Asprich: A Novel Aspartic Acid-Rich Protein Family from the Prismatic Shell Matrix of the Bivalve *Atrina rigida*

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Almost all mineralized tissues contain proteins that are unusually acidic. As they are also often intimately associated with the mineral phase, they are thought to fulfill important functions in controlling mineral formation. Relatively little is known about these important proteins, because their acidic nature causes technical difficulties during purification and characterization procedures. Much effort has been made to overcome these problems, particularly in the study of mollusk-shell formation. To date about 16 proteins from mollusk-shell organic matrices have been sequenced, but only two are unusually rich in aspartic and glutamic acids. Here we screened a cDNA library made from the mRNA of the shell-forming cells of a bivalve, Atrina rigida*, using probes for short Asp-containing repeat sequences, and identified ten different proteins. Using more specific probes designed from one subgroup of conserved sequences, we obtained the full sequences of a family of seven aspartic acid-rich proteins, which we named "Asprich"; a subfamily of the unusually acidic shell-matrix proteins. Polyclonal antibodies raised against a synthetic peptide of the conserved acidic1 domain of these proteins reacted specifically with the matrix components of the calcitic prismatic layer, but not with those of the aragonitic nacreous layer. Thus the Asprich proteins are constituents of the prismatic layer shell matrix. We can identify different domains within these sequences, including a signal peptide characteristic of proteins destined for extracellular secretion, a conserved domain rich in aspartic acid that contains a sequence very similar to the calcium-binding domain of Calsequestrin, and another domain rich in aspartic acid, that varies between the seven sequences. We also identified a domain with DEAD repeats that may have Mg-binding capabilities. Although we do not know, as yet, the function of these proteins, their generally conserved sequences do indicate that they might well fulfill basic functions in shell formation.*

Introduction

Unusually acidic macromolecules are components of the organic matrices of mineralized tissues from many different organisms.[2–4] The first such protein was discovered in vertebrate dentin.[5] The acidic nature of these proteins, their intimate association with the mineral phase and widespread distribution, suggest that they fulfill key functions in the mineralization process.[6–8] Aspartic and/or glutamic acid usually constitute between 30 and 40 mole percent of the matrix protein.[6,9]

There are few observations that indicate that the acidic proteins are directly involved in controlling mineral formation.[10] There is, however, a large literature on in vitro experiments showing that these macromolecules are able to specifically modulate mineral formation. Acidic proteins and peptides generally inhibit crystal nucleation and/or growth[8] when in solution, and when adsorbed on a substrate they can induce crystal formation.[11,12] Thus, certain acidic proteins interact selectively in vitro with calcite crystal faces,[13,14] resulting in modifications in the shape of the growing crystal.[15] Once bound to the surface, the proteins are overgrown by the crystal and are occluded in the intracrystalline environment.[16] Their presence inside the crystals changes the mechanical properties of the mineral.[17] Acidic macromolecules are promoters or inhibitors of calcium carbonate crystal formation and are also capable of controlling polymorph type.[18,19] Despite the in vitro evidence showing that these molecules do potentially fulfill important functions in mineral formation, very little is known about their primary structures, and almost nothing about their secondary and tertiary structures.

Mollusk-shell organic matrices contain tens of different macromolecules that are soluble after the mineral is removed, not all of which are unusually acidic.[2] Quantitatively, the most abundant protein constituents are the Asp-rich proteins.[20] These can be fractionated by ion-exchange chromatography, but until recently have been difficult to characterize by gel electrophoresis because they readily diffuse out of the gel and are not generally stained by Coomassie Blue.[21]

Some matrix proteins from mollusk shells have been sequenced.[1,22–37] Most of the sequenced proteins contain blocks of repeat sequences. This property most likely allows them to

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interact with either repetitive structures of other macromolecular constituents of the matrix or with the repeating motifs in the crystal itself. Some structural motifs may tentatively be attributed to certain functions, based on the available sequences.[38,39] AG-rich proteins homologous to silk and C-rich hydrophobic proteins are most probably framework proteins.[23,27,30,31] The $(GN)_n$ motif, present in five sequences of mollusk soluble proteins[25,26,40] may be a chitin-binding motif, as it has homology to insect chitin-binding proteins. S-rich domains, found in three mollusk-shell matrix proteins,[22,27,29,34] are similar to those found in Sericin—a protein closely associated with silk.[41] They may thus have a silk-binding function. Two proteins with proven carbonic anhydrase activity are most probably involved in the modulation of carbonate ion supply.[25,26] The basic domains present in three different proteins clearly fulfill some as yet unknown key function.[22,27,32,34] Some proteins, such as MSP1 and Lustrin, have multifunctional domains.[22,27,34]

Only two mollusk-shell proteins that have been completely sequenced to date are really acidic, namely MSP-1[22,34] and Aspein.[1] These proteins are rich in aspartic acid, and their pI's are accordingly very low ($pI \approx 3$). Aspein is more acidic than MSP-1 and also contains continuous runs of Asp residues. Both proteins contain DXD or DXYD repeats of aspartic acid. These repeat several times in the protein sequence. MSP-1 contains two acidic domains with such repeats. Both domains share a high degree of sequence similarity. The existence of acidic repeat sequences in the mollusk-shell soluble proteins was first predicted by Weiner and Hood,[42] who used partial acid hydrolysis of matrix proteins to reveal the existence of $(Asp\text{-}Y)_n$ repeat sequences, where Y represents mainly serine or glycine. Partial sequencing of matrix proteins suggested that polyaspartic acid domains might be present.[43]

We chose to study *Atrina rigida* because it is a large bivalve, and the nacreous and the prismatic-shell layers can easily be separated. Furthermore, the soluble organic-matrix fraction of the aragonitic layer (nacre) contains 45 mol% acidic residues (Asx + Glx),[21] and the prismatic calcitic layer contains 53 mol% Asp and 11 mol% Glu.[44] Thus Asp-rich proteins are major constituents of its organic matrix. Here we identified and characterized a novel acidic protein subfamily at the RNA level. Antibodies produced against synthetic peptides with conserved sequence segments from the identified proteins showed that these proteins are indeed constituents of the prismatic shell layer.

Results

The strategy

In order to isolate and characterize the primary sequences of members of the Asp-rich family of proteins, we assumed that the short Asp-containing repeat sequences were common and therefore used appropriate oligonucleotides to screen the cDNA library. The library was made from the mantles (the organ responsible for shell formation) of *Atrina rigida* specimens harvested during mid-summer, when growth, and, hence, shell formation, is at a maximum.[45] The first screen with PCR was broad and was performed with short degenerate oligonucleotides that were designed according to the acidic repeats in the two published mollusk-shell acidic-protein sequences.[1,22,34] We then isolated a specific subgroup of cDNAs encoding Asp-rich proteins, and used their conserved sequences as a probe for hybridization and fluorescent cDNA screening. Finally, we used polyclonal antibodies against synthetic peptides derived from the sequences to show that these proteins are components of the prismatic shell layer of *Atrina*.

