Title

Ran and Calcineurin Can Participate Collaboratively in the Regulation of Spermatogenesis in Scallop

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Running title

Collaboration of Ran and calcineurin in testis

Key words

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Abstract

Calcineurin is a calcium/calmodulin-dependent protein phosphatase that plays important roles in the transduction of calcium signals in a variety of tissues. In addition, calcineurin has been implicated in the process of spermatogenesis. A novel calcineurin-binding protein, CaNBP75, has been identified in scallop testis. The C-terminal region of CaNBP75 is homologous to the C-terminal region of RanBP3, a Ran binding domain-containing protein. A small G-protein Ran has been involved in spermiogenesis by virtue of the fact that its localization in spermatids changes during spermiogenesis. The current study was performed to investigate the functions of Ran and CaNBP75 in the regulation of calcineurin in testis to further understand the basic functions of calcineurin during spermatogenesis. First, cloning and sequencing of a scallop Ran cDNA isolated from testis revealed that scallop Ran is well-conserved at the amino acid level. Secondly, direct binding of Ran to CaNBP75 was demonstrated in an *in vitro* pull-down assay. Thirdly, analysis of the tissue distribution of Ran, CaNBP75 and calcineurin showed that these proteins are abundantly expressed in testis. Fourthly, comparison of the expression profiles of Ran and CaNBP75 with that of calcineurin in scallop testis during the maturation cycle revealed that Ran and CaNBP75 mRNA levels increase during meiosis and spermiogenesis, similar to calcineurin. Finally, co-immunoprecipitation analysis suggests that Ran, CaNBP75 and calcineurin interact in scallop testis during maturation. These results suggest that Ran, CaNBP75 and calcineurin may act in a coordinated manner to regulate spermatogenesis.

Introduction

Calcineurin (CaN) is a calcium/calmodulin (CaM)-dependent protein phosphatase comprised of a catalytic subunit (CaNA) and a regulatory subunit (CaNB) (Stewart et al., 1982; Klee et al., 1998). CaN is highly expressed in brain, immune cells and muscle, and plays important roles in calcium signal transduction in these tissues (Su et al., 1995; Mansuy et al., 1998; Dell'Acqua et al., 2006; Clipstone et al., 1992; Feske et al., 2003; Chin et al., 1998; Friday et al., 2000). In addition, CaN has been involved in spermatogenesis. In testis, expression of CaN fluctuates during the maturation cycle, peaking at spermiogenesis (Su et al., 1995; Muramatsu et al., 1992; Uryu et al., 2000). Additionally, CaN localizes to the nuclei of round spermatids in mouse testis and the postacrosomal region of dog sperm (Moriya et al., 1995; Tash et al., 1988). To date, however, details of the function of CaN in testis have yet to be fully elucidated.

Scallop is a marine bivalve that is cultured commercially and is readily available in Hokkaido, in the northern part of Japan. The maturation cycle of the scallop gonad is annual, and spermatogenesis proceeds synchronously throughout the testis (Maru, 1976). Thus, harvesting of testis tissue at each developmental stage gives a sampling of each stage of spermatogenesis. This is in contrast to the mammalian testis, which is quite intricate in structure and is heterogeneous in terms of germ cell stage at any given point. Thus, the scallop testis represents a useful model system for biochemical and molecular characterization of the maturation cycle.

A gonad-specific CaN-binding protein, CaNBP75, has been identified that is highly expressed in scallop testis and represents as a potential regulator of CaN (Nakatomi and Yazawa, 2003). CaNBP75 is similar in its C-terminal region to one of the human Ran-binding proteins, RanBP3. It is possible that CaNBP75 also possesses Ran-binding activity because the region of homology with CaNBP75 in RanBP3 contains a Ran-binding domain (Nakatomi and Yazawa, 2003).

Ran is a Ras-related small-G protein that modulates nucleocytoplasmic transport within cells during interphase (Drivas et al., 1990; Görlich, 1998). RanBP3 functions as a cofactor in Ran-dependent nuclear export (Lindsay et al., 2001). During mitosis, Ran also functions in spindle formation and nuclear envelope reassembly (Kalab et al., 1999; Suntharalingam and Wente, 2003).

