THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 27, Issue of July 7, pp. 20667–20675, 2000

Printed in U.S.A.

Mucins and Molluscan Calcification

MOLECULAR CHARACTERIZATION OF MUCOPERLIN, A NOVEL MUCIN-LIKE PROTEIN FROM THE NACREOUS SHELL LAYER OF THE FAN MUSSEL *PINNA NOBILIS* (*BIVALVIA*, *PTERIOMORPHIA*)*

Received for publication, April 10, 2000
Published, JBC Papers in Press, April 17, 2000, DOI 10.1074/jbc.M003006200

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A cDNA expression library constructed from mantle tissue mRNA of the Mediterranean fan mussel *Pinna nobilis* was screened with antibodies raised against the acetic acid-soluble shell matrix of the same species. This resulted in the isolation of a 2138-base pair cDNA, containing 13 tandem repeats of 93 base pairs. The deduced protein has a molecular mass of 66.7 kDa and a isoelectric point of 4.8. This protein, which is enriched in serine and proline residues, was overexpressed, purified, and used for producing polyclonal antibodies. Immunological *in situ* and *in vitro* tests showed that the protein is localized in the nacreous aragonitic layer of *P. nobilis*, but not in the calcitic prisms. Because this protein of the nacre of *P. nobilis* exhibits some mucin-like characteristics, we propose the name mucoperlin. This is the first paper reporting the cloning of a molluscan mucin and the first molecular evidence for the involvement of a mucin in molluscan calcification. This finding corroborates our previous hypothesis that some of the proteinaceous constituents of the molluscan shell matrix would derive from mucins, common to many metazoan lineages of the late Precambrian (Marin, F., Smith, M., Isa, Y., Muyzer, G. and Westbroek, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1554–1559). The adaptation of an ancestral mucin to a new function, the regulation of the mineralization process, may be one of the molecular events, among others, that would explain the simultaneous emergence of organized calcification in many metazoan lineages during the Cambrian explosion.

The skeletons produced by molluscs to protect and support their soft bodies are organo-mineral amalgamates (1, 2) exhibiting a high degree of order at the nanoscale (3). As a rule, the mineralized structure has mechanical properties far superior to each of its constituent components. A well known example is mother-of-pearl or nacre. Its micron-sized compact brick wall texture is more than 1000 times tougher than the chemically precipitated counterpart, aragonite (4).

A complex biochemical machinery is required for the production of these highly ordered biominerals. Specialized microenvironments must be created to accommodate the growing minerals (1, 5). The constituent ions as well as macromolecules capable of directing and fine-tuning the crystallization must be supplied, while waste products of crystallization have to be removed and deleterious precipitations avoided. The actual biomineralization process follows as a remarkable case of self-organization, with the densely packed organic-inorganic composite emerging as the final product. Furthermore, the organisms must deploy upstream the calcification, signal transmitters (6, 7), and transcription factors (8, 9) able to switch their biomineralizing machineries on and off.

Despite its complexity, it would appear as though the skeleton-forming machinery evolved with remarkable facility. The skeletons of many animal phyla were installed in less than 30 million years, since the beginning of the Cambrian, 544 million years ago (10, 11). Phylogenetic evidence derived from fossils and morphology and DNA-sequencing of extant animals indicate that major steps in animal diversification had already been made prior to the "Cambrian explosion" (12–16). To explain how a function as complex as skeleton formation could emerge in many independent stocks almost simultaneously, we suggested that components of the mineralizing machinery were already available in the soft bodied ancestors but that they served other functions than calcification. Subsequently, during the Cambrian explosion, they only had to be co-opted for organized biomineralization (17, 18). One indication supporting this "preadaptation" hypothesis came from the observations by Lopez and collaborators that molluscan nacre can induce bone production in human osteoblasts (18–20). These experiments suggest a common Precambrian ancestry for the responsible signaling system in both taxa.

Another point to mention was our finding that secreted muci and soluble shell matrix strongly cross-reacted with antisera against these two antigens in various molluscs as well as in the coral *Galaxea fascicularis* (17). Both the freshly extracted muci, which were secreted by noncalcifying epithelia into the ambient seawater, and the matrix fractions were shown to inhibit the *in vitro* crystallization of calcium carbonate. We explained these observations by assuming that the "anticalcifying" mucous secretions protected the soft bodied ancestors from spontaneous incrustrations in the highly supersaturated Precambrian ocean waters (17). At the onset of calcification, the same inhibitory molecules could then be incorporated into the calcifying machinery to fine-tune the crystallization process. Clearly, this "anticalcification hypothesis" calls for a more

* This work was supported in part by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, Den Haag). This work was also supported by the "Fondation des Treilles" (Paris), the "Société de Secours des Amis des Sciences" (Paris), the "Fondation Singer-Polignac" (Paris) during the period 1996–1999, and the Fondation Simone et Cino Del Duca (Paris) for the year 2000. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF 145215.

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detailed study of the macromolecular fractions in the mucous secretions and the mineralized matrix.

In this paper, we report the isolation of a new gene encoding a water-soluble protein of the nacroprismatic shell of the protected mollusc *Pinna nobilis*, the largest bivalve in the Mediterranean. The protein that we identified is acidic and enriched in serine and proline residues. It is further characterized by the presence of 13 tandem repeats with 31 amino acid residues and a C terminus containing three unique cysteine residues. The presence of tandem repeats, the enrichment in serine residues, and its consequent potential of high glycosylation strongly suggest that this protein is a mucin. The protein is localized in the nacre but not in the prisms of *P. nobilis.* We chose to name it mucoperlin, and we believe that its skeletal association is important evidence in support of our anticalcification hypothesis.

MATERIALS AND METHODS

Tissue and Shell Collection—Mantle tissue fragments from actively calcifying, juvenile *P. nobilis*, grown in aquaria, were obtained. Tissue collection was performed according to the European directive 92-43-CEE concerning protected species (91). This operation did not affect the viability and filtration capacity of the animal. The isolated tissue (about 0.5 g) was immediately frozen in liquid nitrogen.

Shell material was collected from two different batches of juvenile specimens at CERAM (Centres d'Etudes des Ressources Animales Marines). The two layers of the shells (*i.e.* outer prismatic calcitic layer and inner aragonitic mother-of-pearl) were separated by abrasion and by dissociation of the calcitic prisms in dilute sodium hypochlorite.

Construction of the cDNA Expression Library—Total RNA was extracted from 0.5 g of mantle tissue using the RNAgents® Total RNA Isolation System (Promega). The extraction yielded about 70 μg of RNA. Poly(A)-mRNA was subsequently isolated with Oligotex™ Resin (Qiagen). Approximately 1.5 μg mRNA was obtained and immediately processed using the ZAP™ Express cDNA synthesis kit (Stratagene), eventually yielding double-stranded *Xho*I–*Eco*RI cDNA fragments. Approximately 70 ng of cDNA was ligated to 1 μg of *Xho*I/*Eco*RI-predigested dephosphorylated ZAP Express vector. Subsequent packaging of recombinant phages was performed with Gigapack® II Gold Packaging Extract (Stratagene). The resulting phage library consisted of 350,000 clones, of which at least 95% were found to be recombinant. Prior to the antibody screening, the library was amplified.

