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Molecular Cloning of the Crustacean *DD4* cDNA Encoding a Ca^{2+}-Binding Protein

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A cDNA, named *DD4,* was identified in the prawn *Penaeus japonicus* in a search for genes that were expressed during calcification of the crustacean exoskeleton. *DD4* transcripts were detected in the epidermal cells underlying the exoskeleton specifically during the postmolt stage, when the calcification takes place. In the *DD4* cDNA an open reading frame of 542 amino acids was found. The deduced protein was acidic and proline-rich, and exhibited similarity to the *Drosophila* Ca^{2+}-binding protein calphotin in the amino acid sequence and composition. The *DD4* cDNA was expressed in *Escherichia coli* to characterize Ca^{2+}-binding of the encoded protein, and Ca^{2+} was found to bind to a central segment of 186 amino acids. The DD4 protein is suggested to play a role in the calcification of the crustacean exoskeleton.

Key Words: **calcification; crustacean; molt; calcium; cuticle; exoskeleton; calphotin.**

Many animal species have developed skeletal structures which are hardened by deposition of inorganic mineral crystals. Exoskeletons mineralized with calcium carbonate crystals are commonly found among invertebrates such as protozoas (e.g., foraminiferans), cnidarians (e.g., corals), mollusks (e.g., shellfish), and arthropods (e.g., crustaceans) (1). However, little is known about the molecular mechanisms of the calcification processes in invertebrates, which is in contrast to the more well-characterized vertebrate hard tissues which are mineralized with calcium phosphate (1).

The exoskeleton of crustaceans offers an advantageous system for molecular dissection of the calcification process, as it takes place specifically during the postmolt stage of the molt cycle. Growth in crustaceans is associated with periodic casting (molting) and subsequent reconstruction of the exoskeleton. The construction of a new exoskeleton starts during the premolt stage when the two outer layers of the exoskeleton, the epicuticle and exocuticle, are formed. The formation of the exoskeleton continues during the postmolt stage, as the endocuticle, the inner layer, is formed and the new exoskeleton becomes calcified with calcium carbonate crystals (1). There exists variation in the extent of exoskeletal calcification among crustaceans, as both the exo- and endocuticle are calcified in crabs, whereas in penaeid prawns significant calcification can be observed only in the exocuticle (2).

The authors conducted a search for genes which are expressed in the crustacean integument specifically during the postmolt stage, on the premise that some of the genes thus identified are involved in the calcification of the exoskeleton. Differential display and Northern analyses were employed in the search to identify four postmolt-specific mRNA species from the prawn *Penaeus japonicus* (3, 4). Three of the identified mRNAs, *DD9A*, *DD9B* and *DD5*, encode members of a family of arthropod cuticular proteins which contain variants of the Rebers-Riddiford consensus sequence (4; T. Ikeya, P. Persson, and T. Watanabe, unpublished observation). In the present report, we describe analysis of another mRNA, named *DD4*, which encodes a new crustacean Ca^{2+}-binding protein.

MATERIALS AND METHODS

Isolation of RNA and differential display analysis. Live adult prawns *P. japonicus* caught in the Hamanako Lake (Shizuoka, Japan) were purchased from a local commercial source, and kept in 500 l tanks with through-flowing brackish lake water until sampled. Based on the cuticular morphology of the mandibular expedite (5), prawns were grouped into the following four molting stages: postmolt (stages A and B), intermolt (C), early premolt (D_{0-2}) and late premolt (D_{3-4}). The tail fan and blade were collected from the staged animals, and total RNA was isolated as previously described (4) and subjected to differential display analysis. Poly(A)$^+$ RNA used in the 5′ rapid amplification of cDNA ends and Northern analysis was derived from the tail fan alone by the same procedure, and was prepared as previously described (4). Differential display analysis was carried out as previously described (4), except that the following primer set was used to isolate a cDNA fragment of 72 bp (see Results): 5′-AAGCTTCGACTGT-3′ and 5′-AAGCTTTTTTTTTTTA-3′.

