Characterization of Fibrosurfin, an Interfibrillar Component of Sea Urchin Catch Connective Tissues*

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The Sea Urchin Fibrillar (SURF) domain is a four-cysteine module present in the amino-propeptide of the sea urchin 2α fibrillar collagen chain. Despite numerous international genome and expressed sequence tag projects, computer searches have so far failed to identify similar domains in other species. Here, we have characterized a new sea urchin protein of 2656 amino acids made up of a series of epidermal growth factor-like and SURF modules. From its striking similarity to the modular organization of fibropellins, we called this new protein fibrosurfin. This protein is acidic with a calculated pI of 4.12. Eleven of the 17 epidermal growth factor-like domains correspond to the consensus sequence of calcium-binding type. By Western blot and immunofluorescence analyses, this protein is not detectable during embryogenesis. In adult tissues, fibrosurfin is colocalized with the amino-propeptide of the 2α fibrillar collagen chain in several collagenous ligaments, i.e., test sutures, spine ligaments, peristomial membrane, and to a lesser extent, tube feet. Finally, immunogold labeling indicates that fibrosurfin is an interfibrillar component of collagenous tissues. Taken together, the data suggest that proteins possessing SURF modules are localized in the vicinity of mineralized tissues and could be responsible for the unique properties of sea urchin mutable collagenous tissues.

Collagens are a large family of extracellular matrix proteins present in all animal phyla. Among the 19 collagen types hitherto identified, five of them, types I-III, V, and XI, constitute the fibrillar collagens (1). Each procollagen molecule is made of three α chains, each of which can be identical or not. Each α chain contains a triple helical region of 1014 amino acids constructed of an uninterrupted series of GXY triplets. Two non-collagenous regions, the amino- and the carboxyl-propeptide flank this domain. During extracellular maturation of procollagen into collagen molecules, the N- and the C-propeptides are generally removed by the action of specific proteases. The resulting collagen molecule consists of a central triple helix flanked by two short non-collagenous segments, the N- and the C-telopeptides (1, 2). Although the size of the central triple helical region is conserved, with one glycine residue for every three amino acids, the sequence of the C-propeptide domain is the most conserved among the α chains. In contrast, the N-propeptide domain is the most variable region among procollagen molecules. Three different N-propeptide configurations have been characterized in vertebrates (3), and a fourth structure has been defined in sea urchin (4). All of them contain a short triple helical region at the carboxyl terminus. In sea urchin, the N-propeptide consists, from the amino to the carboxyl terminus, of a cysteine-rich region or tsp-2 module, 12 repeats of a four-cysteine domain, and a short triple helical region connected to the N-telopeptide. The four-cysteine module or SURF,1 for Sea Urchin Fibrillar, domain has been described for the first time in the 2α fibrillar collagen chain, but the sea urchin genome possesses at least one other region that could potentially encode several SURF modules (4, 5). The consensus sequence of this 140–145 amino acid motif is $X_{40}GX_{1}LWX_{1}GXGX_{30}CX_{2}CX_{3}/L(F)X_{30}CX_{4}CX_{1}$ (where the numbers in parentheses represent an average number of residues). In situ hybridization reveals that 2α transcripts are detected in mesenchymal cells at the late gastrula stage and in spicule- and gut-associated cells in plutei (6). Immunostaining indicates the presence of this protein around the skeleton spicules and as a thin meshwork in the extracellular matrix surrounding mesenchymal cells (7). In adults, collagen fibrils have been detected in the soft connective tissues of the test, the dermal outer appendages or spines, the Aristotle’s lantern or echinoid jaw, the tube feet, and the peristomial membrane that bridges the gap between the jaw and the skeleton (8–12).

In this study, we sought to obtain new information concerning SURF modules in sea urchin. We characterized a new gene coding for a multidomain protein of the extracellular matrix consisting of a series of EGF-like and SURF modules. Its general structure is reminiscent of sea urchin fibropellins. This new protein is present in several soft tissues of the mineralized part of the adult. Its co-localization with the 2α fibrillar collagen chain, its biochemical properties, the presence of EGF-like motifs that might bind calcium, and its interfibrillar localization suggest a function for this protein in the so-called mutable collagenous ligaments of sea urchin.

