An Arthropod Cuticular Chitin-binding Protein Endows Injured Sites with Transglutaminase-dependent Mesh*

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In mammals, the cornified cell envelope forms beneath the plasma membrane in epithelia and provides a vital physical barrier consisting of insoluble proteins cross-linked by transglutaminase (TGase). In the horseshoe crab Tachypleus tridentatus, TGase is stored in hemocytes and secreted in response to the stimulation of bacterial lipopolysaccharides. Here we characterized a TGase substrate designated as caraxin that was identified in horseshoe crab cuticle. One of the homologs, caraxin-1, possessed a unique domain structure consisting of N- and C-terminal heptad repeats and a central domain with a tandem-repeated structure of a pentapeptide. Western blotting showed the specific localization of caraxin-1 in sub-cuticular epidermis. Moreover, we identified the pentapeptide motif to be a chitin-binding unit. Analytical ultracentrifugation revealed that caraxin-1 exists as an oligomer with 310–350 kDa, which is ~20-mer based on the molecular mass of the monomer. The oligomers were cross-linked by TGase to form an elaborate mesh with honeycomb structures, which was electron-microscopically found to be different from the clotting mesh triggered by lipopolysaccharide-induced hemocyte exocytosis. We determined several cross-linking sites in the N- and C-terminal domains of caraxin-1. The replacements of Leu to Pro at positions 36 and 118 in caraxin-1 reduced the α-helix content, which destroyed the TGase-dependent mesh, thus indicating the importance of the N- and C-terminal domains for the proper mesh formation. In arthropods, TGase-dependent protein cross-linking may be involved in the initial stage of host defense at the sub-cuticular epidermis, as in the case of mammalian skin.

Transglutaminase (TGase),3 which requires Ca2+ for activation, catalyzes various post-translational reactions, such as the protein cross-linking involved in blood coagulation, skin-barrier formation, and other important biological processes, by forming an isopeptide bond between Lys and Gln residues to form the cross-linking of the ε-(γ-glutamyl)lysine bond (1–8).

For example, in the mammalian coagulation system, fibrin polymers are stabilized through fibrin cross-linking induced by plasma TGase, factor XIII (9, 10). Also, in crustaceans, such as shrimps and lobsters, the hemolymph coagulation depends on the TGase-mediated cross-linking of specific clotting proteins (11, 12). However, it is still unclear whether TGase is involved in clot formation in Drosophila melanogaster (13).

In the horseshoe crab, no TGase activity has been found in plasma, with the majority of TGase being expressed in hemocytes (14–16). Horseshoe crab hemocyte is highly sensitive to lipopolysaccharides (LPs), which are cell wall components of Gram-negative bacteria. Stimulation by LPS prompts exocytosis through a GTP-binding protein-mediating signaling pathway, which triggers the secretion of granular components, including serine protease zymogens, involved in hemolymph coagulation, a clottable protein known as coagulogen, protease inhibitors, lectins, and antimicrobial peptides (17–21). The coagulation cascade is composed of the clottable protein coagulogen and four serine protease zymogens. Factor C, one of the zymogens, functions as a biosensor for LPS and activates the coagulation cascade, leading to the conversion of coagulogen to coagulin (18, 20, 21). The resulting coagulin interacts with itself to form a homopolymer through self-polymerization. LPS also could release the cytosolic TGase from hemocytes through an unknown mechanism (22). Horseshoe crab TGase shows significant sequence similarity with the mammalian TGase members (14, 15): human keratinocyte TGase (37.6% identity), human factor XIIIa subunit (34.7%), and guinea pig liver TGase (32.7%). It contains 764 amino acid residues in total with a unique N-terminal extension sequence of 60 residues without a consensus N-terminal signal sequence for secretion. TGase neither catalyzes monodansylcadaverine (DCA) incorporation into coagulin nor cross-links coagulin, whereas TGase promotes the cross-linking of coagulin with proxin, a hemocyte-derived proline-rich protein, resulting in a stable coagulin polymer (22, 23).