Screening the *Atrina* cDNA library with short oligonucleotides—PCR method

Two sets of sense primers encoding DDGSDD and DDGDDD were designed. These degenerate sets contain oligonucleotides of all the possible codons for each amino acid. Each set was reacted separately against the antisense RNA polymerase promoter sequence T3 located downstream from the multiple cloning site of the Lambda zap vector. Numerous DNA fragments resulted from each PCR reaction, and smear bands were obtained on an agarose gel. Selection and isolation of the DNA fragments were achieved by molecular cloning of the PCR products.

About 300 clones were isolated, and each insert DNA was sequenced. Sequence segments corresponding to either the subcloning vector or to the Lambda zap vector were omitted. Eleven different sequences were identified as sequences that encode acidic proteins. One of these was similar to DNA binding proteins. None of the remaining ten sequences (Figure 1) was found to have significant similarity to any protein in the NCBI protein data bank.

Sequence 4 in Figure 1 shows unusual continuous repeats of aspartic acid, which are rarely interrupted by other amino acids. This sequence, containing 53.2 mol% aspartic acid, was the most acidic deduced sequence identified at this stage of the screening. Sequences 1 to 3 (Figure 1) have an average of 92% identity. The average calculated pI of these translated proteins is 3.1. All three sequences contain identical hydrophobic domains prior to the acidic domain. The acidic domain contains an average of 35.4 mol% aspartic acid (D) and 9.3 mol% glutamic acid (E), separated by hydrophobic amino acids. The remaining sequences, although all different, are unusually rich in acidic residues.

Sequences 1, 2, and 3, although less acidic than sequence 4, are highly conserved in their N-terminal region. The N-terminal sequence is composed of a relatively hydrophic portion followed by a rather basic sequence. We decided to take advantage of this unusual region and use these sequences for further screening of the library.

Screening the *Atrina* cDNA library with a fluorescent probe

The DNA sequence encoding for the first 64 amino acids in the identical domains of sequences 1 to 3 (Figure 1) was chosen to be a probe for further cDNA screening (highlighted with gray background in Figure 3, below).

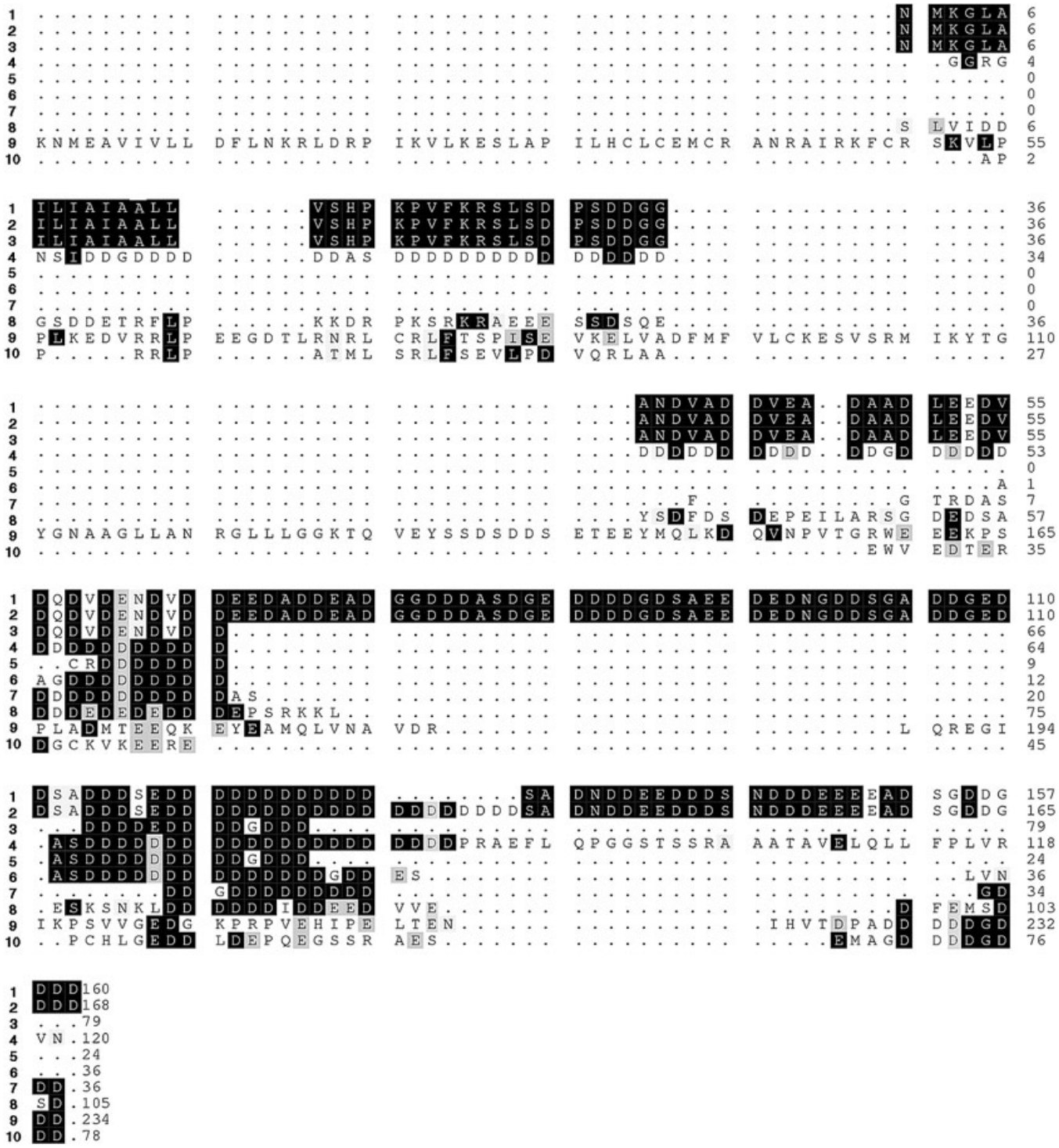

Figure 1. *Clustal W multiple sequence alignment of the deduced unique proteins found by screening the Atrina rigida cDNA library by the PCR method. The black squares indicate identical amino acids. The gray squares indicate similar amino acids. The numbers indicate amino acid residue positions. The dots are gaps, which were opened to align the sequences. Sequences 1 to 3 were used to construct the DNA probe for the hybridization and cDNA fluorescent screening. Sequence 4 is the most acidic sequence to be identified.*

Seven DNA sequences encoding acidic proteins were found (shown schematically in Figure 2). The DNA sequences are homologous and share similar domains. However, they vary in length and are not identical; this shows that they are different mRNA products. The sequences were repeatedly checked by using specific internal primers from both directions, and therefore sequencing errors can be ruled out. The nucleotide sequences are different, especially, but not only, in the noncoding regions (Figure 2 and the NCBI data base).