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Sequence analysis of cDNA clones. A library of cDNA prepared from the tail fan of postmolt prawns (4) was screened using a probe of the 72 bp cDNA. Positive lambda phage plaques were purified in two rounds of screening, and recombinant pBluescript SK− phagemids were rescued from the phage clones according to the instructions of the manufacturer (Stratagene). Four subfragments of clone 4–3 (corresponding to nucleotide positions 787–967, 968–1612, 1613–2274 and 2275–2500 in Fig. 1) were generated by restriction digestion and subcloned into the pBluescript SK− vector. They were sequenced on both strands using the universal forward and reverse primers. Sequencing reactions were performed as previously described (6), and the products were electrophoresed and analyzed using the SQ-3000/32 (Hitachi) or DSQ-2000L (Shimadzu) automatic DNA sequencer.

5′ rapid amplification of cDNA ends (RACE). To obtain the *DD4* cDNA sequence which was not represented in clone 4–3, 5′ RACE analysis was performed. First, an antisense primer (inverse-complementary to nucleotide positions 922–941 in Fig. 1) was annealed to the postmolt tail fan poly(A)$^+$ RNA, and the first strand cDNA was synthesized using M-MLV Reverse Transcriptase RNaseH$^-$ (TOYOBO) and poly(A)-tailed using terminal deoxynucleotidyl transferase (TOYOBO). The cDNA was subjected to the following PCR reaction using an antisense primer (inverse-complementary to nucleotide position 853–872 in Fig. 1) and two sense primers (5′-GAGTCGACTCGAGAATTCT$_{17}$-3′ and 5′-GAGTCGACTCGAGAATTC-3′): initial denaturation at 95°C for 5 min; 30 cycles of PCR reaction at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min; and final extension at 72°C for 15 min. PCR products were subcloned into the pCR2.1 vector (Invitrogen) and sequenced.

Northern analysis and in situ hybridization. The Northern blot was prepared as previously described (4). The filter was hybridized with a *DD4* probe (corresponding to nucleotide position 1613–2274 in Fig. 1) labeled with [a-^{32}P]dATP. The filter was reprobed for *PjL26* transcripts, which was expressed constitutively during the molt cycle (7), to ensure comparable loading of the four poly(A)$^+$ RNA samples. Procedures for hybridization and subsequent washing were the same as previously described (7). To identify cells expressing *DD4, in situ* hybridization analysis was carried out according to the previously described procedure (4). Digoxygenin (DIG)-labeled sense and antisense riboprobes (corresponding to nucleotide position 787–972 in Fig. 1) were hybridized to tissue sections of a postmolt (stage A) tail fan.

Protein expression in Escherichia coli and $^{45}Ca^{2+}$ overlay analysis. Three overlapping segments of the DD4 protein were expressed in *E. coli* and their Ca^{2+}-binding was characterized. First, cDNA fragments corresponding to a N-terminal (amino acid (aa) position 16–224 in Fig. 1), central (196–381) and C-terminal (363–542) region were amplified using PCR. The N-terminal cDNA fragment was amplified using the cloned 5′ RACE product with the longest insert as the template with the following two primers: NtF (5′-CGGGATCCACTCCTGCTGCGCCTGT-3′) and NtR (5′-CGGGATCCTAATCTACCCCTTCGACCT-3′). Clone 4–3 was used as the template to amplify the central and C-terminal fragments. The primer pairs were CeF (5′-CGGGATCCGATGAGTTCGTAGAGGT-3′) and CeR (5′-CGGGATCCCTAGTCAGGTTCAAGTGTCT-3′) for the central fragment and CtF (5′-CGGGATCCCCTGTTGAGCCTATTGCATA-3′) and CtR (5′-CGGGATCCTGGCTCATGGCCTGG-3′) for the C-terminal fragment. The three PCR products were cloned into the *Bam*HI site of the PET-5a expression vector (Promega), and the recombinant constructs were introduced into the BL21(DE3) strain of *E. coli*. Expression of the three partial DD4 proteins was induced in 1 mM of isopropyl-β-thiogalactoside (IPTG) for six hours. The three proteins contained at the N-termini a segment of 14 aa (Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Gly-Ser) derived from the vector. Expressing cells were harvested by centrifugation, and boiled for 10 min in a sample buffer (25 mM Tris–HCl (pH 6.8)), 5% glycerol (w/v), 1% SDS, 2.5% 2-mercaptoethanol, and 0.01% bromophenol blue). The cell extracts and calmodulin (400 ng) were run in duplicates on a 13% SDS–polyacrylamide gel. After electrophoresis one set of the samples in one half of the gel was stained with the fluorescent dye Sypro Red (FMC Bio Product) to quantify proteins using a FLA 2000 computerized densitometer scanner (Fuji Film). A replica in the other half was blotted onto a PVDF membrane at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol and 0.03% SDS using a Mini Trans-Blot electrophoretic transfer cell (BIO-RAD). After the blotting, the gel was stained with Sypro Red to detect proteins remaining in the gel; most of the protein bands including the partial DD4 proteins and calmodulin were not detectable.