Recently, we extensively determined the sequences of horseshoe crab cuticular chitin-binding proteins and grouped these proteins into classes based on their approximate isoelectric points and predominant amino acid compositions (16). Interestingly, we observed TGase-dependent polymerization of sev-
eral cuticular chitin-binding proteins, a finding that suggests that TGase-dependent cross-linking plays an important role in host defense in the arthropod cuticle. Several of the horseshoe crab cuticular proteins contain the so-called R & R consensus found in arthropod cuticular proteins (24), and some proteins contain a Cys-rich domain with a sequence similar to those of insect peritrophic matrix proteins and chitinases (25). In contrast, basic QH4 and QH10 contain no consensus sequences found in known chitin-binding proteins. In this study, we characterized the recombinant proteins of basic QH4 and QH10, respectively named caraxin-1 and caraxin-2, to signify that they are carapace-derived chitin-binding proteins for protein cross-linking.

**EXPERIMENTAL PROCEDURES**

**Cuticular Chitin-binding Proteins**—Extraction and purification of cuticular chitin-binding proteins were performed as previously described (16).

**Preparation of Recombinant TGase**—A DNA fragment encoding horseshoe crab TGase (pTG74) (15) was created using PCR and was subcloned into the NotI and EcoRI sites of the expression vector pFastBac

**Homotypic Interaction between Caraxin Molecules**—Homotypic interaction between caraxins was investigated by pull-down assay using nickel-nitrilotriacetic acid affinity beads. Caraxins with and without His tag were mixed with nickel-agarose beads in 50 mM Tris-HCl, pH 8.5, containing 0.1 M NaCl, and incubated at 4 °C for 1 h. The resulting samples were centrifuged and divided into bound and unbound fractions. The bound fraction was washed with the same buffer, the protein bound to nickel-agarose beads was eluted with the same buffer containing 100 mM imidazole, and the aliquots of each fraction were subjected to SDS-PAGE. As a negative control, His-tagged complement control protein, a recombinant protein derived from a complement control protein-domain of horseshoe crab factor C (21), was used.

**Analytical Ultracentrifugation**—Sedimentation velocity and equilibrium experiments were conducted with an Optima XLI (Beckman-Coulter, Fullerton, CA), using a 4-hole An60Ti rotor at 20 °C (29). The sample solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl, and the dialysate was used as an optical reference. For sedimentation velocity experiments, a sample solution (400 μl) at A

**Identification of Cross-linking Sites of Caraxin-1**—To determine Gln residues susceptible for TGase-dependent cross-linking, DCA at 5 mM was incubated with non-tagged caraxin-1 (0.5

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**Characterization of Caraxins**

with His tag at their N terminus, DNA fragments encoding full-length caraxins-1 and -2 were created using PCR and were subcloned into the Ndel and BamHI sites of the expression vector pET-15b (Novagen). To construct an expression vector for non-tagged caraxin-1 and caraxin-2, DNA fragments encoding full-length caraxins-1 and -2 were created using PCR and were subcloned into the NcoI and EcoRI sites of the expression vector pET-28a (Novagen). To construct expression vectors for the caraxin-1 mutants, PCR-based site-directed mutagenesis was performed from Leu 36/118 to Pro using oligonucleotides. PCR-based site-directed mutagenesis for the creation of mut-3QN and mut-4QN of caraxin-1 was performed from Gln 30/31/32/115 to Asn. All constructs were verified by sequencing. These recombinant proteins were expressed in the *Escherichia coli* strain BL21(DE3)/pLysS and purified using chitin-affinity column chromatography. The expression and refolding of recombinant proteins were carried out by the published method with slight modifications (28).
Characterization of Caraxins