Six sequences are terminated by a poly-A tail. A search was performed with an open-reading-frame finder program (NCBI database). The right open reading frame was selected accord-

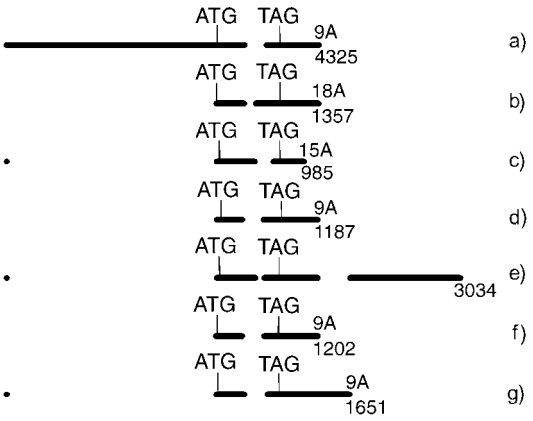

Figure 2. *Comparison of Asprich DNA sequences isolated by hybridization and fluorescent screening of the* Atrina *cDNA library. The solid lines indicate identical DNA sequences. The gaps within the sequences were opened in order to align the identical domains to one another. Vertical lines mark the positions of the ATG initiation codon and the TAG stop codon. The numbers under the solid lines indicate the length of each sequence in bp. The numbers above the solid lines indicate the poly-A regions.*

ing to the poly-A tail direction. All seven sequences contain open reading frames in the same domain. These DNA sequences encode acidic proteins that are highly homologous and share identical domains (sequences a to g in Figure 3) flanked by untranslated sequence regions. All the sequences encode a stop codon prior to the open reading frame; this, together with the poly-A region, implies that the complete deduced protein has been located. The proteins have variable lengths (209–244 amino acids) and a high degree of similarity. No N-glycosylation sites were found. The sequences might, however, contain O-glycosylation and phosphorylation sites. These need to be validated by direct investigation.

Immunodetection of the deduced proteins in different shell extracts

The cDNA library of *Atrina rigida* mantle cells includes sequences coding for many different functions. In order to determine whether the isolated cDNAs encode extracellular proteins located in the shell, two sets of polyclonal antibodies were raised separately against two synthetic polypeptides. These polypeptides were derived from the deduced protein sequence. One comprises the first 20 amino acids of the deduced proteins (MKGLAILIAIAALLAVSHPK), while the sequence of the second follows the former (SLSDPSDDGGANDVADDVEADAADL) and is much more acidic. The two sets of antibodies were tested against either the nacre or the prismatic soluble proteins extracted from the mollusk shell, and the specificity of this interaction was detected by using ELISA. The deduced proteins were only detected by the antibodies against the acidic sequence in the prismatic-matrix proteins and not in the nacreous-matrix proteins (Figure 4). Furthermore, no interaction was detected between the antibodies raised against the first 20 amino acids in either the nacre proteins or the prismatic proteins. We conclude that the deduced proteins are components of the *Atrina rigida* prismatic-shell-layer organic matrix, and that the first 20 amino acids are not a component of the expressed proteins in the shell. More specific identification of individual proteins, such as is routinely performed for nonacidic proteins by Western blot, is most problematic (Albeck, PhD thesis, unpublished) probably because these highly charged, hydrophilic macromolecules freely diffuse out of SDS gels.[21]

Discussion

Ten aspartic acid-rich proteins have been identified and their amino acid sequences determined. Three sequences were only determined by PCR and cDNA screening, and might be incomplete. Seven sequences, determined by hybridization and fluorescent screening of the cDNA library, are complete. They have molecular weights between 20 and 30 kDa, and are composed of 51–61% acidic amino acids (39–50% D and 10–13% E). They have highly homologous sequences, including sequences with unusual continuous stretches of aspartic acid (Figure 3). These seven proteins are present in the organic matrix of the calcitic prismatic shell layer of the mollusk *Atrina rigida.* They thus constitute a family of shell matrix proteins and are named "Asprich (a–g)".

The Asprich proteins can arbitrarily be divided into six domains, schematically represented in Figure 5.

The fact that one of the domains has a highly variable sequence, suggests that the Asprich (a–g) family of proteins might well be the products of alternative RNA splicing of the same gene. The complete sequences of proteins 1–3 listed in Figure 3 are not known as yet, and they differ from each other and the Asprich family. Alternative RNA splicing might be very common in the mollusk-shell proteins, since these proteins are thought to be closely related to one another and to contain variations of characteristic motifs.[46]

The N-terminal hydrophobic domain is present in all ten proteins. It is predicted to be a signal peptide by SignalP server.[47]- The most likely cleavage site is between P19 and K20. The signal peptide contains a core of 14 hydrophobic amino acids preceded by a basic amino acid (K). This pattern, specifically located at the N terminus, is common to other signal peptides that direct proteins to the endoplasmic reticulum.[48,49] Moreover, all the deduced proteins lack a targeting sequence at their carboxyl terminus (KDEL or KKxx) that marks proteins destined for retention in the endoplasmic reticulum. They are therefore destined for transport to the Golgi and to be secreted out of the cell. This is consistent with the extracellular location of the mollusk-shell organic matrix. Our ELISA results show that the signal peptide is not present in the extracellular matrix proteins, as no interaction was detected between the antibodies raised against the hydrophobic signal polypeptide and the nacre proteins or the prismatic proteins. Interestingly, the N-terminal signal peptide has significant similarity to that found in Aspein (Figure 6B),[1] one of the two acidic shell matrix proteins identified to date.