Ca^{2+}-binding of the partial DD4 proteins was characterized using the $^{45}Ca^{2+}$ overlay analysis (8). Briefly, Western blots on PVDF membranes were incubated in a buffer containing $^{45}Ca^{2+}$ (2 mM) for ten min and subsequently washed in 50% ethanol for 30 s, and $^{45}Ca^{2+}$ bound to protein bands was quantified on a relative scale using a FLA 2000 computerized densitometer scanner (Fuji Film). The relative Ca^{2+}-binding capacity of a protein band was determined by dividing the level of bound $^{45}Ca^{2+}$ by the protein amount estimated from the level of fluorescence of the band stained with Sypro Red. The level of the fluorescence was found to be proportional to the amount of the protein in the range of 100 ng and 2000 ng, using phosphorylase b. Thus, the amounts of the loaded partial DD4 proteins and calmodulin were set within this range.

RESULTS

Identification of the DD4 mRNA. Total RNA was prepared from the tail fan and blade, consisting of mainly the exoskeleton and underlying epidermis, of prawns at the following four molting stages: postmolt, intermolt, early premolt and late premolt. The four RNA samples were subjected to a differential display analysis to identify postmolt-specific transcripts. One cDNA fragment of 72 base pairs (bp), excluding the primer sequences, was amplified from the postmolt sample, but not from the intermolt, and early and late premolt samples (data not shown), suggesting that it was derived from a postmolt-specific transcript. As described below, the stage specificity of expression was confirmed using Northern analysis.

To isolate cDNA clones of the putative postmolt-specific transcript, the cDNA fragment was used as a probe in a screen of a library of cDNA which was prepared from the postmolt tail fan. Eight positive clones were isolated and the lengths of the inserts were assessed using agarose gel electrophoresis (data not shown). The clone with the longest insert (named clone 4–3) was selected for sequence analysis. Its insert was 1,714 bp (nucleotide positions 787–2500 in Fig. 1) excluding the poly(A) tail with an open reading frame of 347 amino acids (amino acid (aa) position 196–542 in Fig. 1).

Since the size of the insert was significantly shorter than that of the transcripts detected in Northern analysis (see below), 5′-RACE was performed to obtain additional nucleotide sequences on the 5′ side. The PCR products were subcloned, and the inserts of the three longest clones were sequenced. The longest insert was 852 bp (nucleotide position 1–852 in Fig. 1), and the other two clones were apparently derived from the

