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Identification and Characterization of a Novel Calcineurin-Binding Protein in Scallop Testis

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Calcineurin has been inferred to function in meiosis and spermiogenesis in testis. Here, we identified a calcineurin-binding protein in scallop testis by Far-Western blot analysis using purified calcineurin as a probe. The molecular mass of the binding protein estimated on the blot was 75 kDa. The isolated cDNA clone encoded a novel 474-residue protein, named CaNBP75. The region between T6 and A210 of CaNBP75 was responsible for the interaction with calcineurin. CaNBP75 was predominantly expressed in testis and ovary of scallop. Thus, CaNBP75 may modulate the physiological function of calcineurin in the testis and ovary of scallop, such as in spermiogenesis or meiosis.

Key words: calcineurin, meiosis, Ran-binding motif, spermiogenesis, testis.

Abbreviations: AKAP, protein kinase A–anchoring protein; CaNA, calcineurin catalytic subunit; CaNB, calcineurin calcium–binding subunit; CaNBP75, calcineurin-binding protein of 75 kDa; CBB, Coomassie Brilliant Blue; GST, glutathione-S-transferase; 2-ME, 2-mercaptoethanol; PMSF, phenylmethane sulfonyl fluoride; 5′-RACE, 5′-rapid amplification of cDNA ends; RanBD, Ran-binding domain; RanBP3, Ran-binding protein 3.

Calcineurin, also known as serine/threonine-specific protein phosphatase 2B, is a calcium/calmodulin-dependent phosphatase that plays an important role in the transduction of calcium signals in a variety of cell types in from fungi to vertebrates (*1*, *2*). Calcineurin is a holoenzyme, consisting of a 55-kDa catalytic subunit (CaNA) and a 19-kDa calcium-binding subunit (CaNB). In testis, expression of calcineurin fluctuates during the cycle of spermatogenesis, peaking after meiosis (*3*, *4*). Calcineurin has been detected immunohistochemically in the nuclei of round or elongated spermatids (*4*). Furthermore, calcineurin has also been detected in the postacrosomal region and the flagella of mammalian spermatozoa (*5*). Therefore, it has been thought that calcineurin in testis is related to spermiogenesis and later to the regulation of flagellar motility.

Recently, several calcineurin-binding proteins have been isolated from various tissues. Protein kinase A-anchoring protein AKAP79 is an endogenous inhibitor of calcineurin, which binds to protein kinase A, calcineurin and protein kinase C in post-synaptic densities, and is considered to regulate the substrate specificity of calcineurin (*6–8*). Calsarcins bind to calcineurin and tether it to α-actinin at the z-line of the sarcomere of cardiac and skeletal muscle cells (*9*, *10*). Cain/cabin 1 interacts with calcineurin and inhibits its activity in a phosphorylation-dependent manner (*11*, *12*). Studies on these calcineurin-binding proteins indicated the possibility of a regulatory mechanism for calcineurin phosphatase activity different from the calcium/calmodulin system, whereas the presence of calcineurin-binding proteins in testis remains obscure.

Previously, we identified a calcineurin isoform similar to the mammalian brain-type in scallop testis (*4*, *13*). Here, we attempted to identify a potential regulator of calcineurin in scallop testis, and found a gonad-specific calcineurin-interacting protein named CaNBP75. The cDNA of CaNBP75 encodes a novel 51.9-kDa protein with a calcineurin-binding domain in its N-terminal region.

Preparation of scallop testis calcineurin, calmodulin, anti-calcineurin antiserum, and single-stranded cDNA, and construction of a scallop testis cDNA library were performed as described previously (*4*, *13*).