mg/ml) in the presence of TGase in TA-Ca at 37 °C for 1 h. After incubation, the labeled protein was digested by Asp-N protease (Roche Applied Science) in 50 mM Tris-HCl, pH 7.5, containing 2 mM urea at 37 °C for 16 h. The resulting peptides were separated by rpHPLC. To determine Lys residues that are susceptible to cross-linking, a biotin-labeled peptide containing a Gln residue was synthesized as a probe; biotin-DEQAAL was synthesized based on the sequence corresponding to Asp112–Leu117 of caraxin-1 with an amino acid replacement of Lys to Ala at position 115. The biotin-labeled probe (1 μM) was cross-linked with non-tagged caraxin-1 (10 nM) in TA-Ca at 37 °C for 1 h, and digested by Asp-N protease and separated by rpHPLC as described above. Aliquots of the resulting peptides were adsorbed to microtiter plates, and the peptides cross-linked with the probe were identified by horseradish peroxidase-conjugated streptavidin (GE Healthcare). The enzyme activity of horseradish peroxidase was detected with o-phenylenediamine at 490 nm by using a plate reader. The peptides cross-linked into the recombinant proteins was quantitated by fluorescence measurements with excitation at 355 nm and emission at 425 nm.

**Quantitative Measurement of DCA Labeling**—DCA at 0.5 mM was adsorbed into the recombinant proteins by TGase in TA-Ca at 37 °C. Aliquots were taken at 10-min intervals from 0 to 60 min and treated with 10% trichloroacetic acid. The resulting precipitates were dissolved in 1 ml of 50 mM Tris acetate, pH 7.5, containing 8 mM urea and 0.5% SDS, and DCA incorporation into the recombinant proteins was quantitated by fluorescence measurements with excitation at 355 nm and emission at 425 nm.

**Optical Microscopy**—For scanning electron microscopy, fixed samples were transferred into tert-butyl alcohol and freeze-dried. The dried samples were gold-coated in an ion coater and examined in a Hitachi S-3000N scanning electron microscope.

**Immunofluorescence Staining**—Non-tagged caraxin-1 was incubated with the TGase in TA-Ca buffer at 37 °C for 16 h. To form a coagulation mesh, 1 ml of horseshoe crab hemolymph was collected into 50 ml of pyrogen-free 10 mM HEPES, pH 7.0, containing 0.5 M NaCl, and the diluted hemolymph was plated on a slide glass. After a 20-min incubation, the attached hemocytes were stimulated with 1 μg/ml LPS for 1 h. The proteins were fixed in 3.7% formaldehyde and incubated with ethylene glycol chitin at 1 mg/ml for 1 h. The bound chitin on the proteins was treated with tachylectin-5A (1 μg/ml), and then incubated with anti-tachylectin-5A antibody and Rhodamine-conjugated swine anti-rabbit IgG (Dako).

**Amino Acid Composition and Sequence Analyses**—Amino acid analysis was analyzed by an AccQ-Tag system (Waters Associates, Milford, MA). Amino acid sequence analysis was carried out using an Applied Biosystems 491 protein sequencer.

**RESULTS AND DISCUSSION**

**Identification of Sensitive Substrates for TGase in Cuticular Chitin-binding Proteins**—To identify sensitive protein substrates for TGase, horseshoe crab cuticular chitin-binding proteins were incubated with DCA in the presence of TGase and subjected to Tricine-SDS-PAGE. The cuticular proteins of 16 and 13 kDa were highly reactive for DCA labeling (Fig. 1A). The labeling reaction was completely inhibited by EDTA, which indicated the TGase-specific incorporation of DCA. The protein bands labeled with DCA were cut off from the gel and...
Figure 1. DCA labeling of cuticular chitin-binding proteins by TGase. A, cuticular chitin-binding proteins were incubated with DCA in the presence of TGase. Aliquots were subjected to Tricine-SDS-PAGE, and DCA-labeling proteins were detected by UV illumination. The resulting peaks were subjected to Tricine-SDS-PAGE, and DCA-labeled peptides were detected by UV illumination. The two Leu residues at positions 36 and 118 for the site-directed mutagenesis are indicated by bold letters. The numbers on the right indicate amino acid residue numbers. The two Leu residues at positions 36 and 118 for the site-directed mutagenesis are underlined. C, schematic models of the domain structures of caraxin-1 and caraxin-2. Numbers indicate amino acid residue numbers. A single underline represents a chitin-binding fragment. Circled Gln residues, DCA-labeled Gln residues; a circled Lys residue, a Lys residue susceptible for cross-linking with the biotin-labeled peptide probe; dotted, the Gln-rich N-terminal domain; gray, the tandem-repeated structure of a pentapeptide; open, the C-terminal domain.