On the other hand, Ran has been involved in spermiogenesis and meiosis. In testis, high levels of Ran mRNA are observed from the late pachytene spermatocyte stage to the early round spermatid (López-Casas et al., 2003). Additionally, the intracellular localization of Ran changes in spermatid during spermiogenesis (Kierszenbaum et al., 2002). Testis-specific isoforms of Ran have been identified in mouse and rat (Coutavas et al., 1994; Kierszenbaum et al., 2002); however, details of the role of Ran in testis have yet to be fully explored.

Because CaNBP75 has CaN-binding activity and contains a region of homology with Ran-binding domain, we were interested in assessing whether Ran interacts with CaNBP75; thereby, regulating CaN through or in conjunction with CaNBP75. Here, we investigated the functions of Ran and CaNBP75 in the regulation of CaN in testis to increase our understanding of the basic functions of CaN during spermatogenesis. A scallop Ran cDNA was cloned and sequenced, and the Ran-binding activity of CaNBP75 was investigated by *in vitro* glutathione-S-transferase (GST) pull-down assay. Analysis of the tissue distribution of Ran, CaNBP75 and CaN showed that all three proteins are abundantly expressed in testis. The expression profiles of Ran and CaNBP75 during the annual maturation cycle of the scallop testis were determined by northern blot analysis. The mRNA expression levels of Ran and CaNBP75 increased during the stages of meiosis and spermiogenesis, and this pattern was similar to the expression profile of CaN. Analysis of co-immunoprecipitation indicated that Ran, CaNBP75 and CaN interact in scallop testis during maturation.

Materials and Methods

Materials

The cultured scallop *Mizuhopecten yessoensis* was collected from Lake Saroma (Hokkaido, northern Japan) in April, 2003 and used as the source material for degenerate PCR, 5'-rapid amplification of cDNA ends (5'-RACE) and northern blot analysis of scallop Ran mRNA. Total RNA was extracted from scallop testis using the acid-guanidinium-phenol-chloroform method and stored at −80°C until use (Chomczynski and Sacchi, 1987). Total RNA used for northern blot analysis of expression during the maturation cycle was previously described (Uryu et al., 2000). The cultured scallop was collected in February, 2009, stored at −80°C, and used as a source material for co-immunoprecipitation analysis.

CaN and CaM were purified from scallop testis as described previously (Kitagawa et al., 2011; Kobayashi et al., 1991).

Degenerate PCR and 5'-RACE

Single-stranded (ss) cDNA was prepared from scallop testis total RNA using oligo(dT) primer and a First-Strand cDNA Synthesis Kit (GE Healthcare). The degenerate nucleotide primers 5'GGNAARACNACNTTYRTNAA3' (F1), 5'GGNGANTTYGARAARRARTAY3' (F2), and 5'GGYTTYTCRAARTTRTARTT3' (R1) were designed based on the following conserved amino acid sequences of Ran across various species: GKTTFV/MK (corresponding to positions 22–28 of mouse Ran), GEFEKK/EY (positions 33–39), and NYNFEKP (positions 154–160), respectively (see Fig. 2). First round PCR was performed with Ex Taq polymerase (Takara) using primers F1 and R1 and ss cDNA as the template. The initial phase of denaturation (1 min at 96°C) was followed by 30 cycles of 96°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec, and then a final 10 min extension step at 72°C. Second round PCR was performed using primers F2 and R1 and a 1/100 dilution of the first-round PCR product as a template. The thermal conditions for amplification were the same as for the first round of PCR.

The 5'-terminal region of scallop Ran cDNA was amplified by 5'-RACE. A poly-C tail was added

to the 3’-end of the ss cDNA using terminal deoxynucleotidyl transferase (Takara), and then the product was used as the template for PCR with oligo(dG) and R2 (5'TATCACCTCTGGTTGTGCAG3'; see Fig. 1) as the primers. The initial phase of denaturation (1 min at 96°C) was followed by 30 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 4 min, and then a final 10 min extension step at 72°C.

PCR products were separated by 1% agarose gel electrophoresis and then subcloned into pGEM-T easy vector (Promega) using a DNA Ligation Kit Ver. 2 (Takara). Nucleotide sequences were determined using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 system (Applied Biosystems).

Screening of a scallop testis cDNA library

The digoxigenin (DIG)-labeled scallop Ran DNA probe was prepared by PCR using a DIG DNA Labeling Mix (Roche Diagnostics). The template was a cDNA fragment corresponding to nucleotides 133–474 of scallop Ran inserted into pGEM-T Easy vector (Promega); the two primers corresponded to T7 promoter and SP6 promoter sequences located in the vector.