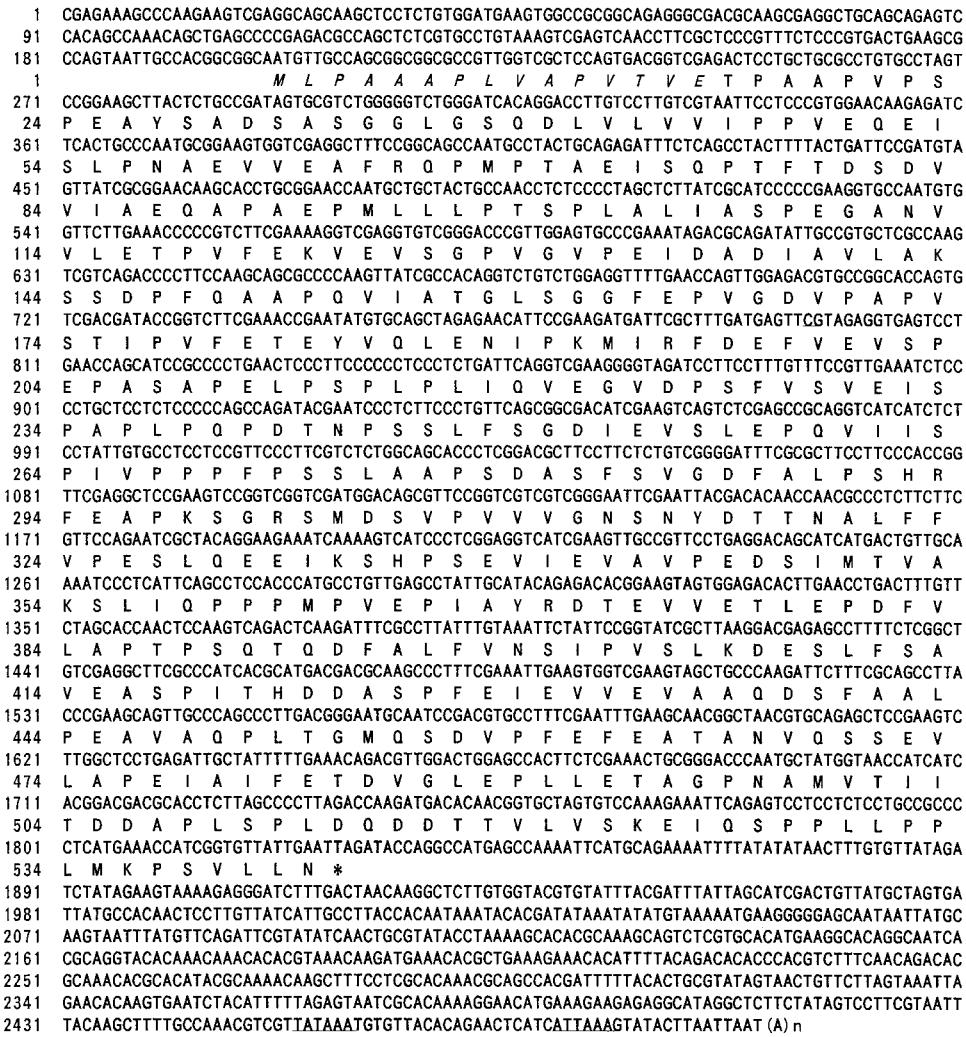

FIG. 1. Nucleotide sequence of *DD4* and amino acid sequence of the conceptual DD4 protein. Sequences (TATAAA and ATTAAA) similar to the consensus polyadenylation signal (AATAAA) are underlined. C^{795} (underlined) is changed to T in the 5′-RACE product. The potential signal peptide (Met^{1}-Glu^{15}) is italicized.

same transcript (data not shown). In the region where the longest RACE product overlapped with the clone 4–3 insert (nucleotide positions 787–852), only one mismatch was found (Fig. 1) which did not result in amino acid substitution in the conceptual translation (see below). We presume that this minor difference was derived from genetic polymorphism among the wild prawn population. The merged cDNA sequence will hereafter be referred to as *DD4*. Although no consensus polyadenylation signal was present within 100 bp from the polyadenylation site, two similar sequences (TATAAA and ATTAAA) were found (Fig. 1).

Conceptual DD4 protein. The *DD4* cDNA sequence was conceptually translated and an open reading frame of 542 amino acids was found (Fig. 1). Results of a computer-aided prediction for the presence of a signal peptide (9) were equivocal. According to three measures (maximum Y, maximum C and maximum S scores), the conceptual DD4 protein was unlikely to contain a signal sequence. Conversely, the mean-S score, which is considered to give a better prediction as to the presence of signal peptides than the other three measures, suggests that DD4 possesses a signal peptide with the most likely signal cleavage site between Glu^{15} and Thr^{16}. See Discussion for further argument as to the presence of a signal peptide.