Antibody Screening of the cDNA Expression Library—The amplified library was screened with antibodies raised against acetic acid-soluble extracts of the nacreous layer of *P. nobilis* (21). About 500,000 phages were used in the initial screening. After infection of *Escherichia coli* XL1-Blue MRF′, cells were plated and grown for 4 h at 42 °C. The plates were covered with isopropyl-1-thio-β-D-galactopyranoside-saturated nitrocellulose membranes (BA85; Schleicher & Schuell) and incubated at 37 °C for an additional 5 h. Membranes were first blocked in 1% gelatin/Tris-buffered saline/Tween solution and then incubated in blocking solution containing the antiserum (diluted 1:3000), NaN3 (500 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml). An *E. coli* extract (1 mg/ml) was added to decrease background staining. The membranes were then incubated with the conjugated antibody GAR/AP (Sigma) and subsequently with the chemoluminescent substrate CSPD® (Roche Molecular Biochemicals) before being exposed to X-Omat films (Eastman Kodak Co.). Eleven positive clones were identified, and three of them were rescreened to purity. In a two-step *in vivo* excision, the pBK-CMV phagemids (Stratagene) with their cDNA inserts were excised from the purified ZAP Express phage clones and converted into the plasmids pPNC1–pPNC3.

Nucleotide Sequence Analysis—Enzyme restriction analysis of plasmids pPNC1–3 showed similar *Pst*I and *Xho*I restriction patterns consisting of a 2100-base pair (bp)[1] *Pst*I and a <100-bp *Pst*I–*Xho*I fragment. Partial nucleotide sequences determined from the 5′- and 3′-termini further confirmed that the three clones contained identical inserts. The sequence of the cDNA insert of pPCN1 was determined using 5′-deletions made with the Erase-a-Base® System (Promega). Sequences were compiled and analyzed with the Wisconsin Package (version 9.0) of the Genetics Computer Group. The GenBank™ accession number for the sequence of the cDNA insert of pPNC1 is AF 145215.

Polyclonal Antibodies against the Recombinant Mucoperlin—The cDNA insert of pPNC1 was cloned as a *Bam*HI–*Kpn*I fragment in vector pQE-32 (QIAexpress® Expression System; Qiagen). In the resulting construct, pPNC4, the putative start codon on the cDNA insert is cloned in frame with the start codon of the T5 promoter transcription-translation system of pQE-32. Cloning in ZAP Express (pBK-CMV) and the above described subcloning in pQE-32 resulted in the addition of an extra fragment of 33 bp to the 5′-end of the original cDNA. This fragment encoded a 11-amino acid peptide (MRGSHHHHHHG) with a molecular mass of 1.5 kDa. The His-tagged recombinant protein was expressed from pPNC4 in *E. coli* M15 and purified using Ni^{2+}-nitrilotriacetic acid chromatography (Qiagen). A complete purification of the recombinant protein was achieved by using a preparative electrophoresis (Bio-Rad; 491 Prep Cell, 7% acrylamide). On denaturing gels (SDS-polyacrylamide gel electrophoresis), the fusion protein migrated as an 80-kDa band, in good agreement with the molecular weight as deduced from the sequence (Fig. 1). Recombinant mucoperlin was desalted by ultrafiltration (Amicon, YM10 membrane), lyophilized, and used to generate polyclonal antibodies in a rabbit.

Mucoperlin in Extracts of Nacre and Prisms—To investigate whether native mucoperlin is present in either the nacreous or in the prismatic layer of *P. nobilis* shell or in both, ~100-mg portions of both cleaned shell materials (batch 1) were powdered and dissolved overnight in 6 ml of 20% (w/v) EDTA, pH 8.0. Insoluble material was removed by centrifugation (5 min, 13,000 rpm). Demineralization with EDTA instead of acetic acid permitted direct analysis of the supernatant by ELISA and dot-blot (22), using anti-mucoperlin.

To study the intimacy of association between the native mucoperlin and the mineral phase (23) and possible contamination of shell matrix by soft tissues (24), nacre powder was submitted to a pretreatment with sodium hypochlorite (2 g of active chlorine/liter) for 70 h, washed with water, dried, and dissolved in EDTA. The resulting extract was compared with EDTA-extracts of nonpretreated material in ELISA assays.

For electrophoretic analysis, 3 g of hypochlorite-treated powders from juvenile nacre and prisms was decalcified overnight at 4 °C with 5% (v/v) acetic acid, pH 4. Acid decalcification precluded the formation of aggregates during the subsequent purification steps, a phenomenon often observed when EDTA is used (25). The solutions were centrifuged, and the supernatants were desalted by ultrafiltration (Amicon, YM10) and lyophilized. The pellets were thoroughly rinsed with water and lyophilized. The lyophilized preparations were denatured in Laemmli sample buffer for 5 min at 100 °C (26). After a short centrifugation, the denatured samples were applied on 12% acrylamide gels. Gels were either stained with silver (27) or electroblotted on Immobilon™-P (Millipore Corp.) (28). Immunodetection of blotted proteins with anti-mucoperlin was performed using the chemoluminescent substrate Luminol (Sigma catalog no. A4685) (29).

Assays of Nacre Matrix Glycosylation—The second batch of shell material was used to determine the degree of glycosylation of nacre matrix. Because the amount of this batch was limited, only a few assays could be performed. Part of the acetic acid-soluble extract of hypochlorite-treated nacre was run on a 12% SDS-polyacrylamide gel and subsequently blotted as described above. The blot was stained with the Immun-Blot kit for glycoproteins (Bio-Rad catalog no. 170-6490). The procedure involves periodate oxidation, labeling with biotin, incubation with streptavidin-AP, and staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. In one sample of nacre matrix, the periodate oxidation was omitted to ascertain the specificity of the staining (negative control). Fetuin was used as a positive control.

The other part of the acid-soluble matrix was submitted to chemical deglycosylation with TFMS (Sigma catalog no. T 1394) for 5 h, as described by Edge *et al.* (30). To prevent peptide bond hydrolysis, all procedures were performed at 0 °C under N_2. The deglycosylated matrix was desalted and concentrated by ultrafiltration and lyophilized. The matrix was then analyzed on Western blots with anti-mucoperlin as described above. Fetuin was deglycosylated by the same method to check the efficiency of the procedure.

Interaction with Calcium and Inhibition of Calcium Carbonate Precipitation—Acetic acid-soluble nacre matrix extracted from the second batch was also tested for ^{45}Ca binding capacity, according to a standard protocol (31). The assays were performed after dot-blotting the whole matrix on polyvinylidene difluoride membranes at various concentrations as indicated in the legend of Fig. 7. GPA, a calcium-binding protein from a unicellular calcifying alga (32), was used as a positive control. Furthermore, the capacity of the nacre matrix to inhibit calcium carbonate precipitation *in vitro* was also tested (33).

[1] The abbreviations used are: bp, base pair(s); ELISA, enzyme-linked immunosorbent assay; MCP, mucoperlin; rec-MCP, recombinant mucoperlin; TFMS, trifluoromethanesulfonic acid.

