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The Carbonic Anhydrase Domain Protein Nacrein is Expressed in the Epithelial Cells of the Mantle and Acts as a Negative Regulator in Calcification in the Mollusc *Pinctada fucata*

Hiroshi Miyamoto[1,2*], Fumiko Miyoshi[1] and Jun Kohno[1]

[1]*Faculty of Biology-Oriented Science and Technology, Department of Molecular Genetics, Kinki University, Uchita, Wakayama 649-6493, Japan*
[2]*Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence, Japan Science and Technology Agency*

ABSTRACT—Signals and organic matrix proteins secreted from the mantle are critical for the development of shells in molluscs. Nacrein, which is composed of a carbonic anhydrase domain and a Gly-X-Asn repeat domain, is one of the organic matrix proteins that accumulates in shells. In situ hybridization revealed that nacrein was expressed in the outer epithelial cells of the mantle of the pearl oyster *Pinctada fucata*. The recombinant nacrein protein inhibited the precipitation of calcium carbonate from a saturated solution containing $CaCl_2$ and $NaHCO_3$, indicating that it can act as a negative regulator for calcification in the shells of molluscs. Because deletion of the Gly-X-Asn repeat domain of nacrein had a significant effect on the ability of nacrein to inhibit the precipitation of calcium carbonate, it is conceivable that the repeat domain has a primary role in the inhibitory function of nacrein in shell formation. Together these studies suggest that nacrein functions as a negative regulator in calcification in the extrapallial space between the shell and the mantle by inhibiting the precipitation of $CaCO_3$.

Key words: nacrein, carbonic anhydrase, calcification, mollusc

INTRODUCTION

To protect and support their soft bodies, many invertebrates produce hard skeletons made of crystallized biogenic minerals. At least 60 different types of minerals have been reported, including $CaCO_3$, SiO_2, hydroxyapatite, and so on (Lowenstam and Weiner, 1989). Since the Cambrian diversification, animals possessing skeletons have been successful in adapting to the environment, resulting in the increased complexity and diversity of morphology in skeletal elements. In molluscs, biominerals of $CaCO_3$ are formed as shells with extremely divergent morphologies among species, enabling classification and identification of species. Shells are genetically regulated biominerals which consist of an external periostracal layer and an inner calcareous layer which is composed of several layers, such as a foliate, crossed lamellar, prismatic and nacreous layer. These layers are composed of $CaCO_3$, but their crystal types vary according to species; in most pearl oysters, calcite in the prismatic layer and aragonite in the nacreous layer (Wilt *et al.*, 2003). The aragonitic nacreous layer is known as mother-of-pearl and consists of compact tablets of several hundreds nm in diameter (Nakahara, 1983). It is reported that its toughness is 1000 times greater than simple aragonite crystals produced by a chemical procedure (Jackson *et al.*, 1988). In addition to $CaCO_3$, mollusc shells contain as minor components organic matrix proteins, which are thought to be responsible for the toughness on the nacreous layer and to have some critical roles in calcification (Crenshaw, 1972; Weiner and Hood, 1975; Wheeler *et al.*, 1981; Weiner, 1983; Greenfield *et al.*, 1984; Wheeler and Sikes, 1984; Addadi and Weiner, 1985; Cariolou and Morse, 1988; Wheeler *et al.*, 1988;). Direct evidence for the importance of organic matrix proteins in calcification was obtained by Falini *et al.* and Belcher *et al.*, who showed that macromolecules extracted from the nacreous layer induced aragonite formation in vitro (Belcher *et al.*, 1996; Falini *et al.*, 1996).

Several proteins or their cDNAs have been isolated from molluscs. Of these proteins, MSP-1, MSI31, and prismalin-14 are calcite-associated (Sudo *et al.*, 1997; Sarashina and Endo, 2001; Suzuki *et al.*, 2004), and MSI60, pearlin

* Corresponding author. Phone: +81-736-77-3888;
Fax : +81-736-77-4754;
E-mail: miyamoto@waka.kindai.ac.jp