Including the potential signal peptide, the predicted molecular weight of the protein was 56,957, and the predicted isoelectric point (pI) was 3.53. The fraction of acidic amino acids (Glu and Asp) was not very high (15.1%), but that of basic amino acids (His, Arg and Lys) was low (3.2%) (Table 1), resulting in a low pI. Hydrophobic amino acids comprised 59.8% of the total protein, with Pro, Val and Ala being predominant (Table 1). Among neutral and hydrophilic residues, Ser and Glu were the most common (Table 1).

TABLE 1

Comparison of Amino Acid Compositions (in Percentage) of the DD4 and Calphotin Proteins

	DD4*	Calphotin
Hydrophobic		
Gly	3.3 (3.4)	0.7
Ala	10.2 (9.7)	20.4
Val	11.8 (11.6)	16.8
Leu	8.3 (8.2)	3.1
Ile	5.5 (5.7)	6.0
Met	1.9 (1.7)	0.2
Phe	4.6 (4.7)	0.1
Trp	0.0 (0.0)	0.0
Pro	14.2 (14.0)	20.6
Neutral		
Ser	10.3 (10.6)	6.2
Thr	5.0 (4.9)	9.5
Asn	2.0 (2.1)	1.0
Gln	3.9 (4.0)	1.7
Cys	0.0 (0.0)	0.2
Hydrophilic		
Asp	5.7 (5.9)	2.8
Glu	9.4 (9.5)	8.8
Lys	1.7 (1.7)	1.3
His	0.6 (0.6)	0.1
Arg	0.9 (1.0)	0.4
Tyr	0.7 (0.8)	0.1

* Numbers in parentheses indicate composition in the DD4 protein excluding the potential signal peptide.

A protein sequence database (DNA Databank of Japan 'Protein All' database (version 3.0t84) which included PIR and Swiss Prot) was sought using the FASTA program (10) to find proteins homologous to DD4. Calphotin, a Ca^{2+}-binding protein expressed in photoreceptor cells of *Drosophila melanogaster* (11, 12), was found to be the most similar, though the similarity score was not very high (optimized similarity score and z-score were 304 and 315.3, respectively). In the optimized alignment in which gaps were allowed, 26.7% identity was found at the amino acid level over the entire DD4 protein, but no regions of high similarity were found (data not shown). Calphotin and DD4 were similar in their amino acid compositions (Table 1). Calphotin was rich in hydrophobic amino acids such as Pro, Ala and Val, and the fraction of the basic amino acids were low, resulting in a very low predicted pI (3.43). Unlike calphotin, however, the DD4 protein did not possess the leucine zipper-like motif.

DD4 expression. Stage specificity of *DD4* expression during the molt cycle was examined using Northern analysis (Fig. 2). Strong hybridization was seen to a band of about 3.0 kb in the postmolt sample, but not in the intermolt and premolt samples. Weaker hybridization was also detected at about 4.0 kb in the postmolt sample, suggesting that more than one mRNA species may be expressed due to alternative splicing, or differential use of promoters or polyadenylation sites. Expression levels of the constitutively expressed *PjL26* gene (7) were similar in all four samples.

In situ hybridization analysis was carried out to identify *DD4*-expressing cells in the tail fan of a postmolt prawn. *DD4* expression was detected in epidermal cells underlying the exoskeleton (Fig. 3A). Signals were also detected in the exoskeleton, which were most likely due to nonspecific adsorption of the riboprobe to the exoskeleton, as similar signals were observed in a control experiment using a sense riboprobe (Fig. 3B). Such nonspecific binding of riboprobes to crustacean exoskeleton has been reported previously (4).

Ca^{2+}-binding of the encoded protein. Sequence similarity between calphotin and the conceptual DD4 protein prompted us to test Ca^{2+}-binding of the latter. *DD4* cDNA fragments cloned in an expression vector were introduced into *E. coli* to express three overlapping segments of the protein (aa position 16–224, 196–381, 363–542; the first 15 amino acids, which potentially constituted the signal peptide, were not included). Attempts to express larger segments resulted in much lower expression levels (data not shown). Proteins extracted from the expressing *E. coli* cells were separated by SDS-PAGE, and the Western blots were subjected to $^{45}Ca^{2+}$ overlay analysis. Levels of Ca^{2+} bound to the partial DD4 proteins were compared to that to calmodulin (Fig. 4).