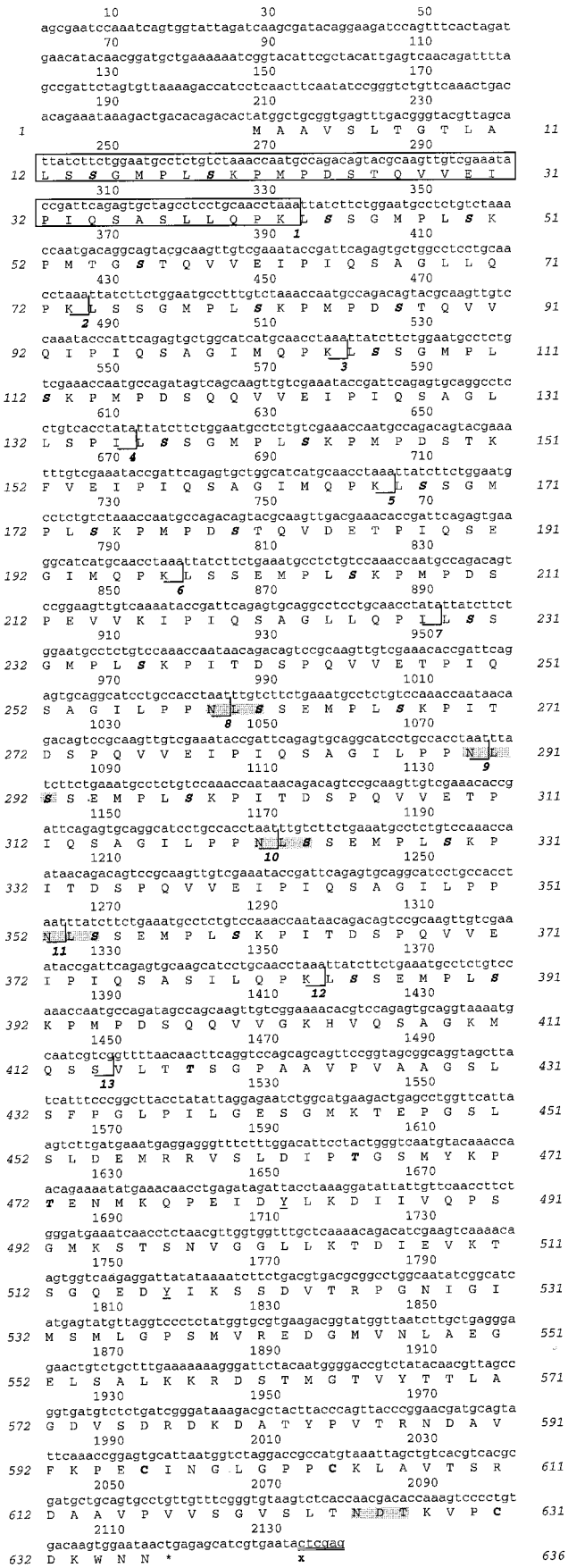

FIG. 1. **Nucleotide sequence of *mcp*.** The amino acid sequence of mucoperlin is shown *under* the nucleotide sequence in one-letter symbols. Mucoperlin contains 13 tandem repeats; the first repeat is *boxed*, and the last amino acid residue of each repeat is indicated and numbered 1–13. The three cysteine residues in the C terminus are indicated in *bold*, and the two potentially sulfated tyrosine residues are *underlined*. The *Xho*I restriction site at the 3′-end is *double underlined* and indicated with *x*. Serine and threonine residues representing possible *O*-glycosylation sites (see Ref. 35) are indicated in *boldface italic type*. *N*-Glycosylation motifs are indicated by *gray rectangles*.

In Situ Immunohistological Localization of Native Mucoperlin—Juvenile and adult *P. nobilis* cleaned shell fragments were polished with micropolish powder (0.05 μm), thoroughly rinsed with water, and slightly etched with EDTA 1% (w/v) during 15 min, to expose antigenic determinants. The material was then immunostained as described previously (17, 34) and viewed by microscope under incident light.

RESULTS

Isolation and Characterization of mcp—Clone PNC1 was isolated from a *P. nobilis* cDNA library using antibodies against the soluble matrix of *Pinna* nacre. Analysis of its insert gave a nucleotide sequence of 2138 bp (Fig. 1). We assume the ATG at positions 208–210 to be the start codon of an open reading frame of 1908 bp, encoding a gene referred to as *mcp*. The cDNA contains a 5′-untranslated region of 208 bp and a truncated 3′-untranslated region of only 23 bp. A poly(A) tail is missing at the 3′-end. This is probably due to an internal *Xho*I restriction recognition site in the original mRNA.

Characterization of the mcp-encoded Protein—The *mcp*-encoded protein mucoperlin (Fig. 1) encompasses 636 amino acid residues and has a calculated molecular mass of 66.7 kDa and a pI of 4.8 (Table I). The two most abundant amino acids are proline and serine (Table I). Mucoperlin comprises three distinct regions (schematically presented in Fig. 2): a short N-terminal part (11 amino acids), a tandem repeat region (403 amino acids), and a C-terminal part (222 amino acids). The tandem-repeat region consists of 13 repeats (Fig. 2). They all contain 31 amino acids, 12 of which are invariant through the repeats. Only few substitutions occur, mostly in the second half of each repeat.

The primary structure of mucoperlin (Figs. 1 and 2) indicates that the natural molecule may be heavily glycosylated, particularly in the tandem repeat region. Four possible *N*-glycosylation sites, characterized by N*X*(S/T) (where *X* may be any amino acid except proline), occur in this region. The tandem repeat region also exhibits many potential *O*-glycosylation sites, as suggested by its high serine and threonine content. A search performed with the NetOGlyc 2.0 program (35) predicts that 27 serines are glycosylated in that region (*i.e.* one to three serines per repeat) (Figs. 1 and 2). Prosite analysis and a search made with NetPhos 2.0 (36) suggested that some of the serine and threonine residues may be phosphorylated, a feature commonly found with proteins associated with mineralized tissues (1). A high content of proline (17% of the residues) is another characteristic feature of the tandem repeat region, suggesting that this part of the molecule has a rigid, rodlike conformation (37, 38).

The C-terminal region of mucoperlin has different characteristics than the repeat region. Glycosylation appears to be less densely distributed (Fig. 1), potentially with only one *N*-glycosylation site at position 625 and three *O*-glycosylation sites through threonine residues at positions 418, 465, and 472. On the other hand, phosphorylation sites may be more densely distributed, according to both Prosite and NetPhos 2.0 analysis. Proline and serine are less common (8 and 10%, respectively, of the C-terminal amino acids), although their abundance is relatively high as compared with values of 5.1 and 6.9% for proteins in general (39). Furthermore, the only three cysteine residues in mucoperlin occur in the C-terminal region, at positions 596, 604, and 631, respectively. Finally, the C-

TABLE I

Amino acid composition of mcp-deduced mucoperlin

n, number of residues. Data shown in parentheses represent the average occurrence of each amino acid, as determined for a large number of proteins (see Ref. 39) (636 total amino acid residues; pI = 4.87; theoretical mass = 66.7 kDa).