(N16), lustrin A, mucoperlin, perlucin, and perlustrin are all found in nacre (Shen *et al.*, 1997; Sudo *et al.*, 1997; Samata *et al.*, 1999; Marin *et al.*, 2000; Miyashita *et al.*, 2000; Weiss *et al.*, 2000). The recently characterized protein aspein is an acidic protein expressed at the outer edge of the mantle (Tsukamoto *et al.*, 2004). Previously, we identified the water-soluble protein nacrein which is present in the nacreous layer of the pearl oyster *Pinctada fucata* (Miyamoto *et al.*, 1996). Nacrein had a domain similar to carbonic anhydrase (CA) and showed a clear enzymatic CA activity. One unique characteristic of nacrein in many animal phyla is that a Gly-X-Asn repeat sequence, which probably binds calcium ions, is inserted into the CA homologous domain. The nacrein gene is also found in *Pinctada maxima* (Kono *et al.*, 2000), which is a congeneric species of *P. fucata*, and in the gastropod *Turbo marmoratus* (Miyamoto *et al.*, 2003), suggesting that nacrein is conserved and plays an important role in shell formation in nacreous layer-producing species in the bivalve and the gastropod. In this paper, to further understand the function of nacrein, we showed its expression in the mantle of *P. fucata* and demonstrated its inhibitory action in the crystallization of $CaCO_3$.

MATERIALS AND METHODS

In situ hybridization

Adult specimens of the pearl oyster, *P. fucata*, were obtained from the nursery Miyuki (Wakayama, Japan). The mantles were excised and rinsed in artificial seawater (ASW) following fixation in 4% paraformaldehyde in ASW for 6 hours. The fixed mantle was dehydrated through an ethanol series, and embedded in paraffin. Paraffin blocks were sectioned at 7 μm thickness. After treatment with proteinase K (10 μg /ml) at 37°C for 30 min, the sectioned mantles were hybridized with antisense nacrein RNA probes labeled with digoxigenin. The sectioned mantles were washed in 50% formamide-2X SSC at 42°C, and then incubated with an anti-digoxigenin antibody coupled to alkaline phosphatase (1:5000 dilution in PBS containing 1% blocking reagent [Roche]). After washing in 100 mM Tris (pH7.5)-150 mM NaCl, signals were visualized with a substrate NBT (4-nitroblue tetrazolium chloride) / BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) according to the manufacturerís instructions (Roche).

Production of the recombinant nacrein protein in *E. coli*

For wild-type of nacrein and the deletion mutants Nac233 and NacR, PCR products amplified using oligonucleotides containing *EcoR* I or *Not* I sequences as primers were ligated into the expression vector pGEX-6P-1. The mutant in which the Gly-X-Asn repeat domain was deleted was generated by joining the N-terminal and C-terminal sequence with a *Pst* I site. The mutant fragment was ligated into the expression vector pGEX-6P-1 as mentioned above. The plasmids were introduced into *E. coli* XL1-blue, and fusion proteins with GST were induced by IPTG. After 4 to 6 hr of culture, cells were pelleted and resuspended in 50 mM Tris (pH 7.5), 0.1 M NaCl, 0.5% Triton X-100, and 5 μg/ml leupeptin. After lysis by sonication, the supernatant was collected and mixed with glutathione-sepharose beads (Amersham Biosciences). The fusion proteins were eluted with 5 mM glutathione and analyzed on a SDS-10% polyacrylamide gel.

$CaCO_3$ precipitation assay

Solutions (10 ml) containing 10 mM $NaHCO_3$, 10 mM $CaCl_2$ and GST-nacrein fusion proteins were prepared at time 0. The pH of the solution was recorded every 15 seconds with the pH meter F22 (Horiba Inc.) at about 25°C

RESULTS

Nacrein is expressed in outer epithelial cells of the mantle

As shown in our previous paper, the nacrein protein was isolated from the shell and its cDNA was cloned from the cDNA library constructed using the poly(A)$^+$ RNA puri-