An extract of partially thawed scallop testis prepared with ten volumes of buffer A (20 mM imidazole-HCl, 1 mM EGTA, 5 mM $MgCl_2$, 50 mM NaCl, 14 mM 2-ME, 1 mM benzamidine, 1 mM PMSF, pH 7.0) was fractionated with ammonium sulfate, between 20 and 40% saturation, and the precipitate was homogenized with a small volume of buffer A. The dialysate obtained with buffer B (20 mM Na-phosphate, 1 mM EGTA, 5 mM $MgCl_2$, 14 mM 2-ME, pH 5.7) was clarified by centrifugation and adjusted to 10 mM NaCl, and then loaded onto a DEAE-Cellulofine column equilibrated with buffer C (buffer B containing 10 mM NaCl). The column was washed with buffer C and then developed with a linear gradient of 10 to 310 mM NaCl in buffer B (Fig. 1A). Calcineurin-binding proteins were detected by Far-Western blot analysis using purified calcineurin and anti-calcineurin antiserum. Samples were subjected to SDS–PAGE (*14*) and then transferred to a nitrocellulose membrane. The blot was preincubated in blocking buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 5% skim milk, pH 7.5), followed by incubation overnight in the blocking buffer supplemented with 0.25 μM calcineurin and 2 μM calmodulin. After washing with the blocking buffer, the blot was incubated with rabbit anti-scallop calcineurin antiserum,

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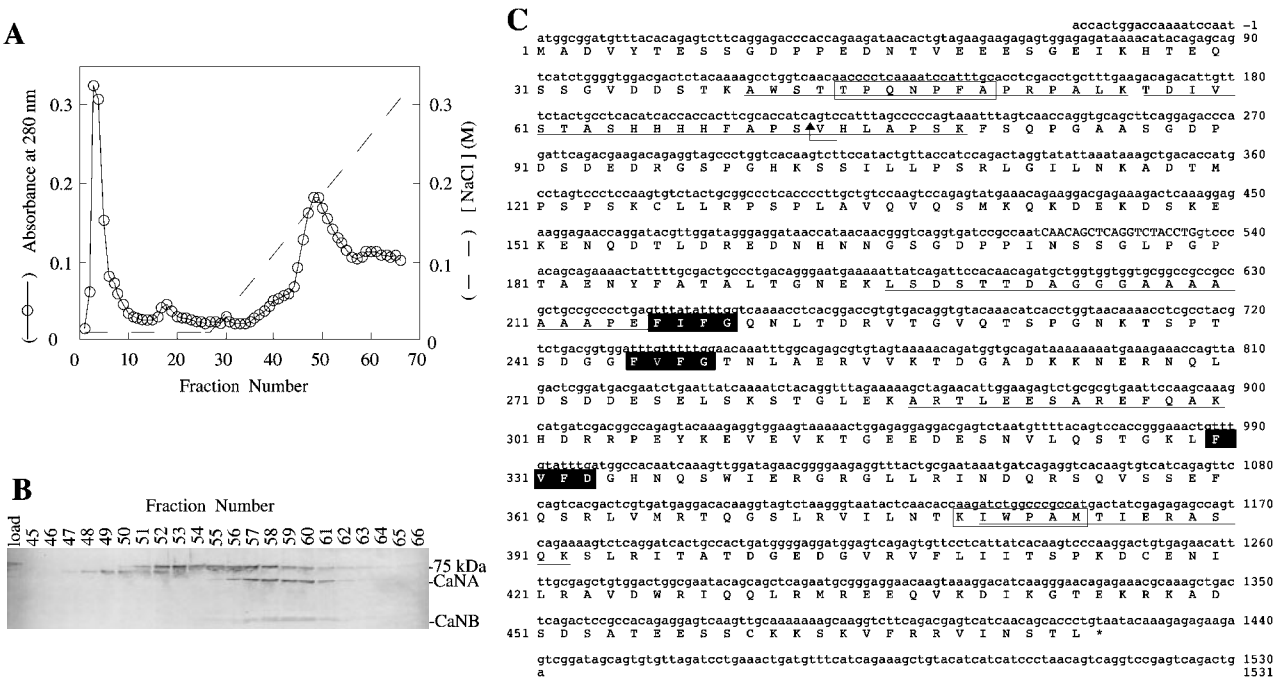