Amino acid	*n*	Mole	%	Amino acid	*n*	Mole	%
Ala	27	4.25	(8.3)	Met	36	5.66	(2.4)
Cys	3	0.47	(1.7)	Asn	13	2.04	(4.4)
Asp	30	4.72	(5.3)	Pro	84	13.21	(5.1)
Glu	29	4.56	(6.2)	Gln	38	5.98	(4.0)
Phe	3	0.47	(3.9)	Arg	8	1.26	(5.7)
Gly	42	6.60	(7.2)	Ser	93	14.62	(6.9)
His	1	0.16	(2.2)	Thr	34	5.35	(5.8)
Ile	46	7.23	(5.2)	Val	46	7.23	(6.6)
Lys	38	6.00	(5.7)	Trp	1	0.16	(1.3)
Leu	59	9.28	(9.0)	Tyr	5	0.79	(3.2)

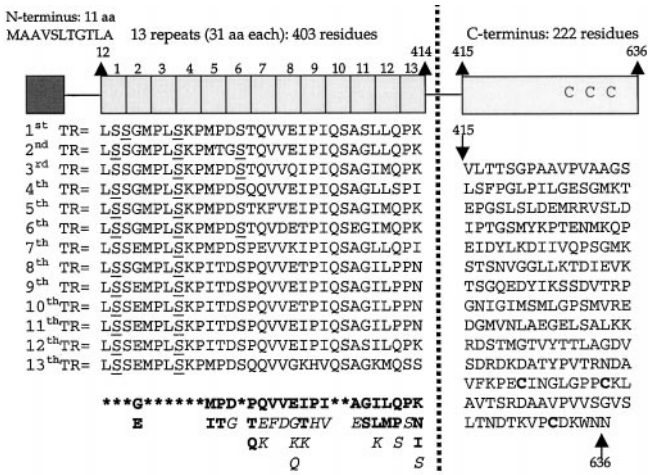

FIG. 2. **Schematic representation of mucoperlin.** The protein comprises a short 11-amino acid N terminus, a tandem repeat region (residues 12–414), and a C terminus (residues 415–636) containing 3 cysteine residues (in *boldface type*). The 13 tandem repeats (31 amino acids each) are aligned. *TR*, tandem repeat. *, the 12 amino acid residues that are invariant throughout the repeats. Five of them are serine residues. The potential sites for *O*-glycosylation are *underlined*. In the second position, 10 serines out of 13 would be glycosylated and all of the serine residues in the eighth as well. *Boldface letters* indicate amino acids that occur at least twice in a given position, throughout the repeats. *Italics* represent amino acids that appear only once.

terminal region contains also the five tyrosine residues of the protein, two of them (at positions 482 and 517) showing all of the requirements for tyrosine sulfation (40).

BLAST searches (41) in protein and DNA-translated data bases showed limited homology of mucoperlin with any other known protein. The highest homology was found with PGM, an incompletely characterized pig gastric mucin (42) that exhibits serine-rich tandem repeats of 16 amino acid residues (Fig. 3*A*). The C terminus of mucoperlin did not exhibit any homology with a mucin. A striking homology was observed between a set of 24 residues (positions 425–448) and the N terminus of a chicken homeobox protein Hox-A9 (Ref. 43; Fig. 3*B*).

Mucoperlin in Extracts of Nacre and Prisms of P. nobilis—The results of immunodetection of mucoperlin in EDTA extracts of nacreous and prismatic shell material (batch 1) of *P. nobilis* are shown in Fig. 4, *A* and *B*. The reactions were performed using ELISA and dot-blotting with the antiserum directed against His-tagged recombinant mucoperlin. Mucoperlin was only detected in the nacre and not in the prisms. ELISA analysis on hypochlorite-treated nacre did not affect the reactivity of the antibody with the extract (Fig. 4*C*). We conclude that the immunological signals did not originate from soft tissue contamination. Furthermore, this result suggests that mucoperlin is either strongly adsorbed on the mineral surface or that it occupies an "intracrystalline" position (23).

The nacre specificity of anti-mucoperlin as determined with ELISA and dot-blot analysis was verified with SDS-polyacrylamide gel electrophoresis and Western blotting on acetic acid-soluble extracts from juvenile aragonitic nacre and calcitic prisms (Fig. 5). Laemmli-soluble extracts of the two acetic acid-insoluble fractions were also included in the analysis. Silver staining of the gels showed the presence of numerous discrete thin bands in a background smear (Fig. 5*A*). All extracts showed distinctively different patterns. Western blot analysis proved that mucoperlin was only present in the nacre matrices (Fig. 5*B*). Neither the acetic acid- nor the Laemmli-soluble fractions of the prisms showed any reaction with anti-mucoperlin.

In the acetic acid-soluble fraction of the nacre, a prominent anti-mucoperlin-positive band with an apparent molecular mass around 55 kDa was detected. We assumed this band to represent native mucoperlin. The diffuse smear below this band may indicate degradation or differences in glycosylation of the native protein. The 55-kDa protein is also visible as a major discrete band on silver-stained gels (Fig. 5*A*). In Laemmli extracts of the insoluble nacre matrix, two closely apposed anti-mucoperlin positive bands are visible with apparent molecular masses around 30 kDa. This indicates that mucoperlin-related proteins remain associated with the insoluble matrix after acid extraction of the nacre. These positive bands may represent breakdown products of mucoperlin, generated by the combined hypochlorite and Laemmli treatments, or different proteins closely resembling mucoperlin.

The apparent molecular mass of native mucoperlin (55 kDa, Fig. 5*A*) is smaller than of the *mcp* deduced protein (66.7 kDa). Heavily glycosylated proteins, like mucins, are known to behave anomalously upon electrophoresis (44); their mobility is influenced by their intrinsic negative charge but also by the fact that they bind very little SDS (45). It is also likely that mucoperlin undergoes specific cleavage when incorporated into the nacre or undergoes degradation due to aging of the nacre layer (see below).

Deglycosylation Assays—The amino acid sequence of mucoperlin suggests that the native molecule is heavily glycosylated. In general, molluscan shell matrices have been shown to contain an important sugar moiety (23, 46–48). This was confirmed by a specific glycoprotein staining of the *Pinna* nacre matrix. Strong staining was obtained with many components of the matrix, with the exception of low molecular weight fractions (<20 kDa), which stained negatively (Fig. 6*A*). We assume that at least part of nondegraded native mucoperlin is represented by the stained band migrating slightly faster than the 58.1-kDa marker (Fig. 6*A*, *lanes 5* and *6*). After deglycosylation, no stained bands were visible.