The basic domain is thus the N-terminal part of the secreted protein. No homology was found between this sequence and

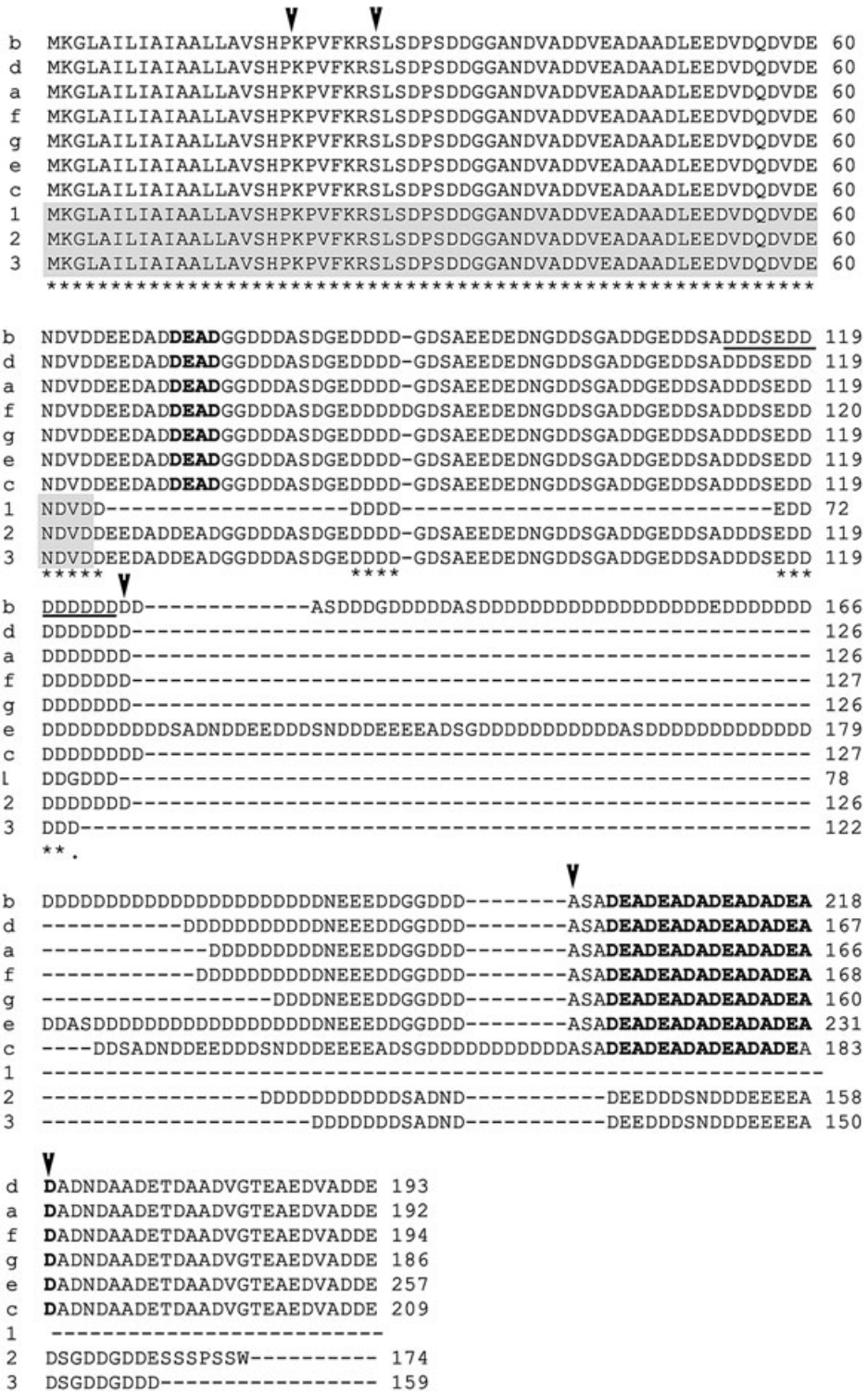

Figure 3. *Multiple alignments among the deduced proteins identified by hybridization and fluorescent screening of the* Atrina *cDNA library (a–g) and PCR cDNA screening (1–3). The translated probe sequence used for hybridization is marked with a gray background. Arrowheads show the borders between the different domains, as described in the discussion (Figure 5). The putative calcium-binding domain identified by the alignment to Calsequestrin is underlined. The DEAD domains are shown in bold. Asterisks indicate identical amino acids. The numbers indicate amino acid residue positions.*

any known protein in several protein data bases (SwissProt, Swall, PDB, PROSITE, NR). As this sequence is common to all the deduced proteins, we can only surmise that it must have an important function, possibly, but not necessarily, related to directing the proteins to a specific region of the matrix.

The acidic domain is identical in all the Asprich proteins (but not proteins 1–3). It is interesting that the sequence of this domain is similar to Calsequestrin, a calcium-binding protein from cardiac and skeletal muscle (Figure 6A). Calsequestrin has 44% identity to the deduced proteins over a 66 amino acid

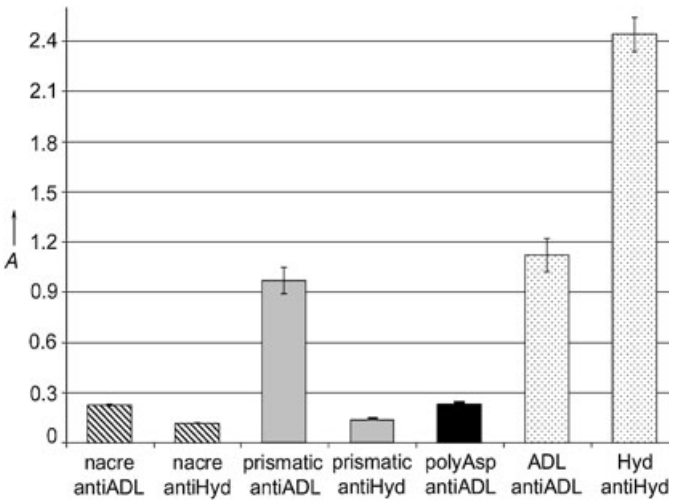

Figure 4. *Graphic presentation of the ELISA results. Each bar represents the absorbance at 405 nm of the ELISA color reaction of two polyclonal antibody mixtures, antiADL and antiHyd, on different substrates. ADL = acidic peptide SLSDPSDDGGANDVADDVEADAADL from the acidic1 domain. Hyd = signal peptide CMKGLAILIAIAALLAVSHPK. Hatched bars: the substrate was matrix protein (10 μg) from the aragonitic nacreous layer of the* Atrina rigida *shell; neither antibody reacted. Gray bars: the substrate was matrix protein from the calcitic prismatic layer (10 μg); the acidic peptide gives a positive reaction. Black bar: control reaction with substrate poly-D. Dotted bars: positive controls of anti-ADL and antiHyd on the polypeptides ADL or Hyd. The strong positive reactions indicate that the antibody titer is high. All antibodies were diluted 1:500 in PBS. ELISA negative controls (not shown) were: 1) preimmune antibody, 2) no antigen, 3) no antibody. The absorbance of all the controls was below 0.2.*

region within the conserved acidic1 domain. When taking into account all the amino acids of the same type, the similarity in this region increases to 68%.[50] The length of the D-rich region of Calsequestrin is important for the Ca^{2+}-binding capacity.[51] Unlike other calcium binding proteins, which bury calcium ions in specific "pocket" motifs, Calsequestrin interacts with calcium ions on its surface. These characteristics are well suited to the acidic matrix proteins associated with mollusk shells, where calcium-binding capacity is essential for building and interaction with the mineral phase.