Fig. 1. **Identification of CaNBP75 and the results of cDNA cloning.** (A) The elution profile on DEAE-Cellulofine column chromatography. A scallop extract fractionated by ammonium sulfate and isoelectric precipitation was applied to a DEAE-Cellulofine column, and the adsorbed proteins were eluted with a linear gradient of NaCl, from 10 to 310 mM, in buffer B. (B) CaNBP75 in fractions in (A) was detected by Far-Western blot analysis using the purified calcineurin as a probe. Subunits of calcineurin in the extract were also detected. The fraction numbers are indicated at the tops of the lanes. (C) Partial nucleotide sequence of the cDNA for CaNBP75 (top) and the deduced amino acid sequence (bottom). The figures in the left and right margins indicate the amino acid and nucleotide positions, respectively. The underlines indicate the sequences determined by peptide sequencing. The asterisk indicates the stop codon. The boxed amino acid sequences were used to design degenerate primers for PCR. The nucleotide sequence shown in capitals indicates the position of reverse primer R1 used for 5′-RACE. FXFG- and FXFX-type sequence motifs are indicated by black boxes. An arrow indicates the position of a nine residue (GTVEVPTIK) insertion, which was identified by peptide sequencing and was confirmed in one of the cDNA clones.

followed by washing and then incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Bio-Rad). Proteins bound to calcineurin were detected with an AP color developing kit (Bio-Rad). A band corresponding to an estimated molecular mass of about 75 kDa, which was not detectable without calcineurin overlaying, was obtained for the fractions eluted with NaCl concentrations of between 130 mM and 230 mM (Fig. 1B, fractions 50 to 63). We tentatively named this protein CaNBP75, *i.e.* calcineurin-binding protein of 75 kDa. Proteins in the eluate were concentrated with Amicon 100 (Millipore) and then subjected to two-dimensional PAGE (*15*). The spots of calcineurin-binding proteins in two-dimensional gels identified on Far-Western blot analysis were dissected out and the proteins in the gel pieces were subjected to cleavage with lysylendopeptidase as described previously (*16*), and then the digests were subjected to reverse-phase HPLC. The amino acid sequences of the separated peptides were determined with a protein sequencer Procise 492 (Perkin-Elmer). A total of 104 residues in six peptide fragments were identified (Fig. 1C, underlined sequences), and a database search did not reveal any homologous sequences in hitherto available data.

Then, we examined cDNA amplification by the PCR method using scallop testis single-stranded DNA and Ex *Taq* DNA polymerase (Takara) with degenerate primers (5′ACNCCNCARAAYCCNTTYGC3′ and 5′GTCATNGCNGGCCADATYTT3′) designed on the basis of the peptide sequences (see Fig. 1C). Nucleotide sequences were determined by the dideoxy chain termination method with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Biosciences) using an automated DNA sequencer (LI-COR LIC-4000). The resulting product (frg68-69) of 985 bp that codes for the amino acid sequence determined on peptide sequence analysis was labeled with digoxigenin (DIG) with a DIG-DNA Labeling Mixture (Roche Diagnostics), and then used to screen the scallop testis cDNA library in lambda gt10. The 5′-untranslated region of the cDNA was amplified by 5′-RACE. A poly-C tail was added to the 3′-end of the cDNA using terminal deoxynucleotidyl transferase, and PCR was performed with primer sets, oligo-dG and reverse primer R1 (see Fig. 1C). Considering the overlapping of the determined sequences, a nucleotide sequence of the cDNA containing an ORF of 1,425 bp coding for an amino acid sequence of 474 residues with a calculated mass of 51,850 Da, a 5′-UTR of 21 bp and a 3′-UTR of 106 bp was obtained (Fig. 1C). The molecular mass of scallop CaNBP75 estimated by SDS-PAGE, *i.e.* about 75 kDa, was greater than the calculated mass, which might reflect the high percentage (29%) of charged residues. The calculated pI of 5.25 agreed with the results of two-dimensional PAGE. One of the clones screened contained