Figure 2. Tissue-specific localization of caraxin-1 and TGase. The tissue-specific localization of caraxin-1 and TGase were investigated by Western blot, using anti-caraxin-1 and anti-TGase polyclonal antibody, respectively. Lane 1, sub-cuticular epidermis; lane 2, hemocytes; lane 3, heart; lane 4, stomach; lane 5, intestine; lane 6, hepatopancreas; lane 7, skeletal muscle; lane 8, nerve; and lane 9, plasma.

Identification of Oligomer Formations of Caraxins—Caraxin-1 and caraxin-2 were each incubated with DCA in the presence of TGase. As expected, DCA was incorporated into caraxin-1 and caraxin-2. Generally, the addition of an excess amount of DCA inhibits protein-protein cross-linking. Interestingly, even in the presence of DCA, the caraxins were cross-linked with each other, suggesting that they exist as non-covalent oligomers that lead to the accession of Lys to Gln between the monomers to be cross-linked readily by TGase (Fig. 3A and 2).

To demonstrate that the caraxins exist as an oligomer, we assessed the interaction between His-tagged caraxin-1 and non-tagged caraxin-1. His-tagged caraxin-1 was incubated with non-tagged caraxin-1 in the presence of nickel-agarose beads (Fig. 3B). As a result, non-tagged caraxin-1 was specifically co-precipitated with His-tagged caraxin-1 but not with a negative control protein, His-tagged complement control protein, indicating the oligomer formation of caraxin-1. Homotypic interaction of caraxin-2 was also demonstrated by the same pull-down assay (Fig. 3C).

To investigate the molecular size of the oligomer of caraxin-1, analytical ultracentrifugation was carried out. Sedimentation velocity experiments revealed a single major peak with a sedimentation coefficient of 12.6 S with a slight shoulder on the high molecular weight side that was probably a dimer. Conversion of c(s) to c(M) gave rise to a molecular weight of 313,500 for the major peak (Fig. 3D). On the other hand, the concentration...
Characterization of Caraxins

gradients obtained for non-tagged caraxin-1 in the sedimentation equilibrium fit well with a single species model with the molecular weight of 349,000, indicating that caraxin-1 exists as an oligomer, ≈20-mer in solution.

α-Helical Structure of Caraxin-1—The multicoil program (34) predicted that caraxin-1 contains two heptad-repeat regions (Gln31 to Leu44 and Ala106 to Glu126) at the N- and C-terminal domains. To test whether the N- and C-terminal heptad-repeat regions form α-helical structures, we designed a proline substitution into caraxin-1 at Leu36 and Leu118 localized at the two heptad-repeat regions, respectively, which was expected to disrupt their structures (35). Wild-type caraxin-1 showed a CD spectrum with minima at 208 and 222 nm with an α-helix content of 20% (Fig. 4). The molar ellipticity at 222 nm for mutant L118P was reduced in comparison with that of the wild type. The double replacements, i.e. mutant L36/118P, caused an additional reduction of the molar ellipticity at 222 nm, and the α-helix content of mutant L36/118P was reduced to 14%.

TGase-dependent Mesh Formation of Caraxin-1—In mammals, the cornified cell envelope is assembled by the incorporation of the fibers of precursor proteins, which are cross-linked by keratinocyte TGase. Caraxin-1 was observed by optical microscope to form a TGase-dependent mesh (Fig. 5A). In contrast, mutant L36/118P lost the TGase-dependent mesh formation and resulted in aggregates of the mutant protein, indicating the importance of the N- and C-terminal domains of caraxin-1 for the proper mesh formation (Fig. 5B).