EXPERIMENTAL PROCEDURES

Embryo Culture and Nucleic Acid Purification and Analysis—Paracentrotus lividus were purchased from the Arago laboratory (Banyuls-sur-mer, France). Gamete collection, fertilization, and embryo culture were done as previously described. Total RNA from embryonic or adult tissues was purified according to a published protocol (13). For

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AJ291489.

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1 The abbreviations used are: SURF, sea urchin fibrillar; EGF, epidermal growth factor; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT, reverse transcription; PAGE, polyacrylamide gel electrophoresis; ASW, artificial sea water; cb, calcium-binding.
adult RNA, a supplementary purification step was performed prior to RACE experiments consisting of pelleting the RNA by ultracentrifugation through a 5.7 M cesium chloride cushion (14). Poly(A) RNA was purified by two passages through an oligo(dT)-cellulose column (Roche Molecular Biochemicals). Northern blot, Southern blot, and screening procedures were done according to conventional techniques. The genomic DNA library was kindly provided by Dr. Christian Gache, marine station, Villefranche-sur-Mer, France. Hybridization and washing of filters with moderate stringency conditions were performed as described (16).

cDNA Synthesis and PCR—For all RT-PCR experiments, 200 ng of plutei poly(A)-RNA were reverse transcribed using random primers and the reverse transcription kit (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. For PCR, several sets of primers were used and 35 cycles of amplification of the target single strand cDNA were done using the Taq Expand polymerase kit (Roche Molecular Biochemicals). The conditions were: 94 °C for 30 s, and 68 °C for 1–2 min. For the last 25 cycles, 15 s were added for each cycle during the elongation step. After PCR, fragments were purified from the gel and cloned using the TA-Topo 2.1 cloning kit from Invitrogen (Groningen, The Netherlands) according to the manufacturer’s instructions. For RACE experiments, we used the 5′ and 3′ RACE kits from Life Technologies, Inc., and total RNA from the test was used instead of poly(A) RNA extracted from plutei. All the oligonucleotides used are listed in Fig. 1 and were synthesized by Isoprism (Toulouse, France). Both DNA strands were sequenced using the dyeoxyrunucleotide chain termination procedure (Sequenase kit, Amersham Pharmacia Biotech), and universal primer or synthetic oligonucleotides.

Computer Analysis—DNA sequences were analyzed by the DNAiLab computer program (17). Blast (18) and Prosite (19) searches were performed using the IBCP site server accessible via the World Wide Web.2 Antibody Production—To prepare anti-fibrosurfin monoclonal antibodies, the DNA insert encoding the SURF module R8 was generated by PCR using the RT-PCR fragment RT3 as template with the primers 5′-CTATGGATCCGCCGTTGAGGTCACAAGCAC-3′ and 3′-CTATCTGCAGACCTGTGCACGTGACAGCTTC-3′ included in the IBCP site, respectively. We used a derivative of pT7/7 (U.S. Biochemical Corp.) as an overproducing plasmid in which six His codons had been included between the PolI and HindIII sites with a stop codon following the last His codon (20). Production and purification were done as previously described (7). Mouse monoclonal antibody production, titration by enzyme-linked immunosorbent assay, and characterization by immunoblotting was performed using established protocols (21).

Protein Detection—Tissues were dissected from adult P. lividus. Test, Aristotle’s lantern, digestive tract, spines, and peristomial membrane were collected. Sequential 24 h extractions at 4 °C in 2 x urea and then in 8 x urea with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide and 0.5 mM dithiothreitol) were performed on embryos or crushed tissues with ~5 ml of extraction buffer of wet material. Supernatants were analyzed by Western blotting. Crude extracts were separated on 6% SDS-polyacrylamide gel electrophoresis (PAGE) followed by electrotransfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore, St. Quentin en Yvelines, France) overnight at 4 °C in 10 mM CAPS pH 11, 5% methanol Blots were exposed to 23-2D4- (anti-SURF module R8, fibroplins) and 11-4E11- (anti-SURF module R2, twoa chain; Ref. 7) purified antibodies at a concentration of 1 μg/ml. Alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad) were used as secondary antibody and developed using the substrate kit from Bio-Rad (Ivy-sur-Seine, France).

