12, 13). This comparison clearly indicated that spot 3 and band 2 corresponded to the 5α chain, whereas band 1 corresponded to the 1α chain. Peptide fragments identified by MALDI-TOF are shown in Fig. 6 and represent 31.8 and 39.8% of the available triple helical sequences, respectively. The ratio between the upper and the lower bands in the 4 M NaCl precipitate (Fig. 5A) suggested that molecules present in this solution had a [[1α]₂5α] stoichiometry. Moreover, data from Fig. 5, A and C, indicate that in sea urchin adult tissues, the 5α chain represents just a few percent of the fibrillar α chains.

**DISCUSSION**

In this report, we have shown for the first time, as in vertebrates, that invertebrates possess major and minor fibrillar collagens that are involved in the formation of heterotypic fibrils. Hence, the 5α chain is the first quantitatively minor invertebrate fibrillar collagen characterized to date. From its N-propeptide maturation, its presence in small amounts in comparison to the 1α and 2α chains, the 5α chain might play a similar structural function to that of the vertebrate type VXI collagens in heterotypic fibrils. In addition, this report confirms that proteins that include SURF modules are located in the vicinity of the mineralized regions of the sea urchin and in adult collagenous tissues.

Two-dimensional gel electrophoresis analysis (Fig. 5C) indicated that 5α represents a small percentage of the fibrillar collagen in test. This low amount and its similarity with the 2α chain (primary structure, co-migration in SDS-PAGE, molecular association with two 1α chains) explain why the 5α chain has not been reported in previous biochemical studies (6–9, 15). Two-dimensional electrophoresis also reveals that the band corresponding to the 1α chain in SDS-PAGE is distributed into several spots. Even though we did not analyze these spots by MALDI-TOF, we suggest that they correspond to distinct post-translational modifications of the 1α chain. These modifications could correspond to different levels of glycosyla-

**FIG. 4.** Ultrastructural analysis of the sutural test ligaments using anti-5α N-propeptide (27-7F10, SURF-R8) antibody (A–C), anti-2α N-propeptide (11-4E11, SURF-R2) antibody (D), and anti-2α N-telopeptide (E and F) is shown. Bar = 200 nm.

**FIG. 5. Isolation of the 5α chain from sea urchin test.** A mixture of pepsinized fibrillar collagen from the test was subjected to two successive salt precipitations, first with 1.5 M NaCl in 0.5 M acetic acid, pH 2.6 (1.5M). The supernatant then was precipitated with 4 M NaCl in 1.5 M Tris-HCl, pH 8.5 (4M). The precipitates were separated by 6% SDS-PAGE followed by Coomassie Blue staining (A) using the anti 2α N-telopeptide antibody. Bovine type I collagen (Hb) was analyzed for comparison. The 1.5 M NaCl precipitate was resolved in a two-dimensional gel electrophoresis system (Fig. 5C). Several spots of molecular mass identical to the 1α chain were detected, suggesting distinct types of post-translational modification to this fibrillar chain. At the molecular mass level of the 2α chain,
tion because it has been shown in three sea urchin species that the \( \alpha_1 \)/H9251 chain is rich in carbohydrate (15).

From their primary structure, the \( \alpha_1 \)/H9251 and the \( \alpha_5 \)/H9251 chains are closely related. Previously, we suggested that the duplication event leading to the creation of their related genes happened prior to the formation of the genomic region encoding the SURF modules R2–R5 of the \( \alpha_1 \)/H9251 chain (14). Similarities between the \( \alpha_1 \)/H9251 and \( \alpha_5 \)/H9251 chains are also shown at the molecular level. Hence, the \( \alpha_1 \)/H9251 chain forms heterotrimeric molecules with two \( \alpha_1 \)/H9251 chains. From Fig. 5A (lane 4M) and the MALDI-TOF analysis, it seems that some molecules may be assembled containing a \( \alpha_5 \)/H9251 chain rather than a \( \alpha_1 \)/H9251 chain to generate \([\alpha_1 \alpha_5]^\alpha1\) molecules. First, the ratio between the upper (\( \alpha_1 \))/H9251 and lower (\( \alpha_5 \))/H9251 bands seems to be compatible with this stoichiometry. Second, from the strong similarity between their C-propeptides (70%), the \( \alpha_5 \)/H9251 chain might replace the \( \alpha_1 \)/H9251 chain during molecular formation. In vertebrates, heterotypic molecules have been shown to include types V and XI chains (26, 27). Hence, \( \alpha_1 \)(XI) might replace the \( \alpha_1 \)(V) chain to form \([\alpha_1 \alpha_1(XI)]\alpha2(V)\) molecule (27).

