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Cuvierian tubules are specialized adhesive defense organs occurring exclusively in some sea cucumber species. Cuvierian tubule adhesion is instantaneous. Our results suggest that the adhesive is in the form of a low molecular weigth precursor protein in the secretory granules of the adhesive cells. Upon release, these proteins instantly polymerize with no enzymatic curing required. The adhesive thus constituted has a composition unique among marine organism bioadhesives and forms relatively strong bonds. The unique characteristics of sea cucumber Cuvierian tubule adhesive could offer novel features or performance characteristics for applications as underwater adhesives.

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GRANT TITLE: Bioadhesion models from marine invertebrates: An integrated study -biomechanical, morphological, biochemical, molecular- of the processes involved in the adhesion of Cuvierian tubules in sea cucumbers (Echinodermata, Holothuroidea)

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OBJECTIVE: Our objectives were (1) the evaluation of the adhesive properties of Cuvierian tubules of various holothuroid species and (2) the characterization of the adhesive proteins in two selected species.

APPROACH: Our research program was divided in two parts. The first part was a comparative study of Cuvierian tubules adhesion in several species of holothuroids, involving adhesion force measurements as well as morphological and biochemical investigations. The second part focused on the characterization of the Cuvierian tubule glue and concerned only two species. This part of the research program, involving the biochemical characterization of the adhesive proteins as well as their molecular cloning and sequencing, was done in collaboration with Dr. J.H. Waite of the University of California at Santa Barbara.

ACCOMPLISHMENTS: Cuvierian tubules are specialized defense organs occurring exclusively in some holothuroid species from the family Holothuriidae. Within the family, these organs differ greatly in terms of their morphology and their mode of functioning. We have estimated the evolutionary path of Cuvierian tubules by the character mapping method and by ultrastructural analyses. A fragment of the mitochondrial genome corresponding to two genes was first sequenced for 20 species of Holothuriidae (3 Actinopyga, 3 Bohadschia, 12 Holothuria, Labidodemas semperianum and Pearsonothuria graeffei) and the relationships between these species were estimated from the molecular data obtained. The methods used to reconstruct those relationships were the neighbour joining, the maximum parsimony and the maximum likelihood. The consensus phylogenetic tree indicates that: (1) the genus Actinopyga is monophyletic and was the first to diverge from the rest of the family, (2) the second diverging group was a clade comprising the 3 Bohadschia, P. graeffei and 4 Holothuria, (3) within this clade the genus Bohadschia is monophyletic, (4) the remaining clade comprises the other species of Holothuria and L. semperianum, (5) the genus Holothuria is paraphyletic. The analysis of the different characteristics of Cuvierian tubules from the viewpoint of this phylogenetic tree strongly suggests that the common ancestor of the Holothuriidae had Cuvierian tubules and that those tubules were ramified, non-adhesive, non-expellable and non-stretchable; that those tubules have evolved to give the non-ramified, adhesive, expellable and stretchable tubules; and that the loss of Cuvieran tubules has occurred several times independently during evolution.

We have then designed a method to measure the adhesion of holothuroid Cuvierian tubules. Tubule adhesive strength was measured in seven species of sea cucumbers belonging to the genera Bohadschia, Holothuria and Pearsonothuria. The tenacities (force per unit area) varied from 30 to 135 kPa, falling within the range reported for marine organisms using non-permanent adhesion. Two species, H. forskali and H.

leucospilota, were selected as model species to study the influence of various factors on Cuvierian tubule adhesive strength. Tubule tenacity varied with substratum, temperature and salinity of the seawater, and time following expulsion. These differences give insight into the molecular mechanisms underlying Cuvierian tubule adhesion. Tenacity differences between substrata of varying surface free energy indicate the importance of polar interactions in adhesion. Variation due to temperature and time after expulsion suggests that an increase of tubule rigidity, presumably under enzymatic control, takes place after tubule elongation and reinforces adhesion by minimizing peeling effects. This was confirmed by measuring tubule breaking strength, stiffness and toughness at various times after expulsion. The results indicate that adhesion in Cuvierian tubules is instantaneous, with no curing required.