Protein extracts (2 x urea) from test were dialyzed against 20 mM Tris, pH 8, and chromatographed on a DE52 anion exchanger. Proteins were eluted with a linear gradient of 0–1 M NaCl.

Immunological Methods—Test and spine bases were dissected from individual P. lividus. Samples were rinsed with artificial sea water (ASW, 480 mM NaCl, 10 mM KCl, 26 mM MgCl2, 29 mM MgSO4, 10 mM CaCl2, 2.4 mM NaHCO3, pH 8) and fixed for 4 h at 4 °C in 2.5% paraformaldehyde in ASW. After rinsing with ASW, calcified tissues were cut on a Reichert-Jung UltraCut ultramicrotome and contrasted with methanolic uranyl acetate and lead citrate. Samples were observed with a CM120 Philips electron microscope at the "Centre de Microscopie Electronique Appliquée à la Biologie et à la Géologie" (CMEABG, Université Claude Bernard, Lyon 1).

RESULTS

The P. lividus Genome Can Potentially Encode Several Proteins Possessing SURF Modules—From previous work we have shown that SURF modules are present in the N-propeptide of the sea urchin twoa fibrillar collagen chain and that another part of the sea urchin genome could encode several SURF modules (4, 5). Until now, however, we have had no evidence that this region is part of an active gene or pseudogene. Moreover, a Southern blot of P. lividus genomic DNA under moderate stringency revealed that several parts of the sea urchin genome could encode SURF modules (data not shown). From these results, we used the same hybridization conditions to screen a P. lividus genomic DNA library. Among 60,000 clones, 54 positive clones exhibiting variable intensities of labeling were detected. Shorter RT-PCR analyses were done on several weakly positive clones, two of which overlap, that possess sequences coding for SURF modules. Blast search analyses revealed that these SURF modules shared 20–30% identity with comparable domains of the twoa chain and the putative twoα protein.

RT-PCR experiments were done using poly(A)+ RNA extracted from plutei embryos. As presented in Fig. 1, six-over...
Northern blot comparisons of fibrosurfin (A) and 2α (B) mRNAs. Probes for fibrosurfin (RT4 cDNA) and 2α (DNA coding for SURF modules R6-R8) were hybridized to plutei (P) poly(A)+ RNA (1 μg) or total RNA (10 μg) from test (T) and adult test (A). The positions of 28S and 18S rRNA markers are indicated on the left.

Fig. 3. Complete amino acid sequence of fibrosurfin. Asterisks indicate the two putative Met. The sequence surrounding the first Met codon (GGTTCATGG) is related more to the Kozak sequence (GCC(A/T)GG) than that surrounding the second Met codon (GGTTCCATGG). The sequence surrounding the first Met, underlined, indicates the putative signal peptide cleavage site. Putative N-linked glycosylation sites are underlined.