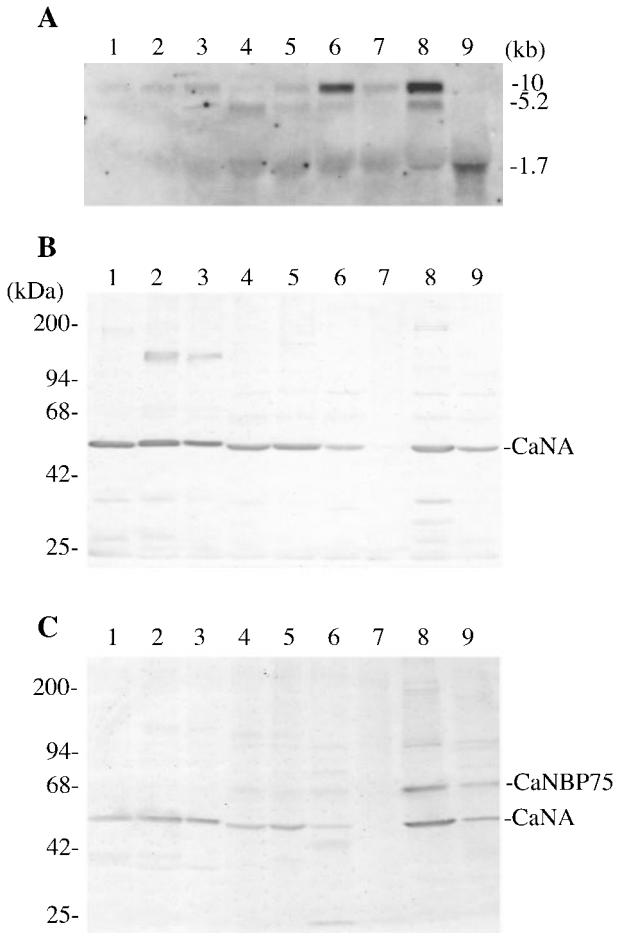

Fig. 2. **Tissue distribution of CaNBP75.** (A) Twenty-five micrograms of total RNA isolated from scallop tissues was separated on an agarose formaldehyde gel and then transferred to Hybond N+ (Amersham Biosciences) for Northern analysis. DIG-labeled CaNBP75 antisense RNA was prepared with the use of the DIG-RNA Labeling Mixture (Roche Diagnostics), SP6 RNA polymerase and cDNA fragment (frg68-69) as a template, and was then used as a probe. (B, C) Thirty μg of proteins in a crude extract was separated by 8% SDS-PAGE and then transferred to a nitrocellulose membrane. Calcineurin in the extract was detected by Western blotting with anti-calcineurin antiserum (B), and CaNBP75 was detected by Far-Western blot analysis (C). Lanes 1–9: striated adductor muscle, translucent adductor muscle, opaque adductor muscle, gill, mantle, kidney, digestive gland, testis and ovary, respectively.

a 27 base insertion coding for 9 residues, which was found in one of the sequenced peptides (Fig. 1C, indicated by an arrow).

We then examined the tissue distribution of CaNBP75 by Northern and Far-Western analyses. Northern blot analysis revealed a major transcript of approximately 10 kb with minor ones of about 5.2 and 1.7 kb. Since the Northern hybridization was performed under high stringent conditions, and, in addition, the cDNA clone encoding a CaNBP75 isoform with a 27 bp (9 amino acid) insertion was detected, it may be reasonable to presume that these transcripts all encode isoforms of CaNBP75. We detected a 10-kb transcript of CaNBP75 highly abundant in testis (Fig. 2A, lane 8), with a lower level in kidney (Fig. 2A, lane 6). The 10-kb transcript could also be detected weakly in all other tissues examined. A transcript of about 1.7 kb was detected specifically in ovary and testis (Fig. 2A, lanes 8 and 9). Far-Western blot analysis revealed prominent expression of CaNBP75 in testis and relatively high expression in ovary (Fig. 2C, lanes 8 and 9). In adductor muscles consisting of striated muscle and smooth muscle (translucent and opaque portions), where calcineurin expression was particularly strong (Fig. 2B, lanes 1–3), CaNBP75 was scarcely expressed. In digestive gland, neither calcineurin nor CaNBP75 was detected at all. In other tissues, CaNBP75 was weakly expressed, and interestingly the expression in kidney (Fig. 2C, lane 6) was also weak, which is not compatible with the clear signal on Northern hybridization (Fig. 2A, lane 6). At present, we cannot explain this discrepancy: some translational regulatory mechanism may inhibit the expression of CaNBP75, or some posttranslational modification may regulate the binding of calcineurin. Future work involving specific antibodies against CaNBP75 may clarify the present discrepancy. The high levels of CaNBP75 protein expression in testis and ovary coincided with the mRNA levels revealed on Northern blot analysis, which suggests that CaNBP75 participates in specific functions of testis and ovary, such as in meiosis, spermiogenesis and oogenesis.