A morphological study of the structure of Cuvierian tubules from all the species investigated in the biomechanical approach was performed. The general tissue stratification was similar in every species. However, one species, H. maculosa, stood apart from the others. In this species, the outer adhesive epithelium is very peculiar, differing greatly from the classical organization of Cuvierian tubules.

The adhesive of several species has also been investigated biochemically. In H. forskali, it is composed of about 60% proteins and 40% carbohydrates. The amino acid compositions of the protein fraction of the adhesive in H. forskali, H. leucospilota, B. subrubra and P. graeffei indicates that their adhesives are closely related. All are rich in small side-chain amino acids, especially glycine, and in charged and polar amino acids. Their compositions differ, however, from those of every other marine bioadhesive investigated so far. Once again, H. maculosa differs from the other species. Its adhesive, rich in acidic amino acids, bears no resemblance with those of the Cuvierian tubules from the other species but, on the contrary, shows some relatedness with the non-permanent adhesives from sea stars and limpets. Polyclonal antibodies were raised against the Cuvierian tubule adhesive of H. forskali and used on tubule histological sections from the different species. These antibodies cross-react with the adhesive cell contents of all species possessing typical Cuvierian tubules, but not with the cells of the tubules from H. maculosa.

In view of the results from the comparative study, the biochemical and molecular approaches have focused mostly on two species: H. forskali and H. maculosa. The lack of a biochemical assay for adhesive substances made it necessary to start the characterization of Cuvierian tubule adhesive with a material that is already considerably enriched in these substances. We used the tubule prints, which consist of patches of material left on the substratum after mechanical detachment of the tubule collagenous core. Tubule print proteins were best extracted using buffers containing both chaotropic and reducing agents. However, even in such buffers, less than 10% of the glue print material was solubilized. The Tricine-SDS-PAGE analysis revealed that the extracts contained about 10 different proteins, with apparent molecular masses ranging from 11 to 220 kD. Five of these proteins that were analyzed had similar amino acid compositions. They were all rich in glycine (16-22%) and in acidic residues (19-22%). Close relatedness between the most prominent tubule print proteins was also corroborated by the occurrence in each amino acid analysis of two undetermined peaks, one of which could correspond to phosphoserine. These two peaks together account for 2 to 4% of the total amino acids. Marine bioadhesives are generally made up of a complex blend of proteins but, to our best knowledge, none comprises so many closely related proteins.

A 45 kDa protein, which is one of the most prominent protein in tubule print extracts, has been purified by electroelution from whole tubules. Amino acid analysis of this protein was identical to that of

the 45kDa protein extracted from the tubule print material, indicating that we were dealing with the same protein. N-terminus sequencing was very limited: only the first amino acid can be removed and it does not correspond to any standard. The protein was digested with endoproteinase Glu-C and the peptides generated were separated by HPLC. Several peptides were sequenced, giving a few N-terminal sequences ranging from 2 to 10 amino acids in length. Similarity between these sequences suggests that the protein may contain repeats. Another peptide sequence gave a perfect match with a peptide from β-actin when submitted to databases. Immunoblotting analyses confirmed that our protein samples were contaminated by β-actin which is a 43-kDa protein. Nevertheless, polyclonal antibodies were raised against the purified 45-kDa protein.

In parallel to the biochemical investigations, a cDNA library has been constructed using the mRNAs extracted from the Cuvierian tubules of 10 individuals of H. forskali. For the extraction, both regenerating and non-regenerating tubules have been pooled. The final titer of the library was $2.7x10^8$ pfu/ml. This library has been screened with degenerate oligonucleotide primers based on the peptide sequences from the 45 kDa protein and a 720 bp fragment of cDNA has been amplified and sequenced. The anti-45kDa protein polyclonal antibodies were also used to screen the cDNA library. Using this method, we have isolated a clone containing a 441 bp insert. These two sequences do not match with any known DNA sequence, including the β-actin cDNA sequence. This suggests a novel protein. However, the two sequences do mot match each other either and further analyses are required to get the complete sequence of the 45kDa protein.