3 and 4 and the replacement of the avdin-lidom-like domain of fibropeptin with a 29-amino acid domain (Fig. 1). From the common modular organization with fibropellin and the presence of SURF modules, we called this protein fibrosurfin. From its primary structure, fibrosurfin is an acidic protein with an estimated isoelectric point of 4.12 and a calculated molecular mass of 276 kDa. The net charge is estimated isoelectric point of 4.12 and a calculated molecular mass of 276 kDa. The net charge is -211 (10.7% of Asp + Glu), but EGF domains are the most anionic part of fibrosurfin (13.15–21.05% of Asp + Glu). From its amino acid composition, fibrosurfin is rich in serine and threonine residues (20.5%). Moreover, the monoclonal antibody used in this study did not cross-react with several previously produced recombinant proteins harboring SURF modules of the 2α chain (data not shown and Ref. 7). These antibodies were used to examine the expression of fibrosurfin in sea urchin tissues. Because the gene
coding for this protein was expressed in test, Western blotting was performed using different protein extracts from demineralized tests (Fig. 4). After urea treatment, several immunoreactive bands were detected between 80–160 kDa. Positive bands with a molecular mass higher than 120 kDa disappeared rapidly upon short term storage at 4 °C or −20 °C (results not shown). Using the chemical properties of fibrosurfin, urea protein extracts from test were submitted to anionic exchange chromatography, and eluted fractions were separated by SDS-PAGE (Fig. 5A). The major bands present in the 0.36 M NaCl fraction were recognized by the anti-fibrosurfin monoclonal antibody (Fig. 5B), whereas 2α immunoreactive bands were detected in the 0.04 and 0.2 M NaCl fractions (Fig. 5C). Edman degradation sequencing of the 0.36 M NaCl bands specific from fibrosurfin was performed, but their amino termini were blocked. Nevertheless, in some experiments, a highest molecular mass band (280–300 kDa) was recognized by the anti-fibrosurfin monoclonal antibody (Fig. 5D, 0.25 and 0.3 M NaCl fractions). As a next step, Western blots were performed using protein extracts from embryos using the anti-fibrosurfin monoclonal antibody (Fig. 6). In these blots, no immunoreactive bands were detected except for the positive control consisting of proteins extracted from test. Finally, several tissues from adult animals were analyzed by Western blotting using anti-fibrosurfin (Fig. 7A) or anti 2α (Fig. 7B) monoclonal antibodies. From these blots, the 2α N-propeptide and fibrosurfin were present in the same tissues, i.e. test, spine ligament, and peristomial membrane. Traces of the proteins were detected in the tube feet, whereas no detectable signals were obtained in extracts of the digestive tract or of spine tips. The 2α chain is also detected in the Aristotle's lantern. As for fibrosurfin, several immunoreactive bands were detected using anti-2α N-propeptide monoclonal antibodies. For both proteins, the patterns of positive bands were slightly different in the different tissues analyzed, especially for fibrosurfin. High molecular mass immunoreactive bands were obtained for both proteins in extracts from the peristomial membrane.

**Immunolocalization of Fibrosurfin and the 2α N-propeptide**—Two positive tissues, the catch apparatus and the test, were analyzed by immunostaining using the same antibodies. In Fig. 8A, a section of the catch apparatus consists of three regions: the mineralized tissues of the spine, the collagenous ligaments, and the external region, which is not depicted and contains mainly muscle cells and the epidermis. The sutural ligaments that link the calcite plates are composed of collagen fibrils (Fig. 8A). We can distinguish the meridional or zigzag suture from the circumferential sutures between the test plates. Using monoclonal antibodies against fibrosurfin or the 2α N-propeptide, immunofluorescence studies indicated that 2α and fibrosurfin were co-localized in the collagenous ligaments of the catch apparatus (Fig. 8, B and C) and in the sutural ligaments (Fig. 8, D and E). Zigzag sutures were more intensively stained than circumferential sutures. As shown in Fig. 8F, a strong autofluorescence was detected within the mineralized plates. To better localize fibrosurfin and the 2α N-propeptide in the spine ligament, preembedding immunoelectron microscopy was performed to preserve antigenicity. For fibrosurfin, gold particles were observed between or in close proximity to collagen fibrils, indicating that fibrosurfin is an interfibrillar component (Fig. 9, A and C). For the 2α N-propep-
tide, gold particles accumulated at the periphery of the bundles made of collagenous fibrils aligned in parallel. These gold particles were generally in the vicinity of cells (Fig. 9D) and rarely observed at the surface of collagen fibrils (Fig. 9E). No signal was observed for the negative control (data not shown).