Interestingly, their C-propeptide presents 73% identity. Third, the sea urchin \( \alpha \) chains lack part of the sequence involved in chain recognition in vertebrates (28), similar to all invertebrate fibrillar collagens characterized to date (29). For this reason, it is tempting to speculate that only the \( \alpha_1 \) chain might form homotrimeric molecules, whereas the \( \alpha_2 \) and \( \alpha_5 \) chains need the \( \alpha_1 \) fibrillar collagen to be included in heterotrimeric molecules. In this model, the ratio between the \([\alpha_1 \alpha_1]^\alpha2\) and \([\alpha_1 \alpha_1]^\alpha5\) molecules will depend on the amount of \( \alpha_2 \) and \( \alpha_5 \) chains synthesized.

Although the \( \alpha_2 \) and \( \alpha_5 \) chains are closely related and seem to make comparable molecular stoichiometries, their maturation in adult tissues is distinct. Hence, the use of monoclonal antibodies specific to the SURF module R8 of the \( \alpha_2 \) or \( \alpha_5 \) chains indicated that the N-propeptide of \( \alpha_2 \) is cleaved in adult tissues, whereas the \( \alpha_5 \) N-propeptide is present on the fibril surface. These results suggest that for the \( \alpha_2 \) chain an N-proteinase absent during embryogenesis cleaves the N-propeptide in a region included between SURF module 9 and the N-telopeptide. A putative Ala-Gln N-proteinase cleavage site is present in the \( \alpha_2 \) N-telopeptide (Fig. 1C) similar to vertebrate types I-III collagens (30–32). This site could be functional even though no sea urchin N-proteinase has been characterized to date. As for the \( \alpha_5 \) chain, the N-propeptide is either unprocessed or cleaved in a region preceding SURF module R8. We could not exclude the possibility that the N-propeptide has been processed but is still associated with the fibrils. From Western blot analysis, using a monoclonal antibody specific to the \( \alpha_5 \) N-propeptide (Fig. 2A), the detection of high apparent
molecular mass bands (higher than 300 kDa, cluster A) argues in favor of retention of the complete N-propeptide in mature 5α collagen chains. Hence, the 5α N-propeptide has a theoretical molecular mass of 160 kDa, whereas its triple helix has an apparent molecular mass of 120 kDa (Fig. 5A, lane 4M). Hence, when the antibody specific to the 2α N-propeptide is used in Western blot experiments, positive bands ranging from 60 to 200 kDa are detected (19) for a theoretical molecular mass of 205 kDa for the 2α N-propeptide. The lack of higher molecular mass bands in this blot is in agreement with the removal of the N-propeptide during 2α maturation. All of these data agree with a distinct maturation of collagen molecules including the 2α or 5α chains. Because of the strong similarity between these two fibrillar chains, it is difficult to understand why 5α does not undergo a similar N-propeptide maturation to the 2α chain. Therefore, like the 2α chain and the 1α chain (10), the 5α chain possesses a putative N-proteinase site in its N-telopeptide (Fig. 1C). One explanation might be that the N-telopeptide of these two chains is the only one region, which is poorly conserved (Fig. 1C). The structure of this region is important in vertebrates for the activity of the N-proteinase (33). It has also been demonstrated that heat denaturation of type I procollagen prevents processing of the N-propeptide by the N-proteinase (34). Furthermore, in a native procollagen, type I molecule, the cleavage of the two first chains is faster than the third because of a partial unfolding of the N-telopeptide region (35).