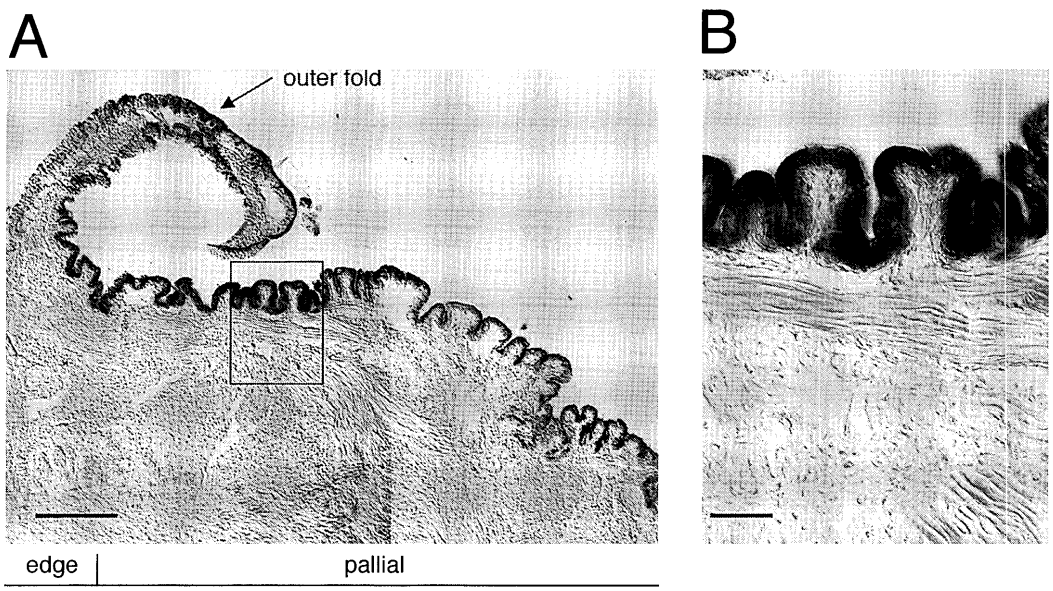

Fig. 1. Expression of the nacrein RNA in the mantle. The mantle edge is to the left and the outer epithelium to the top. (A) Longitudinal sections were hybridized with the digoxygenin-labeled RNA probe. The scale bar is 200 μm. (B) Higher magnification of the boxed region of (A). The scale bar is 50 μm.

fied from the mantle (Miyamoto *et al.*, 1996). The mantle is a thin sheet of tissue surrounding the internal organs of molluscs and contributes to the formation of shells. It is divided into three parts along the dorsal-ventral axis: mantle center, mantle pallial, and mantle edge, which consists of three folds (inner, middle, and outer) in many bivalves. The mantle pallial extends from the mantle edge to the pallial line, which is located at the central zone of the shells. Nacrein expression in the mantle was investigated by in situ hybridization using the antisense nacrein RNA as a probe. Nacrein transcripts were detected exclusively in the outer epithelial cells which line the inner surface of the shell (Fig. 1). When the sense probe of nacrein was used, no signal was detected (data not shown). The positive signals in the outer epithelial cells seemed to be stronger at the mantle edge than in the mantle pallial. In the outer fold of the mantle edge, slight signals were detected in both the outer and inner epithelial cells. It is recognized that the cells of the outer mantle epithelium play the most important role in shell formation (Lowenstam and Weiner, 1989). Together, the results of nacrein expression and physiological information imply that nacrein contributes to calcification in Pteriidae of bivalves as a functional protein in the outer mantle epithelium.

Nacrein acts as a negative regulator in crystallization of $CaCO_3$

We generated the wild-type nacrein (NacW), and

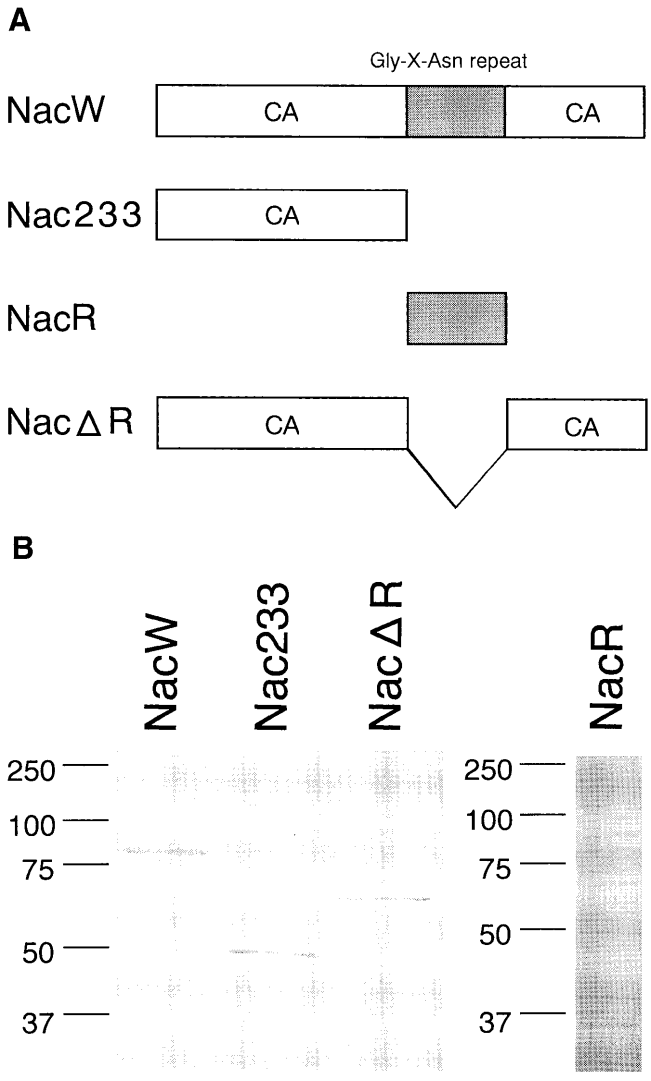

Fig. 2. Production of the GST-nacrein fusion proteins in *E. coli*. (A) Schematic representation of deletion mutants. NacW is the wild type (amino acids 18-447), not including the signal sequence. Nac233 encodes amino acids 18-233. The Gly-X-Asn domain is deleted in the NacΔR mutant. (B) SDS-PAGE of the purified fusion proteins. Molecular mass standards are shown in Kda.