The variable acidic domain sequences differ in all seven of the Asprich family members, although the last 14 amino acids are conserved throughout. The presence of long stretches of poly-D sequences in mollusk-shell-matrix proteins has been reported based on protein-level sequencing.[52]

The DEAD domain, which starts with an ASA triplet, is conserved in all seven members of the Asprich family. The DEAD motif is the most prominent feature in RNA helicases. It is thought to play an important role in ATP binding or ATP hydrolysis. The first two negatively charged residues in the DEAD region are important for Mg^{2+}-coordinated ATP hydrolysis, while the last aspartate plays a role in coupling ATPase and helicase activities.[53] In helicases, the DEAD motif appears once, while the Asprich proteins contain a sequence of four DEAD repeats and one DEAD sequence in the acidic1 domain. Asprich proteins lack other typical features of the helicase family, and thus are not suspected to have helicase activity. If, however, the function of the DEAD motif is similar to that in helicases, these proteins are capable of binding at least five ATP molecules through Mg^{2+}. The possible involvement of Mg^{2+} is in itself incredible, when considering that Mg has a recognized, important, though not well-understood, function in the deposition of calcium carbonate-based mineralized tissues.[54]

The acidic part of Asprich (a–g), comprising acidic domains 1 and 2, the variable acidic domain, and the DEAD box domain, accounts for 85–88% of the sequences. They have an overall composition of 57–68% acidic amino acids (depending on the variable length of the poly-D domain). It is therefore not surprising that Asprich proteins were automatically identified by the search in the different databases as being similar to other proteins containing many acidic residues. The similarity however is limited to the high percentage of aspartic acid residues in the sequence. Some of these acidic proteins are involved in mineralization processes, such as dentin sialophosphoprotein, DSPP,[55] bone sialoprotein-binding protein,[56] the aspartic acid-rich protein aspolin1 found in fish muscle,[57] and Starmaker,[58] a protein associated with aragonite in zebrafish otoliths.

Aspein,[1] the shell-matrix protein from the mollusk *Pinctada fucata*, has 48.6% identity to Asprich (b) over a 276 amino acid overlap, comprising the whole Asprich (b) sequence and approximately $^2/_3$ of Aspein (Figure 6B). Besides the above-mentioned similarity in the signal-peptide region, Aspein contains aspartic acid domains homologous to Asprich (b). Aspein, the Asprich family, and MSP-1 were identified in different pteriomorph bivalves (*Pinctada fucata*, *Atrina rigida*, and *Patinopecten yessoensis* respectively). Interestingly, they are all present only in the calcitic shell layers. No homology exists, however, between MSP1 and Asprich proteins. In particular, MSP1 does not have poly-D domains, and its N-terminal hydrophobic peptide[34] is also different from those of Asprich and Aspein. This indicates that, although there are extensive similarities in the acidic proteins isolated from different bivalves, there are probably many different families that coexist in the prismatic layer, presumably fulfilling different functions.

We can only speculate about the specific function of the Asprich proteins. Some characteristics are, however, evident. The acidic region is probably the functional part of the molecule in terms of mineral formation. It is associated with calcium-bind-

Figure 5. *Schematic diagram showing the six domains present in the Asprich family of proteins: N-hydrophobic (19 residues), basic (9 residues), acidic1 (consensus sequence of 97 residues), variable acidic, DEAD domain (consensus 20 residues), acidic2 (consensus 25 residues) -C. The exact boundaries between these domains are shown by arrowheads in Figure 3.*