Immunolocalization of the 5α chain in adult tissues agrees with our previous observation that proteins that incorporate SURF modules are present in collagenous tissues and are located around mineralized tissues. Hence, in these tissues, fibrils present SURF modules from the 5α chain at their surface, fibrosurfin is located between the collagen fibrils, and the N-propeptide of the 2α chain is present around fibril bundles. SURF modules have been only characterized in sea urchin, but their biological and/or structural functions are still unknown. In mutable collagenous tissues like the spine ligaments, proteins harboring SURF modules might be involved in the variable tensility of these tissues resulting from the modulation of interfibrillar cohesion. However, these proteins are also present in tissues that have not been defined as mutable collagenous tissues, similar to the sutural ligaments (20). It is interesting to note that a clear relationship between sutural loosening and skeletal flexibility during growth has been reported previously (36). Johnson et al. (36) indicated that growth-associated changes in ligamental material properties might explain the sutural loosening. One of the hypotheses is that the properties of mutable collagenous tissues may have had evolutionary origins in tissues that were mutable during growth (36, 37). At this time, the relation of SURF module with some unique properties of sea urchin collagenous tissues is rather speculative. However, the presence of 5α N-propeptides on the fibril surfaces might be important either for linking and/or the correct organization of the collagen fibrils present in these tissues. We show here that sea urchin fibrils are more complex than previously shown and that the formation of heterotypic fibrils arose early during evolution.

Acknowledgments—We thank Dr. Christophe Grangeasse for assistance in two-dimensional electrophoresis experiment and Drs. Michel Becchi and Isabelle Zanella-Cleó for MALDI-TOF analysis. We acknowledge Yvette Descollonges for the production of monoclonal antibodies and Alain Bosch for photographic artwork.