The scallop testis cDNA library (phage library) was previously described (Uryu et al., 2000). Plaques from NZY-containing plates were transferred to Biodyne B nylon membranes (Pall Corporation). Hybridization was performed using the DIG-labeled Ran-specific probe in hybridization buffer (500 mM sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA) at 65°C overnight. The membrane was washed three times in wash-buffer 1 (40 mM sodium phosphate, pH 7.2, 1% SDS) at 65°C for 20 min each time. After washing, positive plaques were detected using a Luminescent Detection Kit (Roche Diagnostics) and X-ray film (RU-X; Fujifilm). Phage DNA purification and subcloning were performed as described previously (Uryu et al., 2000).

Northern blot analysis

The DIG-labeled scallop Ran RNA probe was prepared using a DIG RNA Labeling Mix (Roche Diagnostics), SP6 RNA polymerase, and the scallop Ran cDNA fragment corresponding to

nucleotides 133–474 as a template. The DIG-labeled CaNBP75 RNA probe was prepared as described previously (Nakatomi and Yazawa, 2003). Scallop testis total RNA was separated in a 1% agarose gel containing 1× MOPS (20 mM MOPS-NaOH, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) and 6.2% formaldehyde, and then transferred to a Hybond-N^+ nylon membrane (GE Healthcare). Hybridization was performed in northern hybridization buffer (75 mM sodium citrate, pH 7.0, 750 mM NaCl, 50% formamide, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 2% Blocking Reagent (Roche Diagnostics)) containing the DIG-labeled RNA probe overnight at 68°C (for Ran) or 65°C (for CaNBP75). The membrane was washed twice with wash buffer 2 (30 mM sodium citrate, pH 7.0, 300 mM NaCl, 0.1% SDS) at 25°C for 5 min each time, and then washed twice with wash buffer 3 (1.5 mM sodium citrate, pH 7.0, 15 mM NaCl, 0.1% SDS) at 68°C (for Ran) or at 65°C (for CaNBP75) for 15 min each. Signals were detected as described for screening. Signal intensity was quantified with the LAS-3000 imaging system and Multi-Gauge Version 2.2 software (GE Healthcare) (for Ran) or with NIH-image software (for CaNBP75).

Expression and purification of GST-CaNBP75

The plasmid vector pGEX-2T (GE Healthcare) was modified to contain an NcoI site at the 3'-end of the GST coding region. The cDNA fragments encoding CaNBP75 (AB099485) and the truncated variant of CaNBP75 were inserted into the NcoI and BamHI sites of modified pGEX-2T. Expression of recombinant GST-CaNBP75 in *E. coli* BL21 was induced with 0.1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C. GST-CaNBP75 was purified on Glutathione Sepharose 4B (GE Healthcare) and then dialyzed against buffer A (50 mM Tris-HCl, pH 7.5 at 4°C, 150 mM NaCl, 14 mM 2-mercaptoethanol and 50% glycerol) before being stored at −20°C.

Expression and purification of 6×His-tagged CaNBP75

The cDNA fragment encoding CaNBP75 (AB099458) was inserted into the NdeI and BamHI sites of pET-30b(+). Expression of recombinant 6×His-tagged CaNBP75 full length (amino acids 1–509) or truncated mutant (amino acids 1-462) in *E. coli* BL21(DE3) was induced with 0.1 mM

isopropyl-β-D-thiogalactoside for 3 h at 37°C. His-tagged CaNBP75 was purified on TALON Metal Affinity Resin (Clontech) and then dialyzed against buffer A without 2-mercaptoethanol and glycerol before being stored at −80°C.