Staining with anti-mucoperlin did not reveal such a discrete anti-mucoperlin-positive band as obtained with the first batch of shell (Fig. 5). Instead, a positive smear from 55 to 30 kDa,

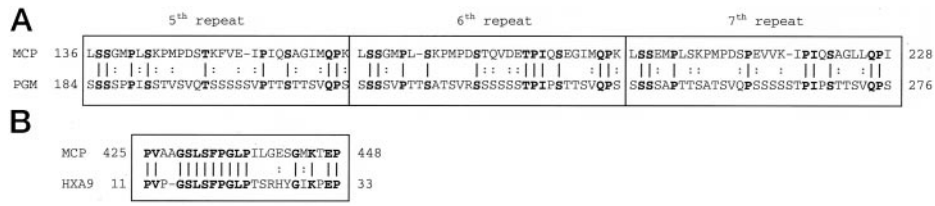

FIG. 3. *A*, alignment of repeats 5–7 of MCP with those of PGM (accession number trEMBL Q29071), a pig gastric mucin. A gap was introduced in each repeat of MCP to fit with the length of the doubled repeats of PGM. Identical ([vert]) and similar (:) amino acid residues are indicated. *B*, a short portion of the C-teminus of MCP is aligned with the N terminus of chicken HoxA9 protein (accession number Swiss-Prot Q98924).

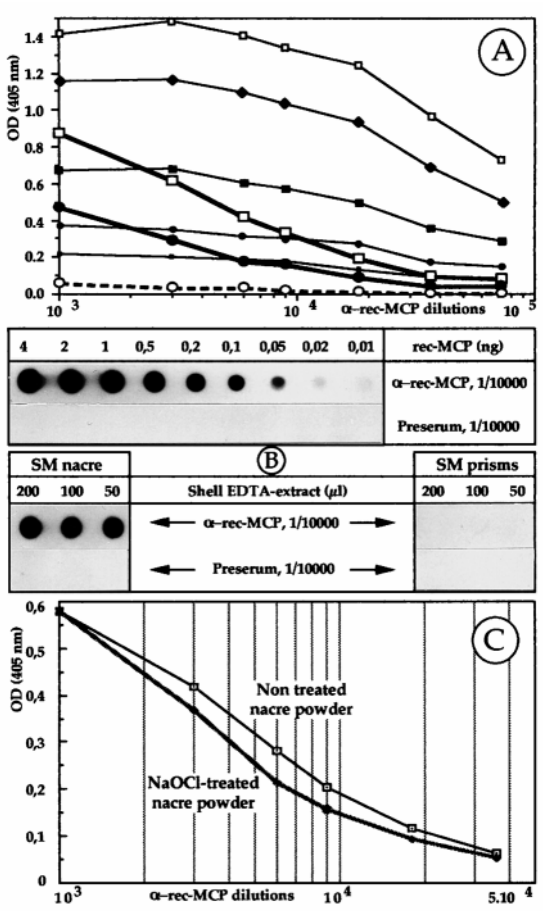

FIG. 4. **Detection of native mucoperlin in different shell extracts.** The anti-recombinant mucoperlin antibody was tested against EDTA-soluble shell matrices of calcitic prisms and aragonitic nacre of *P. nobilis*, via ELISA (*A*) and dot-blot (*B*). For more information, see "Materials and Methods." As a positive control, this antibody was also tested against its target antigen, the recombinant mucoperlin (*rec-MCP*). *A*, □, 1 ng of rec-MCP; ▲, 0.5 ng of rec-MCP; ■, 0.25 ng of rec-MCP; ●, 0.1 ng of rec-MCP; ■, 0.05 ng of rec-MCP; □, SM nacre; ●, SM nacre, diluted 10 times; ○, SM prisms. *C*, the reactivity of the EDTA extract with the anti-rec-MCP antibody was tested before (*black curve*) and after (*gray curve*) a pretreatment of nacre powder with dilute sodium hypoclorite (2 g of active chlorine/liter for 70 h). This pretreatment did not significantly affect the immunological reactivity.

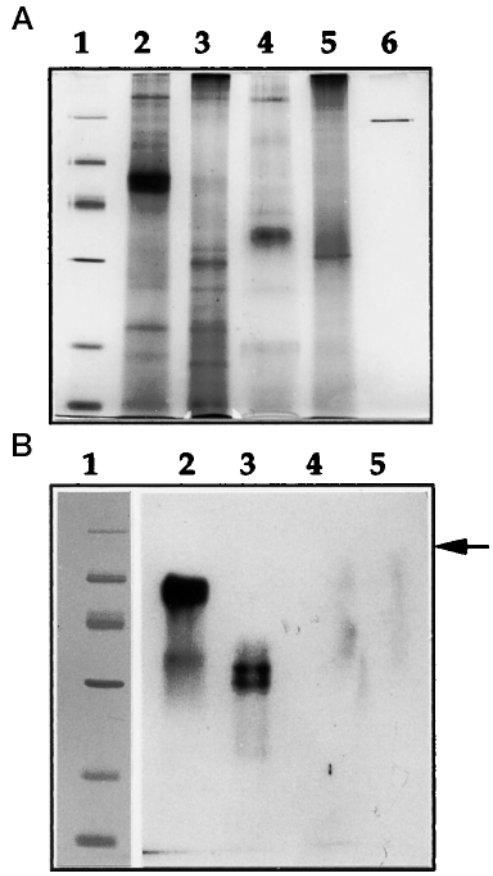

FIG. 5. **Characterization of mucoperlin in CH_3COOH extracts of *P. nobilis* shell on 12% SDS-polyacrylamide gels (*A*) and Western blots (*B*).** The gel was stained with silver; the blot was revealed with the chemoluminescent substrate Luminol. In each lane, equivalent amounts of matrices were applied (about 10 µg). *Lane 1*, molecular mass standards (from *top* to *bottom*): 94, 67, 43, 30, 20.1, and 14.4 kDa; *lane 2*, CH_3COOH-soluble matrix of the nacre; *lane 3*, Laemmli-soluble fraction of the CH_3COOH-insoluble matrix of the nacre; *lane 4*, CH_3COOH-soluble matrix of the calcitic prisms; *lane 5*, Laemmli-soluble fraction of the CH_3COOH-insoluble matrix of the prisms; *lane 6*, recombinant mucoperlin. *B*, the *arrow* on the *right side* of the *blot* indicates the position of the recombinant mucoperlin. *A*, *lane 4* exhibits a strong negative staining with silver. In CH_3COOH-soluble extracts of the nacre, the mucoperlin migrates as a thick band with an apparent molecular mobility between that of bovine serum albumin (67 kDa) and of ovalbumin (43 kDa).

with a maximum between 40 and 30 kDa, was observed (Fig. 6*B*). Apparently, the mucoperlin in this shell batch had undergone more degradation than in the first batch. But clearly, the positive signal shifted to lower molecular weights after deglycosylation (Fig. 6*B*, *lane 2*), indicating removal of sugar residues.