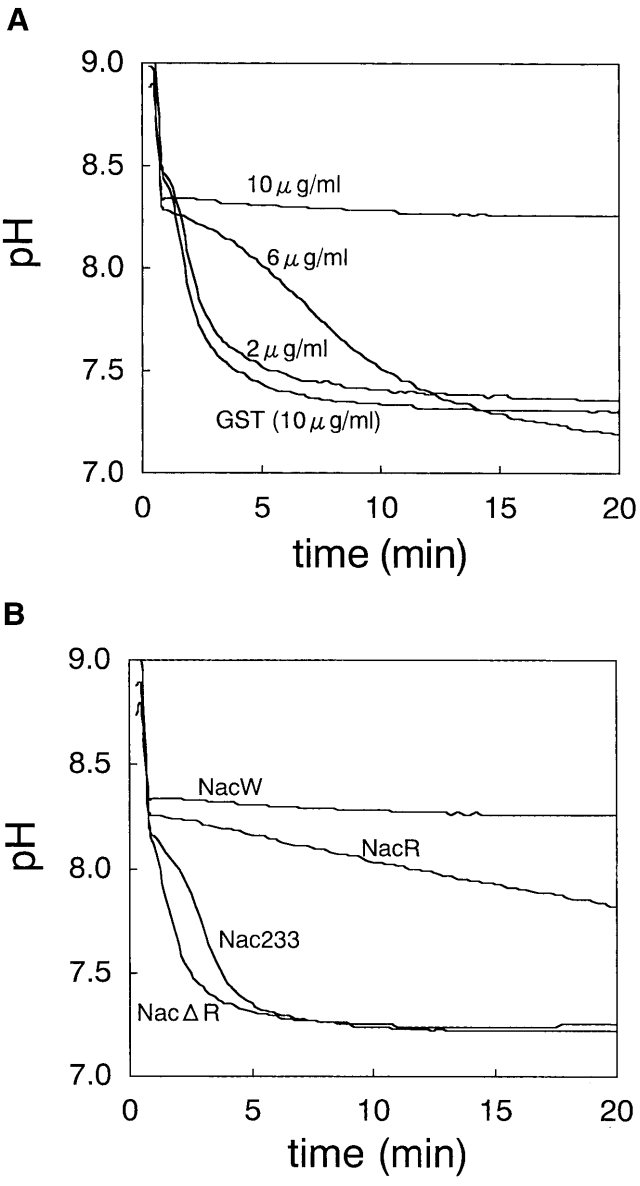

Fig. 3. *In vitro* crystallization in the presence of nacrein. The pH was recorded every 15 seconds. (A) Effect of wild-type nacrein on the precipitation of $CaCO_3$. Notice that nacrein (10 μg/ml) significantly inhibited the precipitation of $CaCO_3$. (B) The deletion mutants (10 μg/ml) of nacrein were used to detect the regulatory region in $CaCO_3$ formation.

mutants Nac233, NacR and NacΔR, as fusion proteins with glutathione S-transferase in *E. coli* (Fig. 2A, B). Nac233 is a C-terminal deletion mutant covering amino acids 18-233, a region which does not include the signal sequence. NacΔR is a Gly-X-Asn domain deletion mutant. Predicted molecular masses of these recombinant proteins (NacW:75 Kda, Nac233: 51 Kda, NacR: 35 Kda, NacΔR: 65 Kda) were almost identical to the molecular masses estimated from bands on SDS-PAGE. The effects of these recombinant proteins were measured in the $CaCO_3$ precipitation assay as described (Wheeler *et al.*, 1981). As shown in Fig. 3A, the wild type nacrein significantly inhibited the lowering of the pH in proportion to the concentration of the protein, which means that nacrein effectively inhibits crystallization of $CaCO_3$. To know where in the nacrein sequence the inhibitory activity resides, three deletion mutants were tested for their ability to inhibit the precipitation of $CaCO_3$. As expected, inhibitory activity was predominantly eliminated when the Gly-X-Asn repeat domain was deleted (Fig. 3B). These results localize the inhibitory activity for crystallization of $CaCO_3$ to the Gly-X-Asn repeat domain.