The fine structure of caraxin mesh was observed by scanning electron microscopy. In the absence of TGase, the oligomers of caraxin-1 were not detectable under these conditions (Fig. 6, A and B). In contrast, in the presence of TGase, caraxin-1 was cross-linked to form an elaborate mesh with a honeycomb structure, indicating that the TGase-dependent covalent cross-linking is essential to form the stable mesh of caraxin-1 (Fig. 6C). The cross-linked fibrils had a rough surface and were 0.3–0.4 μm in diameter (Fig. 6D). TGase treatment of the mutant L36/118P produced no mesh-like structure (Fig. 6E) and caused ball-like aggregates of ≈0.3 μm in diameter (Fig. 6F). Fig. 6 (G and H) showed a clotting mesh generated by the LPS-induced hemo-
cyte exocytosis, and the clotting fibrils had a smooth surface and were \( \sim 0.1 \mu m \) in diameter. A clotting protein, coagulin, forms a thinner fibril of \( \sim 0.01 \mu m \) in diameter through non-covalent head-to-tail and lateral interactions (36, 37). TGase does not cross-link coagulin itself, and the clotting mesh is completed by TGase-dependent cross-linking of the coagulin fibrils with at least proxin (22) and possibly with other proteins, such \( \alpha_2 \)-macroglobulin, C-reactive proteins, and hemocyanin (38). Differences in the electron-microscopic structures between the caraxin and clotting meshes suggest their different physiological functions.

To examine whether caraxin-1 mesh retains the chitin-binding activity, the mesh was incubated with a soluble chitin derivative, ethylene glycol-chitin. The soluble chitin bound to the mesh was visualized by a horseshoe crab lectin, tachylectin-5A, which recognizes the acetyl group of chitin (39). As a result, the network structure of the cross-linked caraxin-1 was profiled by immunostaining against tachylectin-5A (Fig. 7, A and B). Calcofluor, a chitin-binding fluorescence reagent, at 1% concentration, inhibited the binding of the soluble chitin to caraxin-1 mesh. In contrast, the soluble chitin did not bind to the clotting mesh generated from LPS-stimulated hemocytes (Fig. 7, E and F). These results indicated that caraxin-1 mesh retains the chitin-binding activity that may play an important role in the interaction with exposed chitin at injured sites that serves to seal lesions effectively.

**Chitin Binding Region of Caraxin-1**—To identify the chitin binding region of caraxin-1, caraxin-1 was digested with Asp-N protease and trypsin. The resulting fragments were applied to a chitin-affinity column, and a bound fragment was obtained by stepwise elution by 10% acetic acid. The chitin-binding fragment was assigned to the sequence from His56 to Leu112 by amino acid composition and amino acid sequence analysis (Fig. 1C). This chitin-binding fragment contains tandem repeats of the conserved pentapeptide.

**Interaction of Caraxin-1 with Chitin and Other Polysaccharides**—The binding parameter of caraxin-1 with ethylene glycol chitin was determined by surface plasmon resonance analysis, which revealed an association rate constant, \( k_a = 9.52 \times 10^9 M^{-1}s^{-1} \), and a dissociation rate constant, \( k_d = 3.43 \times 10^{-3} s^{-1} \), and consequently, a dissociation constant of \( K_d = 3.60 \times 10^{-8} M \). The binding parameters of caraxin-1 with polysaccharides, including chitin, chitosan, and cellulose, were also determined by quartz-crystal microbalance analysis (Table 1). The binding affinities of caraxin-1 for chitin and chitosan were indistinguishable with \( K_d = 5.0 \times 10^{-8} \), but that for cellulose was reduced to one-third of that for chitin. In contrast, caraxin-1 did not bind to N-acetylchitooligosaccharides such as N-acetylchitoheptaose.

Caraxin-1 and caraxin-2 are the cuticular chitin-binding proteins that do not contain the R & R consensus sequence or the peritrophin-like domain commonly found in insect cuticular proteins (24, 25, 40, 41). The pentapeptide GYYHP showed a binding affinity for chitin with \( K_d = 1.60 \times 10^{-5} \), which is lower by five orders of magnitude than that of caraxin-1. His and Tyr
residues found in several cuticular proteins and chitin-binding antimicrobial peptides are required for chitin binding (42, 43). Therefore, the repeating unit of the pentapeptide in caraxins may be the smallest sugar-binding unit, and the repetition of the pentapeptide may increase the binding affinity for chitin.