Calcium Binding Assay and Inhibition of $CaCO_3$ Precipitation—The specific association of mucoperlin with the nacre layer suggests that the molecule is functional in the calcification process. This might imply that mucoperlin is able to interact with Ca^{2+} and/or to interfere with $CaCO_3$ precipitation. When tested in the $CaCO_3$ precipitation assay as described by Wheeler *et al.* (33), the recombinant mucoperlin did not delay the precipitation of $CaCO_3$. It did not appear to bind Ca^{2+} either when submitted to the Ca^{2+} binding protocol on Western blot as described by Maruyama and co-workers (31). We assumed that potential interactions of the native mucoperlin with Ca^{2+} would be conveyed by its sugar moieties, as is the case for many molluscan shell proteins (47, 48). The combined acetic acid-soluble nacre matrix was able to bind Ca^{2+} when tested on dot-blots (Fig. 7*A*) and strongly inhibited $CaCO_3$

crystallization (Fig. 7*B*). However, individual Ca^{2+} binding components could not be demonstrated on Western blots with the protocol of Maruyama *et al.* (not shown), probably as a result of the denaturing conditions during electrophoresis. This was confirmed by the inhibition of Ca^{2+} binding by SDS and β-mercaptoethanol in the dot-blot assay (Fig. 7*A*). Consequently, we could not determine from these experiments whether native mucoperlin is one of the nacre components that interact with Ca^{2+}.

In Situ Localization of Mucoperlin—The outer, prismatic layer of the *P. nobilis* shell is made of long, thin prisms of calcite with a length of 1–3 mm and a diameter between 50 and 100 μm. Each prism is composed of a pile of thin crystallites, all with identical optical axes (49). The internal, nacreous layer consists of flat aragonite tablets, approximately 0.5–1 μm thick. The tablets are stacked in a characteristic mortar-and-brick structure (3, 50). The transition between the two layers is abrupt, with sometimes an organic sheet (50-μm thickness) in between. The *in situ* experiments on thin transversal EDTA-etched adult shell sections (Fig. 8) confirm the nacre location of mucoperlin. The nacre layer is stained purple as a result of a positive reaction with anti-mucoperlin, whereas the prismatic layer does not stain at all (Fig. 8*B*). Mucoperlin might also be localized in the heavily pigmented organic layer between the two microstructures. However, none of our assays permitted us to visualize the presence of mucoperlin in that layer. The staining pattern of the nacre layer on transverse sections is not uniform. At low magnification, it reveals short diffuse purple lines more or less perpendicular to the growth plane (Fig. 8*B*). At higher magnification, the lower and upper surface of each nacre tablet are finely underlined by the staining, while predominant staining is observed on the short sides of each tablet (Fig. 8*C*). In any case, mucoperlin was seen to form a continuous sheet around each crystallite of the nacre. Furthermore, sections parallel to the growth plane of juvenile nacre show that mucoperlin is concentrated along the outlines of the tablet (Fig. 8, *D* and *E*).

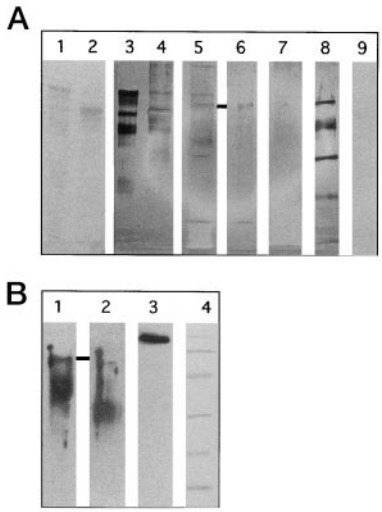

FIG. 6. **Glycosylation study of mucoperlin on Western blots, using the glycoprotein detection kit from Bio-Rad (*A, lanes 3–9*) and the anti-recombinant mucoperlin on acetic acid-soluble nacre extracts (*B, lanes 1–3*), before and after chemical deglycosylation with TFMS.** *Gel A*, *lane 1*, glycosylated fetuin, Coomassie Brilliant Blue-stained; *lane 2*, TFMS-deglycosylated fetuin, Coomassie Brilliant Blue-stained; *lane 3*, glycosylated fetuin; *lane 4*, TFMS-deglycosylated fetuin; *lane 5*, nondeglycosylated acetic acid-soluble extract from nacre, batch 2; *lane 6*, nondeglycosylated acetic acid-soluble extract from nacre, batch 1; *lane 7*, TFMS-deglycosylated acetic acid-soluble extract from nacre, batch 2; *lane 8*, biotinylated markers, respectively 58.1, 39.8, 29, 20.1, and 14.3 kDa; *lane 9*, same as *lane 5*, without treatment with periodate (negative control). The position of mucoperlin is indicated by a *bar*, between *lanes 5* and *6*. *Gel B*, *lane 1*, nondeglycosylated acetic acid-soluble extract from nacre, batch 2; *lane 2*, TFMS-deglycosylated acetic acid-soluble extract from nacre, batch 2; *lane 3*, recombinant mucoperlin; *lane 4*, molecular mass markers, stained with Coomassie Brilliant Blue. The position of mucoperlin is indicated by a *bar* between *lanes 1* and *2*.

DISCUSSION

Calcified skeletons are formed by the intricate interplay between inorganic ions and a variety of organic macromolecules, collectively called the skeletal organic matrix (1, 2, 5). In a previous paper (17), we proposed that some components of the soluble skeletal matrix of corals and molluscs were recruited from mucous secretions of the Precambrian uncalcified ancestors. Originally, these materials would have been involved in the protection of soft tissues against mineral encrustment in the highly supersaturated oceans of the late Precambrian (51). This anticalcification hypothesis was based on immunological cross-reactivities between water-soluble skeletal matrices and mucous secretions as well as on the ability of both materials to inhibit calcium carbonate crystallization *in vitro*. A drawback of this earlier work was the lack of biochemical information on the secreted macromolecules. This paper supports our hypothesis by providing convincing structural evidence that at least some skeletal matrix components evolved from mucous secretions.

We isolated a novel gene (*mcp*) from an expression library of the bivalve *P. nobilis* with antibodies raised against the acetic acid soluble skeletal matrix from *Pinna* nacre. The encoded protein mucoperlin shows homology with mucins, a broad family of proteins (37, 38, 45) and important constituents of metazoan mucous secretions.

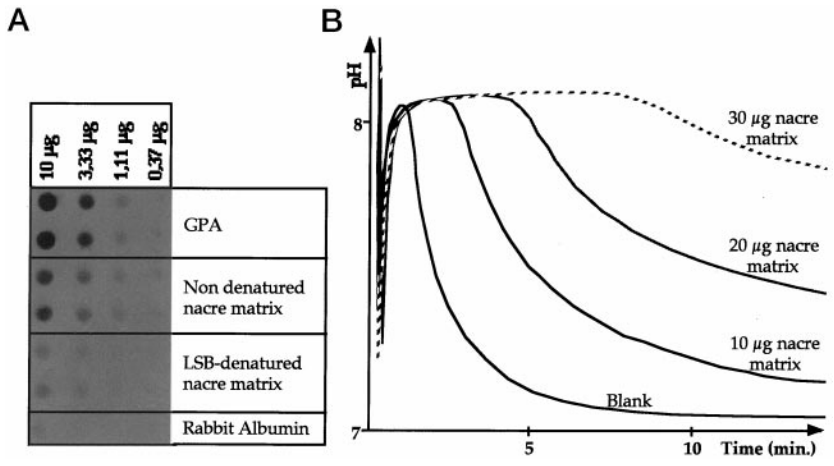

FIG. 7. **Calcium binding (7A) and *in vitro* inhibition of calcium carbonate precipitation (7B) assays.** *A*, the acetic acid-soluble matrix of nacre-bound calcium only in nondenaturing conditions. In presence of Laemmli sample buffer, this effect was drastically reduced. GPA and rabbit albumin are positive and negative controls, respectively. In *B*, the same matrix exhibited a strong inhibiting effect.