Significant Ca^{2+} binding was detected in the protein containing the central segment, but not in the other two. The observed Ca^{2+} binding should not be due to the stretch of 14 aa derived from the expression vector (see Materials and Methods), as the two proteins which did not bind Ca^{2+} also contained it. The level of Ca^{2+} binding (per mol of the protein) to the central segment was estimated to be 15.9 ± 5.4% (N = 4) of that to calmodulin (13). Amino acids involved in Ca^{2+}-binding

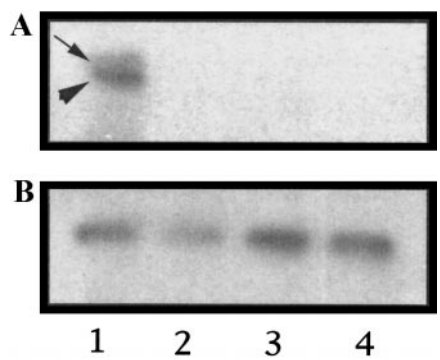

FIG. 2. Northern analysis of *DD4* transcripts. A Northern blot of poly(A)$^+$ RNA samples was hybridized with radio-labeled *DD4* (A) and *PjL26* (B) probes. The RNA samples were prepared from the tail fan at the following four phases of the molt cycle: lane 1, postmolt; 2, intermolt; 3, early premolt; 4, late premolt. Strong hybridization of the DD4 probe was seen to a band of about 3.0 kb (arrowhead in A). A weaker signal was also detected at about 4.0 kb (arrow).

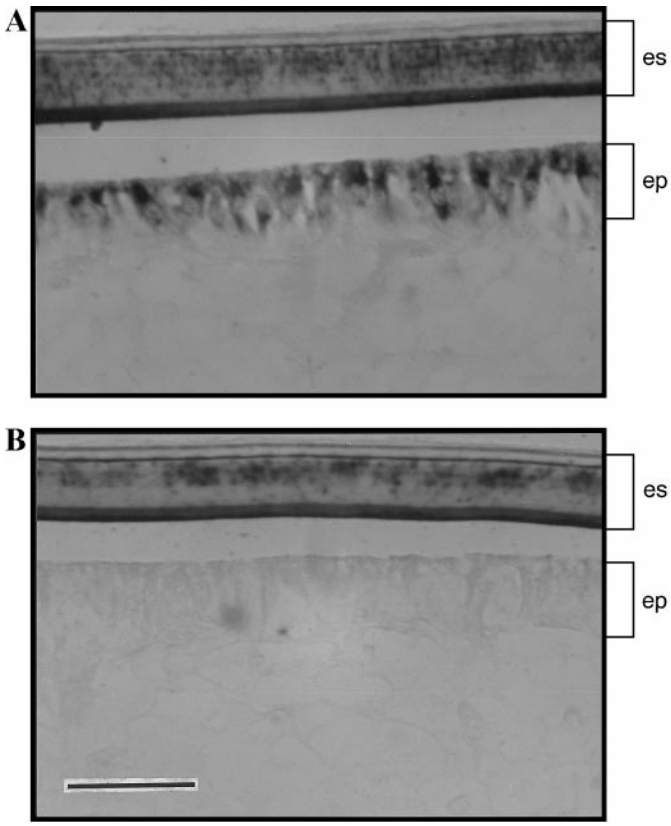

FIG. 3. *In situ* hybridization analysis of *DD4*-expressing cells in postmolt prawn. (A) A DIG-labeled *DD4* antisense riboprobe was hybridized to tissue sections of a proximal part of a tail fan at stage (A). Hybridization signal is detected in the epithelial cells (ep). The space between the epithelial layer and the exoskeleton (es) was artificially generated. (B) Hybridization of a sense riboprobe. Signal is observed in the exoskeleton but not in epithelial cells. Scale bar = 50 μm.

could not be identified, as the DD4 protein did not contain any known Ca^{2+}-binding motif such as the EF-hand. The Ca^{2+}-binding should not be due merely to prevalence of acidic residues, since the predicted pI of the central segment (3.70) is higher than those of the N-terminal (3.42) or C-terminal (3.37) segment.