**Identification of Cross-linking Sites** —To determine the Gln residues susceptible for cross-linking, caraxin-1 was incubated with DCA in the presence of TGase, and the DCA-labeled caraxin-1 was digested by Asp-N protease and separated by rpHPLC. Thirteen peptides were isolated, and they were sufficient to identify all Gln residues present in caraxin-1. Gln32 and Gln115 were not identified by sequence analysis of the two peptides (positions 13–34 and 113–135), which indicates that they were modified by DCA. Sequence analysis of peptide 13–34 also showed partial modification at Gln30 and Gln31. On the other hand, the Gln residues at 18, 21, 22, and 53 were clearly identified by sequence analysis, and no DCA-labeled peptides containing these Gln residues were recovered. In addition, peptide 35–112, which contains Gln53 and Gln109, had no detectable fluorescence. These data suggest that the four Gln residues at positions 30, 31, 32, and 115 are major cross-linking sites of caraxin-1 (Fig. 1C).

To confirm whether these Gln residues are involved in protein-protein cross-linking by TGase, two caraxin-1 mutants were prepared: mut-3QN, in which the three Gln residues at positions 30, 31, and 32 were replaced by Asn; and mut-4QN, in which the four Gln residues were all replaced by Asn. Caraxin-1 (wild type) and the mutants were, respectively, incubated with TGase in the presence of Ca\(^{2+}\) or EDTA, and aliquots were subjected to SDS-PAGE. The wild type was cross-linked to form the high molecular weight oligomers observed on the top of the gel, and EDTA inhibited the oligomer formation (Fig. 8A, lanes 1 and 2). Interestingly, the cross-linking reaction of mut-3QN was not strongly reduced (Fig. 8A, lane 3). In contrast, mut-4QN with the additional replacement of Gln at position 115 resulted in the reduction of high molecular weight oligomers, and the majority remained as non-cross-linked monomers (Fig. 8A, lane 5). These data suggest that Gln115 plays a key role in the TGase-mediated protein-protein cross-linking.

**TABLE 1**

| Protein or peptide | Polysaccharide | \(K_d\)  
|--------------------|----------------|---------|
| BIACore | Caraxin-1 | Ethylene glycol chitin | 3.60 \(\times\) 10\(^{-8}\)  
| Affimmun Q | Caraxin-1 | Chitin | 5.64 \(\times\) 10\(^{-8}\)  
| Caraxin-1 | Chitosan | 5.23 \(\times\) 10\(^{-8}\)  
| Caraxin-1 | Cellulose | 1.46 \(\times\) 10\(^{-7}\)  
| GYYHP | Chitin | 1.60 \(\times\) 10\(^{-3}\)  

**FIGURE 7.** Chitin binding ability of caraxin-1 mesh. Caraxin-1 mesh (A–D) and coagulation mesh (E and F) were treated with ethylene glycol chitin, and the bound chitin was detected by using tachylectin-5A and Rhodamine-conjugated swine anti-rabbit IgG. A and E, brightfield views. B–D and F, immunofluorescence views. C, without tachylectin-5A; D, without the anti-tachylectin-5A antibody. Scale bar, 25 \(\mu\)m.

**FIGURE 8.** Oligomer formation and DCA labeling of the caraxin-1 mutants by TGase. A, caraxin-1 and the mutants were treated with TGase in the presence of Ca\(^{2+}\) or EDTA, and aliquots were subjected to SDS-PAGE. Lane 1, caraxin-1/Ca\(^{2+}\); lane 2, caraxin-1/EDTA; lane 3, mut-3QN/Ca\(^{2+}\); lane 4, mut-3QN/EDTA; lane 5, mut-4QN/Ca\(^{2+}\); lane 6, mut-4QN/EDTA. B, caraxin-1 and the caraxin-1 mutants were incubated with DCA in the presence of TGase. The amounts of DCA in the proteins were monitored by fluorescence measurement with excitation at 355 nm and emission at 525 nm. Bars represent the deviation of three individual measurements. Closed circles, caraxin-1; open circles, mut-3QN; and open triangles, mut-4QN.
doublet band corresponding to the dimer of mut-4QN was also observed (Fig. 8A, lane 5). The lower molecular weight species of the doublet may have been produced by intrachain cross-linking to form a more compact protein structure.