DISCUSSION

The development of shells is a useful model for studying biomineralization at the molecular level, because shells are easy to handle and show remarkably intricate structures such as mother-of-pearl, or nacre (Bevelander and Nakahara, 1969; Wise, 1970; Nakahara, 1991). In addition, it is possible to recognize and isolate the tissue responsible for shell formation, the mantle. Several matrix proteins have been identified from bivalves and gastropods, and their cDNAs have been cloned using the cDNA library constructed from the mantle. Nacrein was sequenced as the first report on soluble matrix proteins found in mollusc shells. The characteristic feature of nacrein is its CA-like structure punctuated with the Gly-X-Asn repeat, which is predominantly Gly-Asn-Asn or Gly-Asp-Asn. Several lines of evidence have indicted that many organic constituents of shells are rich in aspartic acid and glycine (Weiner and Hood, 1975; Weiner, 1979; Wheeler *et al.*, 1981; Weiner, 1983; Weiner, 1984; Cariolou and Morse, 1988; Kawaguchi and Watabe, 1993; Aizenberg *et al.*, 1997). Considering that the nacrein protein is detected by SDS-PAGE as a major band in the soluble proteins extracted from the nacreous layer of *Pinctada fucata*, nacrein is thought to constitute the major part of the Gly- and Asp-rich fraction of the soluble proteins. The result of in situ hybridization analysis presented in this paper indicates that nacrein is highly expressed in the outer epithelial cells of both the mantle edge and pallial, which form the lining of the shell and are involved in the transport of calcium into the mineralizing site and in the secretion of some proteins into the shell. This suggests that nacrein is important for shell formation in both the calcitic prismatic layer and the aragonitic nacreous layer. In this sense, the expression of nacrein in the mantle contrasts with that of shell framework proteins such as MSI60 and MSI31, which are expressed in the pallial region and the mantle edge respectively (Sudo *et al.*, 1997). Based on these observations, we speculate that nacrein is involved in the regulation of shell formation on the fundamental framework scaffolded with MSI60 or MSI31. It is known that the CA activity is present in the mantle of most molluscs (Freeman and Wilbur, 1948) and that calcium ions are concentrated in the epithelial cells of the mantle (Jones and Davis, 1982; Bielefeld *et al.*, 1992). A previous Stains-all staining experiment suggested that the nacrein protein has the ability to bind Ca^{2+}, and we detected CA activity in the purified nacrein protein. Therefore, it is plausible that nacrein would function both as a calcium concentrator and as an enzyme required for production of carbonate ions, which are assembled to $CaCO_3$ at mineralization sites.

This report shows that, as one of its biochemical functions, the nacrein protein inhibits the crystallization of $CaCO_3$. Mucoperlin, which has been isolated from the Mediterranean mussel (*Pinna nobilis*), also has an inhibitory effect on $CaCO_3$ crystallization (Marin *et al.*, 2000). Mucoperlin is localized around aragonitic tablets in the nacreous layer. On the other hand, perlucin (isolated from abalone *Halitotis laevigata*) and MSI7 (isolated from pearl oyster *P. fucata*) nucleate $CaCO_3$ crystals and seem to facilitate calcification (Weiss *et al.*, 2000; Zhang *et al.*, 2003). It might be explained that shell formations in molluscs proceeds by coordinated processes, which involve both nucleating factors such as perlucin and MSI7, and aniticalcification factors such as nacrein and mucoperlin. On the basis of our results demonstrating that in the absence of the Gly-X-Asn repeat domain, the inhibitory effect of nacrein on crystallization of $CaCO_3$ was suppressed, it is clear that the repeat domain acts as a regulator for the negative function of nacrein in mineralization. The nacrein-like protein found in *P. maxima* (a congeneric species with *P. fucata*) has a longer Gly-X-Asn repeat sequence (Kono *et al.*, 2000), and the homologous protein in a gastropod *Turbo marmoratus* has a Gly-Asn repeat (Miyamoto *et al.*, 2003). It is intriguing to consider how these differences in the repeat domains relate to the morphological divergence of shells and the adaptive radiation of mollusc species that produce the nacreous layer in the inner surface of shells.

The formation of shells has been studied at a variety of levels using various mollusc species and is providing many insights into the molecular mechanisms of biomineralization in animal phyla. However, many of the mentioned matrix proteins are unrelated in their amino acid sequences, making it difficult to speculate about the common mechanisms of calcification among invertebrates. Nacrein appears to be conserved in the families Pteriidae and Turbinidae. Therefore, the functional analysis of nacrein will extend our knowledge of how shells are constructed in the two families.

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