To determine the properties of the CaNBP75 protein, a recombinant CaNBP75 protein of full length and various deletion mutants of it were expressed in *E. coli* as His-tagged or GST-tagged fusion proteins (Fig. 3A). A cDNA fragment for full length CaNBP75 with *Nco*I/*Bam*HI sites was produced by PCR. cDNA fragments corresponding to the N-terminal (T6-E296) and C-terminal (E275-L474) regions, CaNBP75(N) and CaNBP75(C), respectively, were excised with *Eco*RI or *Not*I from appropriate cDNA clones of CaNBP75 in the lambda gt10 vector. The cDNAs were cloned into the respective restriction sites of pET-30b(+) (Novagen). A fragment corresponding to A211-L474, CaNBP75(211-474), produced by PCR was cloned into the *Bam*HI site of pGEX-2T (Amersham Biosciences). The proteins were expressed in *E. coli* BL21 (DE3), and purified by TALON Metal Affinity Resin (Clontech) or glutathione Sepharose 4B (Amersham Biosciences) column chromatography. The sizes of the recombinant proteins estimated by SDS-PAGE were 83, 58, 31, and 53 kDa for His-CaNBP75(Full), His-CaNBP75(N), His-CaNBP75(C), and the GST-CaNBP75(211-474) fragment, respectively (Fig. 3B). The estimated molecular mass of His-CaNBP75(Full) is consistent with the value of 75 kDa for CaNBP75 in the crude extract of scallop testis. Since the calculated masses for these recombinant proteins were 56.7, 39.3, 29.3, and 56.0 kDa, respectively, the low mobility of CaNBP75 on SDS-PAGE could be ascribed to the properties of the N-terminal portion (Fig. 3B).

Using these recombinant proteins, the interaction of CaNBP75 and calcineurin was examined. Calcineurin binding of these recombinant proteins was assessed by Far-Western blot analysis using the purified calcineurin as a probe (Fig. 3B). His-CaNBP75(Full), as well as the native CaNBP75 in the crude extract, bound to calcineurin (lanes 1 and 2). Deletion mutant His-