REFERENCES

1. Myllyharju, J., and Kivirikko, K. I. (2001) Ann. Med. 33, 7–21
2. Pace, J. M., Corrado, M., Missiuro, C., and Byers, P. H. (2003) Matrix Biol. 22, 3–14
3. Boot-Handford, R. P., Tuckwell, D. S., Plumb, D. A., Rock, C. F., and Poulson, R. (2003) J. Biol. Chem. 278, 31067–31077
4. Koch, M., Laub, F., Zhou, P., Hahn, R. A., Tanaka, S., Burgessen, R. E., Gerecke, D. R., Ramirez, F., and Gordon, M. K. (2003) J. Biol. Chem. 278, 43236–43244
5. Exposito, J. Y., Cluzel, C., Garrone, R., and Lettisias, C. (2002) Anat. Rec. 268, 302–316
6. Trotter, J. A., and Kebb, T. J. (1994) Comp. Biochem. Physiol. 107, 125–134
7. Omura, Y., Urano, N., and Kimura, S. (1996) Comp. Biochem. Physiol. 115, 63–68
8. Cluzel, C., Lettisias, C., Garrone, R., and Exposito, J. Y. (2000) Matrix Biol. 19, 545–547
9. Paci-Minafra, L., Galante, R., and Minafra, S. (1978) J. Submicrosc. Cytol. 10, 53–63
10. Exposito, J. Y., D’Alessio, M., and Ramirez, F. (1992) J. Biol. Chem. 267, 15359–15362
11. Exposito, J. Y., D’Alessio, M., and Ramirez, F. (1992) J. Biol. Chem. 267, 17404–17408
12. D’Alessio, M., Ramirez, F., Suzuki, H. R., Solursh, M., and Gambino, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9030–9037
13. D’Alessio, M., Ramirez, F., Suzuki, H. R., Solursh, M., and Gambino, R. (1990) J. Biol. Chem. 265, 7050–7054
14. Exposito, J. Y., Boule, N., Delage, G., and Garrone, R. (1995) Eur. J. Biochem. 234, 69–65
15. Tomita, M., Kinosita, T., Izumi, S., Tomino, S., and Yoshizato, K. (1994) Biochim. Biophys. Acta 1217, 131–140
16. Lettisias, C., Exposito, J. Y., and Garrone, R. (1997) Eur. J. Biochem. 245, 434–440
17. Burke, R. D., Boulard, C., and Sanderson, A. I. (1989) Comp. Biochem. Physiol. 94, 41–44
18. Shimizu, K., Amemiya, S., and Yoshizato, K. (1990) Biochim. Biophys. Acta 1038, 39–46
19. Cluzel, C., Lettisias, C., Humbert, F., Garrone, R., and Exposito, J. Y. (2001) J. Biol. Chem. 276, 13108–13114
20. Wilkie, I. C. (2002) J. Exp. Biol. 205, 159–165
21. Leahy, P. S. (1986) Methods Cell Biol. 27, 1–13
22. Cortay, J. C., Negre, D., Scarrabal, M., Rameisier, T. M., Vartak, N. B., Reizer, J., Saier, M. H., and Cozzone, A. J. (1994) J. Biol. Chem. 269, 14885–14901
23. Lettisias, C., Descollonges, Y., Garrone, R., and van der Rest, M. (1993) J. Investig. Dermatol. 101, 92–99
24. Miller, E. J. (1984) in Extracellular Matrix Biochemistry (Pier, K. A., and Haldar, A. H., eds) pp. 41–81, Elsevier Science Publishers B.V., Amsterdam
25. Wilkins, M. R., Lindskog, I., Gasteiger, E., Bairoch, A., Sanchez, J. C., Hochstrasser, D. F., and Appel, R. D. (1997) Electrophoresis 18, 403–408
26. Nomura, Y., Urano, N., and Kimura, S. (1985) FEBS Lett. 181, 314–318
27. Klemann, J. P., Hartmann, D. J., Ramirez, F., and van der Rest, M. (1992) Eur. J. Biochem. 210, 329–335
28. Lees, J. F., Taab, M., and Bulleid, N. J. (1997) EMBO J. 16, 908–916
29. Boot-Hand福德, R. P., and Tuckwell, D. S. (2003) BioEssays 25, 142–151
30. Hojima, Y., Morgelin, M., Engel, J., Boutillon, M. M., van der Rest, M., McKenzie, J., Chen, G. C., Rahi, N., Romanic, A. M., and Prockop, D. J. (1994) J. Biol. Chem. 269, 11381–11390
31. Colge, A., Beschin, A., Samyn, B., Goebels, Y., Van Beeumen, J., Nusgens, B. V., and Lapierre, C. M. (1995) J. Biol. Chem. 270, 16724–16730
32. Wang, W. M., Lee, S., Steiglitz, B. M., Scott, I. C., Lebaros, C. C., Allen, M. L., Brenner, M. C., Takahara, K., and Greenspan, D. S. (2005) J. Biol. Chem. 278, 19549–19557
33. Prockop, D. J., Sieron, A. L., and Li, S. W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13488–13491
34. Berg, J., Tanazawa, K., and Prockop, D. J. (1985) Biochemistry 24, 600–605
35. Johnson, A. S., Ellers, O., Lemire, J., Minor, M., and Luddy, H. A. (2002) Proc. R. Soc. Lond. B Biol. Sci. 269, 235–220
36. Ellers, O., and Telford, M. (1996) Proc. R. Soc. Lond. B Biol. Sci. 263, 39–44
Distinct Maturations of N-propeptide Domains in Fibrillar Procollagen Molecules Involved in the Formation of Heterotypic Fibrils in Adult Sea Urchin Collagenous Tissues

Caroline Cluzel, Claire Lethias, Robert Garrone and Jean-Yves Exposito

J. Biol. Chem. 2004, 279:9811-9817.
doi: 10.1074/jbc.M311803200 originally published online December 9, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311803200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 13 of which can be accessed free at http://www.jbc.org/content/279/11/9811.full.html#ref-list-1