As a second enriched source of Cuvierian tubule adhesive proteins, we have isolated the granular cells (adhesive cells) of H. forskali. These cells were enzymatically dissociated from whole tubules and purified by density gradient centrifugation. Transmission electron microscopy demonstrated that granular cells were readily purified by this method. Extraction of the cells with the denaturing buffer used on tubule prints and electrophoretic analyses revealed a very abundant low molecular weight protein (about 10kDa) that was not present in the print material. The amino acid composition of this protein is almost identical to the one of the whole adhesive. The 10kDa protein could be therefore the constitutive monomer of the adhesive. In this hypothesis, most of the proteins extracted from tubule prints would be polymers of the 10kDa protein.

Tubule prints from H. maculosa have been extracted in the same denaturing buffer as those of H. forskali. In this species, the tubule print material appears less insoluble and proteins are more easily extracted. The SDS-PAGE analysis of the extract shows 3 major proteins with apparent molecular weights of 17, 23 and 33 kDa and and five minor proteins with apparent molecular weights of 16, 27, 58, 67 and 92 kDa, respectively. We obtained the amino acid composition of some of these proteins which are especially rich in acidic residues (up to 35%). Based on solubility characteristics, apparent molecular weights and amino acid compositions, the CT proteins of H. maculosa differ from those of H. forskali. These results, together with those from the morphological approach, suggest that Cuvierian tubule adhesion mechanism in this species could be different from that of H. forskali.

CONCLUSIONS: Cuvierian tubule adhesion really is instantaneous adhesion. Our results suggest that the adhesive is in the form of a low molecular weigth precursor protein in the secretory granules of the adhesive cells. Upon release, these proteins instantly polymerize with no enzymatic curing required. The adhesive thus constituted has a composition unique among marine organism bioadhesives and forms relatively strong bonds.

SIGNIFICANCE: The unique characteristics of holothuroid Cuvierian tubule adhesive could offer novel features or performance characteristics for applications as underwater adhesives.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. Flammang P. 2001. Tube feet and Cuvierian tubules: two different adhesive systems from echinoderms. American Zoologist 41: 1444-1445 (abstract).

2. De Moor S., Waite J.H., Jangoux M. & Flammang P. 2001. Adhesive proteins from the Cuvierian tubules of the holothuroid Holothuria forskali. Gulf of Mexico Science 19: 184-185 (abstract).

3. Flammang P., Ribesse J. & Jangoux M. 2002. Biomechanics of adhesion in sea cucumber Cuvierian tubules (Echinodermata, Holothuroidea). Integrative and Comparative Biology 42: 1107-1115.

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6. Flammang P., Leclercq D., Becker P., Kerr A.M., Lanterbecq D. & Eeckhaut I (in press). Estimation of the evolution of the Cuvierian tubules, defence organs in the family Holothuriidae, by the character mapping method and by ultrastructural analyses. In: Heinzeller T. & Nebelsick J. (eds), Echinoderm München, Balkema, Rotterdam (abstract).

7. Flammang P., Santos R. & Haesaerts D. (in press). Echinoderm adhesive secretions: from experimental characterization to biotechnological applications. In: Matranga V. (ed.), Marine Molecular Biotechnology: Echinodermata, Springer, Heidelberg.

8. Flammang P., Leclercq D., Plumet V., Becker P., Kerr A.M., Lanterbecq D. & Eeckhaut I (in preparation). Evolution of Cuvierian tubules, peculiar defense organs, within a family of sea cucumbers.

9. Flammang P., De Moor S., Becker P., Waite J.H. & Jangoux M. (in preparation). The atypical Cuvierian tubules of Holothuria maculosa: An integrated study.

10. Flammang P., Van Dyck S. & Jangoux M. (in preparation). Mechanical design of the Cuvierian tubules in Holothuria forskali.