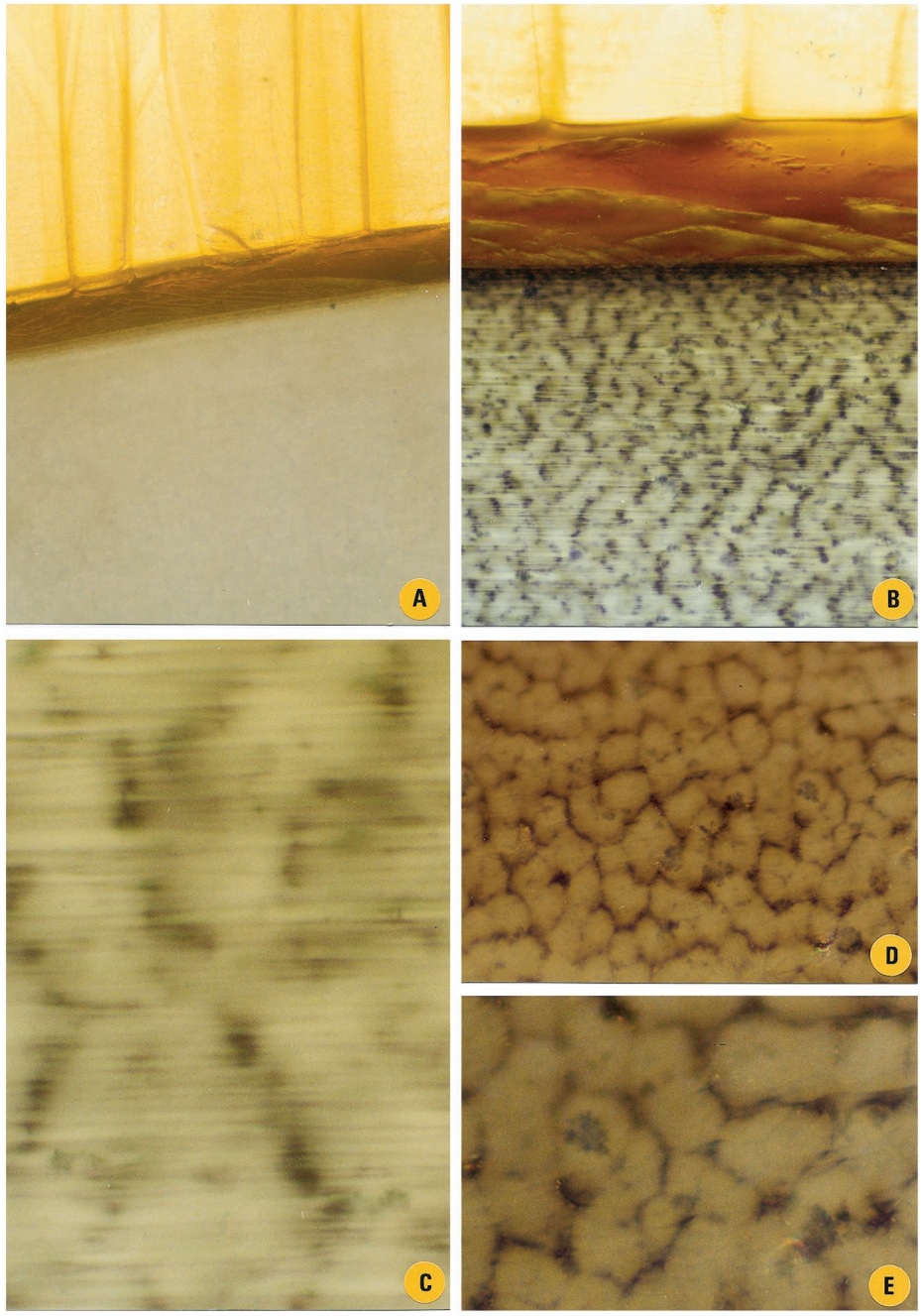

FIG. 8. ***In situ* localization of mucoperlin in the shell of *P. nobilis*, with the antirecombinant MCP antibody.** For technical details, see "Materials and Methods" and Refs. 17 and 34. In transversal section, the shell presents a nacroprismatic microstructure, with long calcitic prisms (Fig. 8, *A* and *B*, *upper yellow compartment*) and a nacreous layer (Fig. 8, *A* and *B*, *lower compartment*). The two calcified layers are generally separated by an organic layer, 50–60 μm thick (*brown line*, Fig. 8, *A* and *B*). The nacreous layer is composed a succession of minute aragonitic tablets (approximately 1-μm thick). *A*, negative control with preserum of anti-rec-MCP, diluted 10^4 times. *B*, staining with anti-rec-MCP, diluted 10^4 times. *C*, same section, high magnification. The staining reveals *horizontal lines*, which delineate the upper and lower surfaces of each tablet. The predominant staining occurs, however, on the short lateral sides of the tablets. *D*, longitudinal section (parallel to the growth plane of the nacre) in a juvenile *P. nobilis* shell. This preparation confirms that mucoperlin is localized around the polygonal elements (size about 15–20 μm) of the nacre. *E*, same preparation, high magnification.

Numerous mucin genes have been determined, from protozoans to vertebrates (52–57), but among the molluscs they have not yet been identified. So far, the single molluscan mucus proteins, which have been genetically characterized are few lectins of the land slug (58, 59). Mucins are heavily glycosylated and sometimes sulfated proteins of which the sugar moiety can amount to 85% of the total weight (38, 56). The protein cores (apomucins) are characterized by the presence of numerous tandem repeats in their central region. The repeats vary in size from 6 (60) up to 169 (61) amino acids. They all contain many serine and threonine residues, a large proportion of which constitute *O*-glycosylation sites. The protein cores of mucins are also rich in helix-breaking proline residues, accounting for the rigid rodlike structure of the proteins (37, 38). Mucins are able to form disulfide-dependent soluble dimers (56, 62) and multimeric insoluble gels through cross-linking of cysteine-enriched domains in the C- and N-terminal parts of the molecules (63, 64). They act as lubricants protecting the underlying epithelial tissues against viruses, bacteria, and other harmful agents. Furthermore, they play a role in selective interactions with the environment and in cellular recognition processes (65). Our idea of an anticalcifying function is well in agreement with the multifunctional nature of mucous materials.

Mucoperlin has many of the structural features characteristic of mucins. The core region has 13 tandem repeats rich in serine, threonine, and proline. These residues, important determinants of glycosylation and protein conformation, all occur at conserved positions (see Fig. 2). Also indicative is the concentration of the 3 cysteine residues of mucoperlin in the C-terminal region. This suggests that mucoperlin may form tail-to-tail dimers.