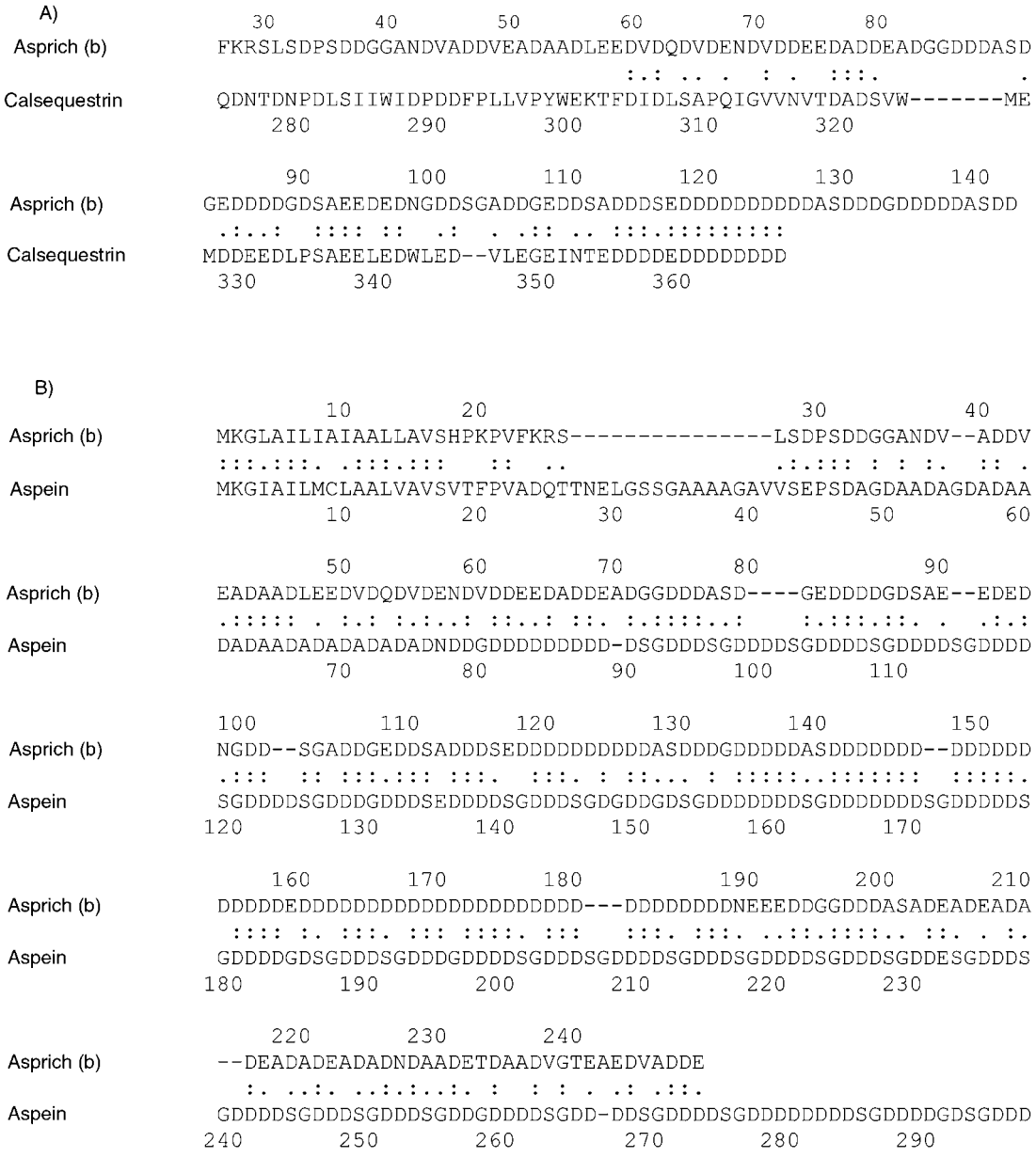

Figure 6. *Comparison between the deduced amino acid sequence of Asprich (b) and A) Calsequestrin and B) Aspein.[1] Identical amino acids are indicated by double dots and similar amino acids are indicated by single dots. The numbers indicate amino acid residue positions.*

ing and possibly magnesium-binding activities. As noted, Asprich proteins are associated with the prismatic layer of *Atrina* shells, but not with the nacre. The proteins associated with the prismatic layer are generally much more acidic then those of nacre. In *Atrina* in particular, the total assemblage of proteins and the intracrystalline proteins of the soluble matrix fraction have a very acidic composition with more than 60% aspartic and glutamic acid. This is similar to the Asprich family. The intracrystalline proteins constitute only about 20% of the total protein, but their compositions are very similar.[44] The intracrystalline proteins are mainly located on planes perpendicular to the prism axes.[59] As each prism is a single crystal of calcite with the *c*-axis oriented along the prism axis, this corresponds to the intracrystalline proteins being in contact with the (001) plane of calcite. The (001) plane of calcite has a very characteristic structure, with alternating calcium and carbonate layers, such that an aspartic acid-rich protein interacting with these planes is well positioned to have the carboxylate groups of aspartic acid interacting with the calcium ions. The continuous Asp-runs may affect both crystal nucleation and crystal growth. It would in fact be very tempting to suggest that they may be adsorbed on the (001) plane of a growing prism, and then induce nucleation of a new crystallite domain.

It must be noted that the peptide injected for producing the antibodies used in the immunoassay, had a sequence taken from the acidic1 domain. The immunoassay was negative for proteins extracted from nacre; this indicated that the acidic1 domain, and thus Asprich, is absent in nacre. This does

not mean, however, that proteins containing poly-D sequences are necessarily absent in nacre.

It is interesting, although admittedly possibly coincidental, that the only acidic proteins sequenced to date are all associated with calcite. This is even more peculiar when taking into account that most investigations on protein sequences concentrated on nacre or were motivated by attempts to understand the proteins present in nacre.

Conclusion

The known properties of the acidic proteins indicate clearly that they must fulfill key functions in controlling mineral deposition, such as in calcium transport, establishment of calcium carbonate supersaturation, crystal nucleation, orientation, and modulation of shape. How these functions are fulfilled by individual proteins, is not clear. With the addition of seven new sequences, the challenge is to elucidate the structure–function relationships and identify the in vivo locations of these proteins in relation to the forming crystal. In contrast to many other proteins that have "sequences in search of a function", we are confronted here with "functions in search of a protein".

Experimental Section

General materials and equipment: DNA fragments were sequenced at the Weizmann Institute (WIS) DNA sequencing unit, which utilizes the BigDye Terminator Cycle Sequencing Kit from Applied Biosystems. Most primers for PCR and sequencing, NaCl, NaOH, Tris·HCl, Na-citrate·2 H_2O, $CaCl_2$, Tween 20 (Polyoxyethylene sorbitan monolaurate), gelatin, and EDTA were from Sigma–Aldrich (Rehovot, Israel). Sequencing primers for T3, T7, and SP6 were supplied by the DNA sequencing unit (Biological services, WIS). Primers were designed and analyzed by using the Oligo-4 program. All PCR reactions were performed with Agarose I biotechnology grade, which was from Amresco (Solon, Ohio, USA). Low DNA mass ladder standard was from Invitrogen (Carlsbad, CA, USA). Uncut Lambda DNA standard was from New England Biolabs (Beverly, MA, USA). IPTG and X-Gal were from Fermentas GmbH (Germany). Plasmid DNA quantity was determined by using a micro (capillary) spectrophotometer (DNA sequencing unit, WIS). Plaque-lifting membranes, circles Nytran N 82 mm 0.45 μm, were from Schleicher and Schuell (Dassel, Germany). Hybridization was performed on Promega TermoHYBAID roller bottle and hybridization oven (Madison, WI, USA). ExAssist helper phage with SOLR strain was from Stratagene (West Cedar Creek, TX, USA). Activated BSA (bovine serum albumin), KLA and IgG purification kit were from Pierce (Rockford, IL, USA). PBS (Dulbecco's phosphate buffered saline), secondary antibody, alkaline phosphatase conjugated goat-anti-rabbit IgG, and *p*-nitrophenylphosphate were purchased from Jakson Immunoresearch (West Grove, PA, USA).

cDNA library construction: The average growth rates of *Atrina rigida* in Florida bay are highest during July–September.[60] Therefore, fresh *Atrina rigida* bivalves were collected in August at Fort Pierce, Florida. The mantle cells were isolated and snap frozen in liquid nitrogen. The cells were shipped to Stratagene on dry ice. Poly-A + mRNA was purified with oligo(dT) and random primers. The cDNA library was cloned in a Lambda Zap II vector. The insert sizes ranged between 0.6–2.5 kb.

Purification of lambda DNA- preparation of template DNA for the PCR cDNA screening: After infection of *Escherichia coli* XL1-Blue MRF′ (Stratagene) with 270 000 pfu (plaque-forming units) of lambda phage, the cells were plated and grown at 37 °C overnight. The plates were overlaid with SM buffer (3.5 mL; 50 mM Tris, pH 8, 0.01 % gelatin, 100 mM NaCl, 10 mM Mg_2Cl_2). The covered plates, sealed with Parafilm, were incubated at room temperature for 3 h. The SM buffer was then removed to sterile 50 mL polypropylene tubes. Chloroform (1/50 volume) was added and mixed in by vortex. The tube was incubated at room temperature for 10 min. The phage DNA was further isolated and purified by high pureTM lambda isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). The purified DNA was examined in 0.4 % agarose gels and compared to uncut Lambda DNA standard.