Moreover, DCA labeling in the wild type and the two mutants was quantitatively analyzed by a fluorometer. The amount of DCA labeling in the wild type or the mutants reached a plateau after a 1-h incubation (Fig. 8B). The levels of DCA labeling in mut-3QN and mut-4QN were, respectively, 50 and 30% of that in the wild type. Namely, the four Gln residues occupied ~70% of the total DCA labeling altogether.

On the other hand, to determine the Lys residues susceptible for cross-linking, caraxin-1 was incubated with a biotin-labeled peptide probe, biotin-DEQAAL, and Lys residues cross-linked with the probe were identified as described under “Experimental Procedures.” As a result, Lys at position 119 was one of the major cross-linking sites of caraxin-1 (Fig. 1C).

**Implications for Host Defense**—In mammals, proteins involved in the cornified cell envelope share functional domains, especially Gln- and Lys-rich domains, which are commonly engaged in intrachain and interchain cross-linking by TGases (44). Several of these proteins contain tandem repeats in the central domain; human involucrin, a key component of the cornified cell envelope, is built of a tandem array of helix-turn-helix folds, which is ideally suited to serve as a scaffold for cell envelope assembly (45).

Although caraxins have no sequence similarity to involucrin, they contain a central domain with tandem repeats of five amino acids, flanked by the N- and C-terminal α-helical domains rich in Gln and Lys residues. We demonstrated here that caraxin-1 exists as an oligomericized protein with 310–350 kDa in solution (Fig. 3D). We assume that caraxin-1 probably forms either a dimer or trimer between the N- and/or C-terminal heptad-repeat regions, and the resulting oligomers with coiled-coil structure may assemble together to form a higher order oligomer with 310–350 kDa. Taken together, these facts imply that TGase could covalently cross-link the higher order oligomers, resulting in the formation of the mesh structure observed by scanning electron microscopy (Fig. 6, C and D).

Mammalian keratinocyte TGase (TGase-1) and its substrates are both localized in keratinocytes and thus have a physiological function. In contrast, only caraxins, and not the horseshoe crab TGase, are localized in the sub-cuticular epidermis (Fig. 2). The horseshoe crab TGase is predominantly localized in hemocytes. Fig. 9 shows a hypothetical scheme for TGase-dependent cross-linking of caraxins. One of the principal functions of the hemocyte is to seal scars in the exoskeleton. This function is fulfilled in part by the adherence of hemocytes to injured sites and in part by the polymerization of a clottable protein coagulogen secreted by LPS-induced exocytosis (46). The horseshoe crab hemocyte possesses active motility. In experimental wounds in the horseshoe crab *Limulus polyphemus*, a coagulation plug is formed within 10 min, and the coagulum is then infiltrated by hemocytes to form a cellular plug within 24 h (47). The horseshoe crab TGase is secreted from hemocytes in response to stimulation by LPS (22, 23). Therefore, TGase may be secreted sufficiently from the recruited hemocytes at injured sites and immediately activated by Ca²⁺ in hemolymph plasma, leading to the cross-linking of caraxins localized in the sub-cuticular epithelial cells. Eventually, caraxins may serve to provide an effective mesh to fix invading pathogens at injured sites in cooperation with the clotting mesh. The repetitive array of the pentapeptide in caraxins shows the binding affinity to chitin, a major component of arthropod cuticles, suggesting that the mesh plays an essential role in sealing the wound and promoting wound healing and sclerotization at injured sites of the cuticle. The sub-cuticular epithelial cells begin to migrate into the wound after 15 days, and the epithelial cells span the wound between the cut ends of the exoskeleton by day 30, and then probably secrete cuticular components to complete the wound repair process (47). We here showed the TGase-dependent mesh formation of horseshoe crab cuticular chitin-binding proteins, caraxins. In arthropods, protein cross-linking by TGase may be involved in the initial stage of wound healing, as in the case of mammalian skin.

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Characterization of Caraxins

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