We did not find N-terminal cysteines, although these occur in virtually all mucins studied. Note that MUC7 (66), a water-soluble mucin from human saliva, and RSM, a rat submandibular mucin (67), contain only two cysteine residues, both localized at the N terminus. Thus, as MUC7 and RSM, mucoperlin

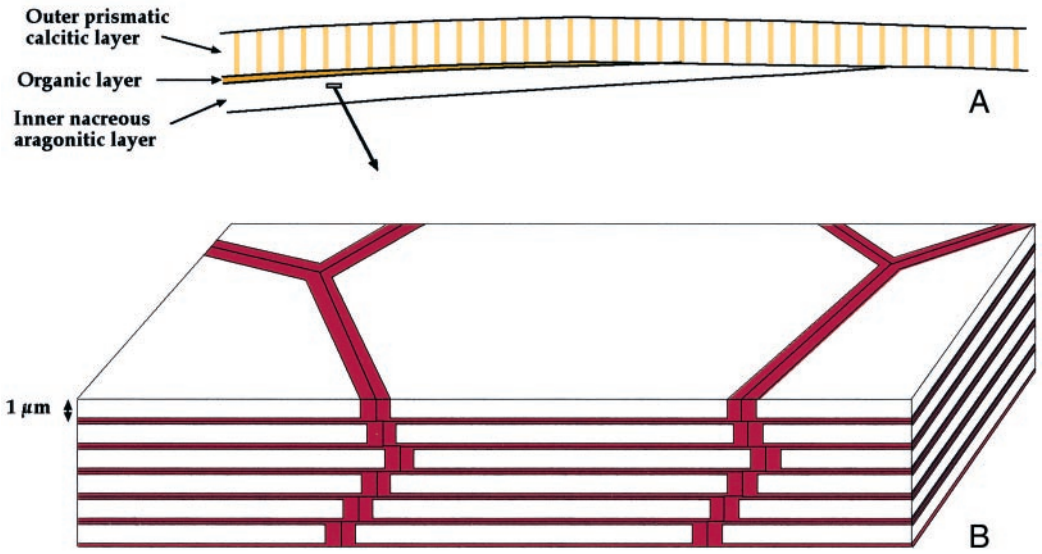

FIG. 9. **Schematic drawing of the localization of mucoperlin in the shell of *P. nobilis*.** *A*, nacroprismatic microstructure of the shell. *B*, mucoperlin (*dark red*) is localized in the nacreous layer, around the tablets. Since nacre tablets grow from a center and extend laterally, mucoperlin may be involved in the lateral control of the growth.

appears to be anomalous in this respect. On the other hand, we cannot exclude the possibility that our isolate of *mcp* is incomplete at the 5′-end. Our identification of the start codon of *mcp* should be confirmed with Northern analysis, reverse transcriptase-polymerase chain reaction amplification of the mRNA 5′ terminus, and genomic cloning. This work could not be carried out for this study because fresh tissue material of the protected species *P. nobilis* can only rarely be made available.

The specific association of mucoperlin with nacre tablets and not with the prisms indicates that the protein plays a functional role in the calcification process and is not a haphazard inclusion in the shell. This observation confirms our previous experiments (17), where we found a specific localization of mucous-homologous soluble matrix components in the shell of the bivalve mollusc *Mercenaria mercenaria.*

What could be the role of mucoperlin in the formation of nacre? Weiner and Traub (68) proposed a hypothetical model that would describe nacre formation (see also Refs. 1, 2, and 69). Insoluble matrix components would form a hydrophobic chamber-like framework to which soluble, acidic proteins rich in Asp/Glu-*X* or Asp/Glu-Ser-*X* motifs would be attached, adopting a β-sheet conformation. These repetitive acidic motifs would induce $CaCO_3$ nucleation and promote epitaxial growth of the crystals or, in contrast, terminate crystal growth when covering growth surfaces (70). Structural information that became available on various molluscan shell proteins in recent years (71–76) indicates that the regulation of crystal growth may be more subtle and complex; proteins such as nacrein (71), MSI60 (72), lustrin A (73), N16 (75), N14, and N66 (76), all from molluscan nacre, are clearly multifunctional. For example, nacrein and MSI60 exhibit both calcium binding domains and in addition a carbonic anhydrase function (for the first one) and a set of hydrophobic motifs (for the second one). N16, a newly discovered protein comprising at least three isoforms, exhibits four short acidic regions and a heparin-binding-like domain. Lustrin A is characterized by an alternation of proline-rich and cysteine-rich modules and a long GS domain putatively forming a "glycine loop." The C terminus of lustrin A presents a short basic domain and a protease inhibitor-like domain. Although lustrin A is thought to be mainly responsible of the toughness of nacre (77), additional functions are predicted from its sequence. Lustrin A may be a member of a protein family, since another protein related to lustrin A has been recently isolated (78). The cloning of mucoperlin, the first mucin-like protein to be localized in a molluscan nacre, extends one step more our view on molluscan calcification. Furthermore, it emphasizes the role of mucins in metazoan mineralization.

Mucins have indeed been shown to be associated with calcification in different mineralizing systems: the buccal cavity, where salivary mucins strongly bind to teeth hydroxyapatite (79, 80) and protect the teeth against demineralization (81); the gallbladder, where the mucin GBM promotes gallstone formation (82) but also delays the precipitation of calcium phosphate (83); and urine, where urinary mucins modulate the shape of calcium oxalates (84) and act as heterogeneous nucleants for calcium salts (85). In molluscs, mucins have been suspected of playing a role in calcification (86), but only little molecular evidence has been given (87).

In the absence of a more comprehensive theory on molluscan calcification than the proposed model, any suggestion for the role of mucoperlin must be tentative (Fig. 9). It has been previously suggested (88–90) that the nucleating macromolecules in nacre were located in the central part of the nacre polygons. Our observation that mucoperlin snugly surrounds the nacreous tablets (see Fig. 8) might indicate that its primary role would be to terminate crystal growth, in a way, similar to the mechanism described by Wheeler *et al.* (70). This hypothesis would be in agreement with the idea that the original function of mucoperlin in ancestral stocks would have been anticalcification, *i.e.* to inhibit spontaneous encrustration of soft tissues. We observed that acetic acid-soluble extracts of nacre of *P. nobilis* containing mucoperlin, could bind calcium, when non-denatured, and strongly inhibited the precipitation of calcium carbonate *in vitro*. It is not yet clear whether these roles are played solely by mucoperlin or are the result of the interaction between different components of the nacre matrix. Further studies are required to isolate large amounts of native mucoperlin from nacre extracts and to characterize this protein *in vitro.* In particular, attention has to be given to its glycosyl moieties. Recent works (47, 48) demonstrated the importance of protein-bound polysaccharides in the regulation of molluscan calcification process. This, in combination with the study of the temporospatial pattern of *mcp* gene expression by *in situ* hy-

bridization, will finally permit elucidation of the function of mucoperlin in molluscan calcification and identification of the cell types that contribute to mucoperlin deposition in nacre tissues.

Acknowledgments—F. M. thanks Dr. D. Sellos and Dr. Van Vormhout (Laboratoire de Biologie Marine du Collège de France) for teaching him the basic techniques of molecular biology.

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J. Biol. Chem. 2000, 275:20667-20675.
doi: 10.1074/jbc.M003006200 originally published online April 17, 2000