The $^{45}Ca^{2+}$ overlay analysis allowed quick identification of a Ca^{2+}-binding protein without the effort of purification. However, for quantitative analysis of the Ca^{2+}-binding (e.g., determination of the number of Ca^{2+}-binding sites or measurement of their affinities for Ca^{2+}), purification of the protein without denaturing it would be prerequisite. Our attempt to solubilize the DD4 protein from the exoskeleton has not been successful, so we plan to establish a baculovirus overexpressing system as an alternative source of the protein for further characterization of Ca^{2+}-binding.

DISCUSSION

Results of the analyses of the *DD4* mRNA and the encoded protein are consistent with the possibility that the DD4 protein is involved in calcification of the exoskeleton. Firstly, he protein was found to bind Ca^{2+}. Secondly, *DD4* expression in the tail fan was detected specifically during the postmolt stage when the exoskeleton becomes calcified. Finally, the transcripts were detected in the epidermal cells which secrete organic component of the exoskeleton. Results of preliminary immunohistochemical analysis argue that DD4 is exported to the exoskeleton, as higher levels of immunological signals were observed in the exoskeleton than the epidermis (H. Endo, Y. Takagi and T. Watanabe, unpublished observation). This observation would argue for the presence of a signal peptide in the DD4 protein. Furthermore, the signals were distributed mainly in the exocuticle, the most heavily calcified layer of the exoskeleton in penaeid prawns, suggesting a positive role of the protein in the calcification process.

DD4 exhibited similarity to calphotin of *D. melanogaster* in amino acid sequence and composition. Calphotin is expressed specifically in photoreceptor cells and localized in cytoplasmic compartments where calcium precipitates of unknown crystalline structure are observed (11). Calphotin binds Ca^{2+}, and therefore is implicated in formation of the Ca precipitates, although its precise role is unknown (11, 12). Similarly, the role of DD4 in the calcification process remains to be elucidated. It may be involved in storage of Ca^{2+} or transport of Ca^{2+} to the exoskeleton. Alternatively, it may promote formation of nuclei of calcium carbonate crystals by interacting with Ca^{2+}, as Ca^{2+}-binding proteins in skeletal matrices are implicated in nucleation of both calcium phosphate and calcium carbonate crystals (reviewed in 14–16), though some Ca^{2+}-binding proteins may be involved in inhibition of crystal growth

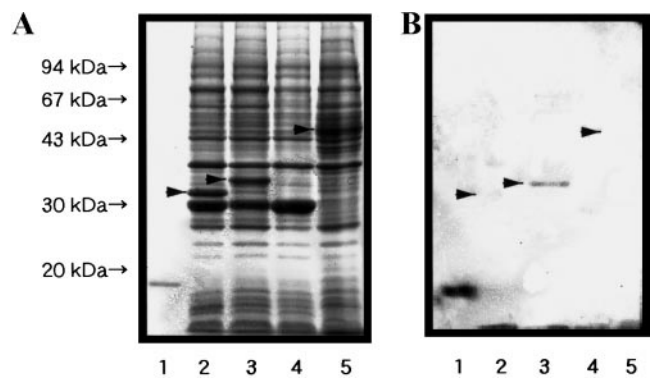

FIG. 4. Ca^{2+}-binding analysis of partial DD4 proteins expressed in *E. coli*. (A) SDS–PAGE of partial DD4 proteins. Lane 1, calmodulin (a positive control); 2, extract of *E. coli* expressing the N-terminal segment (arrowhead); 3, extract of *E. coli* expressing the central segment (arrowhead); 4, extract of *E. coli* without an expression construct (a negative control); 5, extract of *E. coli* expressing the C-terminal segment (arrowhead). Positions of molecular weight markers are indicated on the left. (B) A $^{45}Ca^{2+}$ overlay of a Western blot. Significant binding of Ca^{2+} was detected only in calmodulin (lane 1, arrow) and the central segment (lane 3, arrowhead).

(17). These possibilities shall be addressed in future studies using *in vitro* crystallization experiments and immunohistochemical analyses at the electron microscopic level.

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