**DISCUSSION**

In this report, we clearly demonstrate that several genes in sea urchin could encode SURF modules. In addition to the previously described 2α fibrillar collagen chain (4, 5), we have obtained the primary structure of a new protein, which we call fibrosurfin and contains a series of 13 SURF modules. Immunolocalization and biochemical studies indicate that fibrosurfin, like the 2α chain, is one of the components of the collagenous ligaments that link together the calcite ossicles of the sea urchin skeleton. In addition, preliminary data concerning the previously described COLP5α gene (5), indicate a similar localization of this related protein in adult tissues.3 Taken together, these results suggest that proteins, including SURF modules, seem to be located around the mineralized region of the sea urchin and in so-called adult mutable collagenous tissues.

From Fig. 1, a common origin for genes encoding fibropellins and fibrosurfin is strongly suggested. Firstly, highest identity scores were obtained between these two proteins for two types of modules, the CUB and EGF domains. Secondly, their general structures are closely related with the exception of the carboxyl-terminal domain and the insertion of a series of SURF modules between two EGF motifs (24). Both these features greatly support the notion of exon shuffling (28), which accounts for considerable variety among multimodular proteins. Even though the general structures of these proteins are similar, it is difficult to obtain any co-linearity between their EGF modules as has been observed between sea urchin fibropellins. This suggests that these genes had diverged early during evolution or that they have evolved rapidly. Although the 2α chain and 5α protein are similar, we could not detect any similarities between their SURF motifs and those of fibrosurfin. However, like 2α and 5α, fibrosurfin SURF modules are acidic. One of the particularities of fibrosurfin SURF modules is their high serine and threonine residue content. Several clusters of these amino acids provide potential sites for O-linked glycosylation (29).

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3 C. Cluzel, C. Lethias, R. Garrone, and J. Y. Exposito, unpublished data.
two complement components, C1s and C1r, show high affinity for calcium (34). These two proteins form a tetrameric subunit C1s-C1r-C1r-C1s, and their assembly is calcium-dependent. Thus, the presence of a CUB-cbEGF region in fibrosurfin reinforces the idea that these domains might promote a homotypic association, whereas stretches of cbEGF might be involved in homotypic and heterotypic protein-protein interactions. EGF modules are located at the two extremities of fibrosurfin and correspond to the most anionic part of this protein. The inter-fibrillar matrix of these collagenous ligaments contains several polyanionic glycosaminoglycans (8). Moreover, several acidic glycoproteins that have a strong negative charge seem to be important in the aggregation properties of the collagen fibrils (35, 36). Both fibrosurfin and the 2α N-propeptide (pI 4.55) are also acidic and have a strong negative charge.

From this potential capacity to bind calcium and its localization in collagenous ligaments as an interfibrillar component, fibrosurfin could be one of the factors responsible for the unusual properties of these collagenous tissues. In fact, echinoderm ligaments are quite unique and have been called mutable collagenous tissues or catch connective tissues (8, 37). These animals possess a mechanism to alter the transfer properties of the inter-fibrillar matrix of their ligaments (35, 37), which permits modulation of both the shape and stiffness of collagenous tissues. A recent report indicates than one or more secreted molecules induce the aggregation of fibrils in the presence of calcium. For the sea cucumber dermis, stiparin is one of these stiffening factors (35). Modulation of these properties by anti-stiparin molecules has also been described (36). A more recent study indicates that stiffening and plasticizing factors seem to be located inside the cells of the holothurian dermis rather than in compartments of the extracellular matrix (38). One of their hypotheses is that the effect of these reagents could be amplified by matrix macromolecules like stiparin. From its extracellular matrix location and its biochemical characteristics, fibrosurfin might play a similar function.

From the uniqueness of mutable collagenous tissues in echinoderms, an evolutionary origin for these functions has been proposed (39). SURF modules have been characterized only in sea urchin despite the numerous international genome and expressed sequence tag programs. In *Caenorhabditis elegans*, more than 20 modules seem unique to this phylum (40). Because the three proteins harboring SURF modules appear to be specific to the mutable collagenous tissues, it is tempting to speculate that this module is one of the evolutionary elements responsible for this echinoderm feature. A search of SURF modules in other echinoderms and further analysis of SURF-containing proteins will permit us, in the future, to define more precisely the relationship between SURF modules and the so-called mutable collagenous tissues.

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