Screening the *Atrina* cDNA library with PCR: The *Atrina rigida* cDNA library was screened by PCR. Amplification was performed with Expand High Fidelity enzyme (Roche Molecular Biochemicals, Mannheim, Germany) in an automated thermal gradient cycler by using cycles of 4 min incubation at 94 °C; 30X (1 min incubation at 94 °C, 2 min incubation at 52.5 °C with gradient of $G = 7.5$ °C, 3 min incubation at 72 °C); 7 min incubation at 72 °C. Four sets of degenerate primers were designed (Figure 7). Each set was reacted separately against T3 primer, ATTAACCCTCACTAAAGG.

Set 1: Degenerate primers encoding DDGSDD, sense direction:

1. 5' - GAT GAY GGN AGY GAY GAY - 3' 64 different primers
2. 5' - GAT GAY GGN TCN GAY GAY - 3' 128 different primers

Set 2: Degenerate primers encoding DDGSDD, antisense direction:

1. 5' - RTC RTC RTC NCC RTC ATC - 3' 64 different primers
2. 5' - RTC RTC NGA NCC RTC ATC - 3' 128 different primers

Set 3: Degenerate primers encoding DDGDDD, sense direction:

5' - GAT GAY GGN GAY GAY GAY - 3' 64 different primers

Set 4: Degenerate primers encoding DDGDDD, antisense direction:

5' - RTC RTC RTC NCC RTC RTC - 3' 64 different primers

Where: Y=C or T, N=A, G, C or T, R=A or G.

Figure 7. *Degenerate primer sets used to screen the* Atrina rigida *cDNA library with PCR. The two primers in set 1 were mixed and interacted as one degenerate primer against T3, as were the primers in set 2.*

The concentration of the purified lambda DNA in all the experiments was 50 ng per 50 μL solution. All reactions were performed at two annealing temperatures (57.5 and 60.1 °C). Six sets of reaction were performed for each degenerate primer. In four of them, the T3 primer concentration was 25 pmol per 50 μL solution and the degenerate primers were in variable concentrations (25, 50, 100, and 200 pmol, 50 μL). In the other two reactions, the T3 concentration was 10 pmol per 50 μL and the degenerate primer concentrations were 25 or 100 pmol per 50 μL. The PCR products were examined in 2 % agarose gels and compared to a low DNA mass ladder standard.

cDNA cloning and sequencing: The PCR products were cloned in a pGEM®-T Easy vector system (Promega, Madison, WI, USA). Positive clones, which contain insert DNA were identified as white col-

onies on LB agar plates containing IPTG and X-Gal in contrast to the blue colonies, which did not contain insert DNA. The plasmid DNA was purified by QIAprep® miniprep kit (Qiagen, Valencia, CA, USA). The DNA was examined in 1% agarose gel and compared to DNA mass ladder standard. The plasmid DNA was sequenced by using SP6 primer, CCAAGCTATTTAGGTGACAC. Each DNA sequence was screened with VecScreen program, which is part of the NCBI WWW BLAST server. After the vector sequence had been excluded, the insert sequence was copied to SeqEd editor (Wisconsin package), and six-frame translation was performed on Map program (Wisconsin package). Multiple alignments were performed with the CLUSTALW (1.82) program and viewed with the PrettyBox program. Protein analysis was performed with PEPTIDESORT program. Only one continuous frame, which does not contain a stop codon in the middle and includes the original primer sequence (encoding either DDGSDD or DDGDDD), was chosen to be the representative frame.

Labeling DNA probe for fluorescence screening of *Atrina* cDNA library: *Atrina* cDNA fluorescent screening was performed with the DIG-dUTP system. The PCR DIG probe synthesis kit, DIG wash and block buffer set, DIG easy hyb granules, Anti-Digoxigenin-AP Fab fragments, and CDP-star reagents were purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Probe labeling with DIG-dUTP was performed in two steps with PCR. In the first step the DNA fragment was amplified in the absence of DIG-dUTP. The template DNA was the DNA sequence, which encodes to sequence 1 in Figure 1 (50 ng, 50 μL). The specific primers were: CATCATCGACATCATTTTCATCTA and AACATGAAGGGGTTAGCCATTTT. Amplification was performed with Expand High Fidelity enzyme in an automated thermal gradient cycler by using cycles of 2 min incubation at 95 °C; 10× (30 s incubation at 95 °C, 30 s incubation at 62–0.3 °C per cycle, 40 s incubation at 72 °C); 20× (30 s incubation at 95 °C, 30 s incubation at 59 °C, 40 s incubation at 72 °C), 7 min incubation at 72 °C. The PCR product was observed in 3% agarose gel and compared to low DNA mass ladder standard. At the second step the PCR reaction was performed on the PCR product of the first step (10 pg, 50 μL) with the same primers, but in the presence of PCR DIG labeling mix. The PCR conditions were the same as the first step. As a control, the second-step PCR reaction was performed in the absence of DIG-dUTP. This control product was sequenced to verify the probe sequence.

Plaque lifts: After infection of *E. coli* XL1-Blue MRF′ with 3000 pfu of lambda phage, the cells were plated and grown at 37 °C overnight. The plates were then incubated for 2 h at 4 °C. Transfer membranes marked with three asymmetrical dots were placed on each plate for 1 min. A second control membrane, marked at the same position, was placed on the same plate for 2 min. The membranes were incubated in denaturation solution (100 mL, 1.5 M NaCl, 0.5 M NaOH) for 3 min, transferred to neutralizing solution (100 mL, 1.5 M NaCl, 0.5 M Tris·HCl, pH 7.5) for 5 min, and then incubated in 2xSSC solution (100 mL, 0.3 M NaCl, 0.03 M Na-citrate·$2H_2O$) for 30 s. The membranes were cross-linked on cross-linker DNA and then air-dried.

Plaque hybridization: The plaque-lift membranes were incubated in prewash solution (0.75 M NaCl, 75 mM Na-citrate·$2H_2O$, 0.5% SDS, and 1 mM EDTA, pH 8) for 1 h at 42 °C with shaking. They were then placed in a 24 cm hybridization roller bottle (TermoHYBAID) containing hybridization solution (10 mL). Each bottle contained up to 10 membranes. The roller bottles were incubated (4 h, 42 °C) in a hybridization oven (TermoHYBAID) with rolling. The hybridization solution was changed after 2 h. The DNA probe (25 $ngmL^{-1}$, 10 mL hybridization solution) was incubated at 95 °C (3 min) and then on ice (1 min). This probe was added to the hybridization bottles and incubated while rolling overnight at 42 °C in the hybridization oven. The hybridization solution was discarded and pre-warmed (42 °C) low-stringency solution (30 mL; 0.3 M NaCl, 0.03 M Na-citrate·$2H_2O$, 0.1% SDS) was added to the rolling bottles for 5 min incubation. This step was repeated once more. The membranes were placed on prewarmed (62 °C) high-stringency solution (200 mL; 30 mM NaCl, 3 mM Na-citrate·$2H_2O$, 0.1% SDS) for 1 h incubation at 62 °C with shaking. This step was repeated once more.