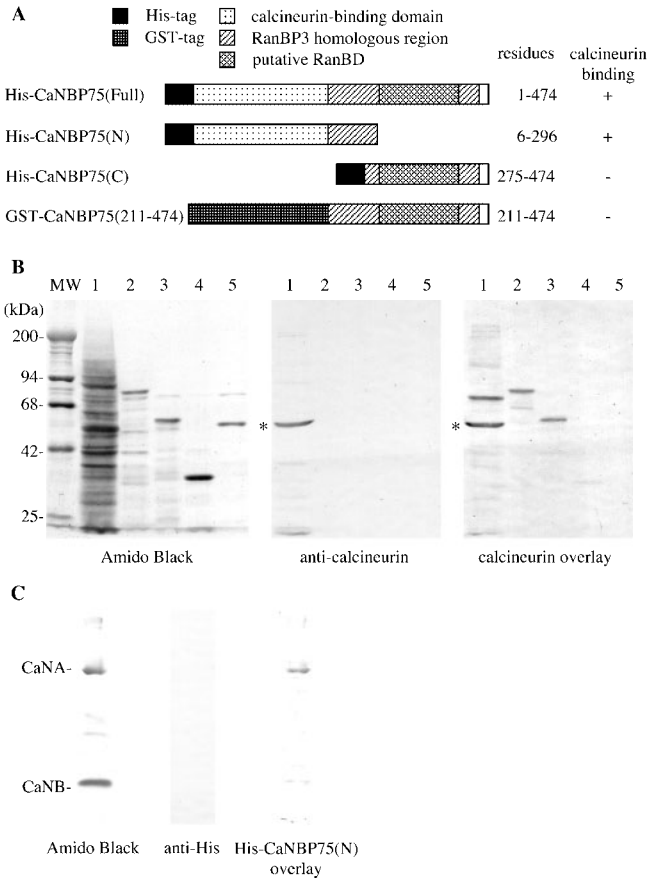

Fig. 3. **Analysis of the CaNBP75-calcineurin interaction.** (A) Schematic diagrams of the recombinant CaNBP75 protein and various deletion mutants of it. (B) Proteins in a scallop testis crude extract (30 μg) and recombinant proteins (1 μg each) were separated by 8% SDS-PAGE and then transferred to a nitrocellulose membrane. Proteins were visualized by means of Amido Black 10B (left), Western blotting with anti-calcineurin antiserum (center), and Far-Western blotting using purified calcineurin as a probe (right). Lane 1, scallop testis crude extract; lane 2, His-CaNBP75(Full); lane 3, His-CaNBP75(N); lane 4, His-CaNBP75(C); lane 5, GST-CaNBP75(211-474). Asterisks indicate the endogenous CaNA subunit in extracts. (C) CaNBP75 interaction with CaNA. Purified calcineurin (10 μg) was separated by 12% SDS-PAGE and then transferred to a nitrocellulose membrane. Proteins were visualized by means of Amido Black 10B (left), Western blotting with anti-His antibodies (center), and Far-Western blotting with His-CaNBP75(N) as a probe (right).

CaNBP75(N) also bound to calcineurin (lane 3), but mutants lacking the N-terminal region did not (lanes 4 and 5), indicating that the region between T6 and A210 of CaNBP75 is responsible for the interaction with calcineurin. To determine which subunit of calcineurin interacts with CaNBP75, Far-Western blot analysis was performed with His-CaNBP75(N) as a probe (Fig. 3C). The purified calcineurin was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. The blot was preincubated in blocking buffer, followed by incubation with 5 μM His-CaNBP75(N) in the blocking buffer. After washing, the blot was incubated with the His-probe (G-18) (Santa Cruz Biotechnology), which recognizes the His-tag sequence of the pET-system, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies. CaNA, but not CaNB, was responsible for the binding to His-CaNBP75(N). This indicates a direct interaction between the calcineurin catalytic subunit and CaNBP75.

To reveal the biochemical effect of this interaction, the protein phosphatase activity of calcineurin was assessed in the presence of the recombinant CaNBP75 protein using the phosphorylated inhibitor-1 as a substrate. Only a weak inhibitory effect (20% inhibition) was observed with the addition of His-CaNBP75(Full) and His-CaNBP75(N) at micromolar concentrations, but not with His-CaNBP75(C). This indicates that CaNBP75 may affect calcineurin activity depending on some post-translational modification like cain/cabin 1 (*11*, *12*), or may have another regulatory function (see Discussion). These possibilities need to be examined further to establish the function of CaNBP75.

DISCUSSION

Several calcineurin-binding proteins, such as AKAP79, calsarcins and cain/cabin 1, have been identified in mammalian tissues (*6–12*). The physiological functions of these calcineurin-binding proteins are suggested to be scaffolding and modulation of phosphatase activity, but they remain obscure. In the present study, we identified a novel calcineurin-binding protein, designated as CaNBP75, in scallop testis. The N-terminal domain of CaNBP75 is responsible for calcineurin-binding, whereas no known sequence motif or sequence homologous to other calcineurin-binding proteins in the available databases was found in this region. Owing to this novel sequence, the expressed N-terminal region showed abnormal mobility on SDS-PAGE, and this structural property affected the whole protein with a similar electrophoretic property (Fig. 3B).

Interestingly, a sequence homology search revealed that CaNBP75 showed similarity in its C-terminal region to one of the human Ran-binding proteins, RanBP3 (Fig. 4) (*17*). In the C-terminal region of CaNBP75, there are two FXFG- and one FXFX-type repeat motifs, which were previously identified in a subclass of nuclear pore complex proteins (*18*). In particular, a segment, A211 to S459, of CaNBP75 closely resembled the C-terminal region of RanBP3 that contains a Ran-binding domain (RanBD) (*19*). A region of CaNBP75 (E311 to I411) shows 70% identity in sequence to the RanBD of RanBP3, which is much higher than the 30% identity to the original RanBD of RanBP1 (Fig. 4). Therefore, it is quite probable that CaNBP75 is regulated through the binding of Ran. Although there is no evidence for Ran-directed translocation of calcineurin, these findings suggest that CaNBP75 regulates calcineurin through its N-terminal region, which could be modulated through binding of Ran with the putative RanBD in the C-terminal region. To address the physiological function of CaNBP75, the binding of Ran to CaNBP75 should be examined.

It has been reported, however, that the expression of human RanBP3 is particularly strong in testis and heart (*17*), and that the expression of Ran is much stronger in testis, while it is expressed in most somatic tissues of mouse (*20*). In addition, Ran is localized in the nuclei of round spermatids and along the microtubules

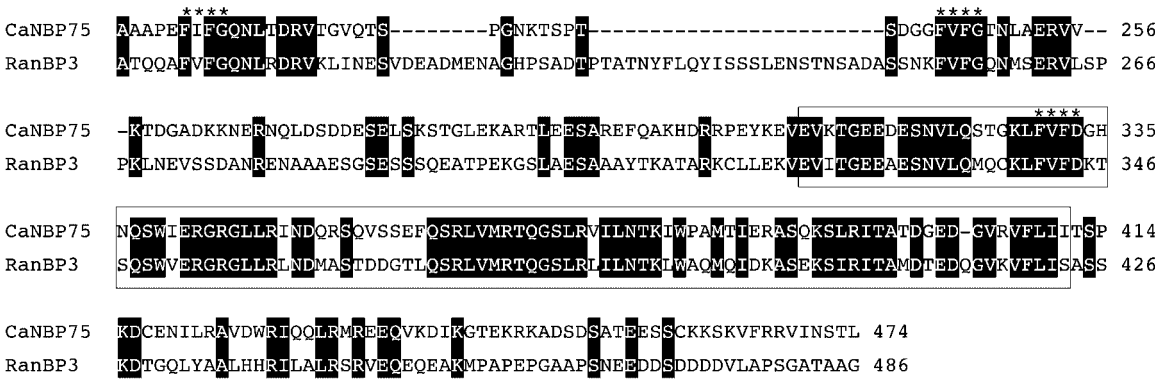

Fig. 4. **Comparison of the amino acid sequences of scallop CaNBP75 and human RanBP3.** The deduced amino acid sequence of scallop CaNBP75 was aligned with that of human RanBP3b (*17*). Conserved residues are shown by black boxes, and the RanBD homologous region is boxed. Asterisks indicate FXFG- and FXFX-type sequence motifs. For the best alignment, gaps were inserted in the CaNBP75 sequence, which are shown by dashes.

of the manchette in elongating spermatids, and in the centrosome region of maturing spermatids when the manchette is disabled (*21*). It has been thought that calcineurin in testis is related to the regulation of spermiogenesis (*3*, *4*), and the flagellar motility of sperm (*5*), since calcineurin is located in the nuclei of mouse spermatids immediately after meiosis, and in the postacrosomal region and the flagella of mammalian spermatozoa. Considering the function of calcineurin in testis, CaNBP75 and Ran are expected to play important roles in its regulation, since the expression of CaNBP75 is prominent in testis and ovary (Fig. 2). Further studies on CaNBP75 will reveal the physiological function of calcineurin in testis.

Nucleotide sequence data reported will appear in the DDDBJ/EMBL/GenBank databases under the accession number AB099485 for calcineurin-binding protein of 75 kDa. The authors wish to thank Ms. Yuriko Yamashita for the bacterial expression and purification of the GST-tagged CaNBP75 protein, Mr. Masanori Iwase for the assaying of calcineurin phosphatase activity, and Mr. Yukichi Abe (Center for Instrumental Analysis, Hokkaido University) for the peptide sequencing. We are also grateful to Dr. Hajime J. Yuasa (Faculty of Science, Kochi University) and Dr. Masumi Eto (Center for Cell Signaling, University of Virginia) for the helpful discussions and technical advice.

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