A 192 bp DNA probe was amplified by PCR in the presence of DIG-dUTP. This labeled probe was hybridized to *Atrina* cDNA inserted in the Lambda phage plaques and visualized with a chemiluminescence substrate, which when dephosphorylated leads to light emission recorded on X-ray film. The phage plaques containing hybridized cDNA, were isolated and hybridized again to the labeled probe. Plaque isolation and hybridization processes were repeated until single plaques were detected. Their insert DNA was then excised and sequenced.

Probe detection: The hybridization membranes were incubated with blocking buffer (30 min). Each membrane, with the agar side facing down, was incubated with DIG antibody (3 mL, 30 min), then twice in washing buffer (15 min each) and then in detection buffer (5 min). The membranes were incubated with the chemiluminescent substrate CPD star (500 μL, 15 min, 37 °C) and then transferred in the dark to a cassette (JPI) containing X-ray film (35X43 cm) for 10 min incubation. The film was developed on a Kodak M35 X-OMAT processor. The spots obtained in each membrane were compared to the second hybridized control membrane, and the spatially separated dots were identified relative to the original plaques. The hybridized plaques were identified and isolated. These plaques were used for the second hybridization and fluorescent detection. The resulting positive plaques were continued to the third hybridization and fluorescent detection until a single plaque was isolated.

DNA excision and sequencing: The pBluscript SK(−) phagemid was excised from the Lambda zap II vector by using ExAssist helper phage with SOLR strain. These plasmid-DNA inserted sequences were determined by backward and forward sequencing with T3 GCGCAATTAACCCTCACTAAAG and T7 TAATACGACTCACTATAGGG primers. These sequences were then completed by sequencing with specific internal primers compatible to each sequence.

DNA sequence analysis: The vector sequences were determined by using the VecScreen program (NCBI WWW BLAST). These sequences were then omitted, and the unique inserts were analyzed by using SequencherTM 4.1 program. This program aligns all the sequences and gathers the homologous sequences into subgroups according to their similar domains. Six-frame translation of the sequences was performed by using the MAP program (Wisconsin package). Protein multiple alignments were performed with the CLUSTALW (1.82) program and viewed with the PrettyBox program. Signal-peptide prediction was performed with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). Database searches for homologous sequences were performed by using SwissProt, Swall, PROSITE, NR web databases (ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics) and the PDB website.

Synthetic polypeptides: CMKGLAILIAIAALLAVSHPK denominated Hyd and CSLSDPSDDGGANDVADDVEADAADL denominated ADL were synthesized by using an APEX 396 Multiple Peptides Synthe-

sizer (Advanced Chemtech, Louisville, KY, USA). The peptides were conjugated to BSA prior to producing polyclonal antibodies in order to stimulate an immune response after the injection to rabbits. The same peptides were conjugated to KLH (keyhole limpet hemocyanin) for binding to a polystyrene dish for ELISA detection.

Polyclonal antibodies against the synthetic polypeptide: Polyclonal antibodies were produced according to the following procedure. New Zealand rabbits (3 months old) were injected subcutaneously with synthetic polypeptide (100 μg) conjugated to BSA in complete Freund's adjuvant. 3 weeks later they were boosted subcutaneously with antigen (100 μg) in incomplete adjuvant, and 3 weeks after that boosted intermuscularly with antigen in PBS (100 μg). The rabbits were bled 10 days later. The presence of antigen was tested. Two boosts were given in PBS at 3 weeks intervals and bled 10 days later. These experiments conformed to the US National Institures of Health ethical guidelines.

The specificity of the polyclonal antibodies was tested with ELISA in which the synthetic polypeptide conjugated to KLH (25 μg) was treated with diluted antibodies (1:100, 1:300, 1:600, 1:900). The specific ELISA conditions and controls are as described below. The antibodies and the preimmune serum were purified by using Immunopure (G) IgG purification kit (Pierce, Rockford, IL, USA).

Enzyme linked immunosorbent assay (ELISA): Soluble proteins were extracted either from the nacreous aragonitic layer or from the prismatic calcitic layer of the shell of the mollusk *Atrina rigida* by using an ion-exchange resin for dissolution of the mineral as describe elsewhere.[21] The proteins were diluted in PBS (containing magnesium and calcium) and incubated in a 96-well multidish overnight (10 μg, 0.1 mL per well, 4 °C). Protein concentrations were based on amino acid analysis. Note that the OD of the antibody solution was 1.0, and that when 5 μg of protein was used, no significant reaction was obtained. The unbound proteins were removed by washing three times with PBS and Tween 20 (0.05 %, *v/v*, 3 min). The wells were then incubated with 0.5 % (*w/v*) gelatin for 1 h to block the exposed region. Purified antibodies diluted in PBS (1:500) were applied to each well (0.1 mL per well), incubated for 1 h, and then washed (×3, 3 min) with PBS and Tween 20 (0.05 %, *v/v*). The secondary antibody, alkaline phosphatase conjugated goat-anti-rabbit IgG diluted 1:1000 in PBS and Tween 20 (0.05 %, *v/v*) was applied to each well (0.1 mL per well) and incubated for 1 h. The unbound secondary antibody was washed (3×, 3 min) with PBS and Tween 20 (0.05 %, *v/v*). For the standard color reaction, *p*-nitrophenylphosphate was dissolved at 1 mg mL^{-1} in substrate buffer (10 % triethanolamine buffer, pH 9.8, containing 0.01 % $MgCl_2$ and 0.02 % NaN_3) and incubated (0.1 mL per well) for 30 min. The color reaction was stopped by EDTA (0.1 M, 0.1 mL per well). Absorbance was measured at 405 nm with a Spectrafluor Plus fluorometer (TECAN, Switzerland). Negative controls were: 1) The pre-immune serum (diluted 1:500), 2) no antigen, 3) no antibody. Positive controls were: ADL synthetic polypeptide conjugated to KLH (25 μg) interacted with antiADL polyclonal antibodies (diluted 1:500) and Hyd synthetic polypeptide conjugated to KLH (25 μg) interacted with the antiHyd polyclonal antibodies (diluted 1:500)

The nucleotide sequences of Asprich (a) to (g) and Asprich 1 to 3 were submitted to NCBI GenBank. The accession numbers are: AY660589, AY660590, AY660591, AY660592, AY660593, AY660594, AY660595, AY660596, AY660597, and AY660598.

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