Purification of Ran protein from scallop testis

Purification of Ran protein from scallop testis was performed as described in Bischoff and Ponstingl (1995) and in Yokoya et al. (1999), with some modifications. One hundred grams of scallop testis tissue were homogenized in 1000 ml of buffer B (50 mM potassium phosphate, pH 7.0 at 4°C, 100 mM NaCl, 1 mM benzamidine-HCl, 0.1 mM phenylmethylsulfonyl fluoride and 14 mM 2-mercaptoethanol) and then subjected to centrifugation. Ran was fractionated from the supernatant with ammonium sulfate at 25–50% saturation. The precipitate containing Ran was dissolved in buffer C (50 mM Tris-HCl, pH 8.0 at 4°C, 75 mM NaCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol) and then dialyzed against buffer C. The solution was clarified by centrifugation and applied to a DEAE Sepharose CL-6B (GE Healthcare) column (46 × 200 mm) that was pre-equilibrated with buffer C. The flow-through fractions containing Ran were pooled and fractionated by 60%-saturated ammonium sulfate. The precipitate containing Ran was dissolved in 10 ml of buffer D (50 mM potassium phosphate, pH 7.0, 10% glycerol, 1 mM 2-mercaptoethanol) and then dialyzed against buffer D. To obtain homogeneous Ran-nucleotide complex, the dialysate was adjusted to 2 mM EDTA, 0.5 mM GDP and then incubated for 30 min, after which it was adjusted to 5 mM $MgCl_2$. The solution was clarified by centrifugation and applied to a Sephacryl S-200HR (GE Healthcare) column (28 × 900 mm) that was pre-equilibrated with buffer E (buffer D containing 2 μM GDP). The fractions containing Ran were pooled and applied to a Gigapite (Seikagaku) column (22 × 103 mm) that was pre-equilibrated with buffer E. The column was washed with buffer E, and then Ran was eluted with a linear gradient of 0 to 1 M KCl in buffer E. The protein was dialyzed against buffer F (20 mM Tris-HCl, pH 8.0 at 4°C, 20 mM NaCl, 2 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 2 μM GDP, 50% glycerol) and stored at −20°C until use.

For the GST pull-down assay, Ran was pre-loaded with GTPγS as follows. Ran protein in solution

was dialyzed against buffer G (50mM Tris-HCl, pH 7.5 at 4°C, 150 mM NaCl, 1 mM $MgCl_2$, 28 mM 2-mercaptoethanol), adjusted to 5 mM EDTA and 1 mM GTPγS, and then incubated with gentle agitation overnight. The solution was adjusted to 10 mM $MgCl_2$ and dialyzed against buffer G before being adjusted to 0.05% BSA and 0.1% Tween 20.

Generation of anti-CaNBP75 antiserum

A truncated variant of recombinant 6×His-tagged CaNBP75 (amino acids 1–462) was used to immunize rabbits. The resulting antiserum (FS0024 and FS0025) specifically recognized scallop CaNBP75, and did not recognize the 6×His-tag or the S-tag derived from the expression vector (pET-30b(+)).

Purification and preparation of antibodies

Purification of anti-CaN and anti-CaNBP75 polyclonal antibody from the antisera was performed as described in Talian et al. (1983) with minor modification. Purified CaN or recombinant 6×His-tagged CaNBP75 (1–509) was transferred to and crosslinked on a PVDF membrane (Immobilon-P, Millipore) with 0.25% glutaraldehyde. The membrane was blocked by 3% gelatin in TBS, and then protein strips were cut from the membrane and cut into pieces. After washing with TBS, the pieces were incubated with the antisera diluted by Can Get Signal Immunoreaction Enhancer Solution 1 (1:50, Toyobo) for overnight, and then wash with TBS. Bound antibodies were eluted with 0.2 M glycine-HCl, pH2.8, neutralized with 1.88 M Tris, and diluted with PBS.

Normal rabbit IgG was purified from preimmune rabbit serum using Thiophilic-Superflow Resin (Clontech).

Purified polyclonal antibodies and normal rabbit IgG were concentrated by ultrafiltration using 5k Apollo 20mL (Orbital Bioscience).

Preparation of horseradish peroxidase (HRP)-conjugated anti-CaNBP75 polyclonal antibody was performed as described previously (Avrameas and Ternynck, 1971).

GST pull-down assay

The GST pull-down assay was performed as described by Lindsay et al. (2001) with some modifications. GST-fusion proteins (0.4 nmol) were incubated with 25 µl of Glutathione Sepharose 4B beads (GE Healthcare) that had been preincubated with 3% BSA/PBS and then equilibrated with buffer H (buffer G containing 0.05% BSA, 0.1% Tween 20). The beads were washed three times with buffer H and then incubated with GTPγS-loaded Ran (0.2 nmol) in 200 µl of buffer H with gentle agitation overnight. The beads were washed four times with buffer H without BSA and bound proteins were eluted with buffer I (20 mM glutathione, 50 mM Tris-HCl, pH 8.0 at 25°C, 200 mM NaCl). Proteins were separated by SDS-PAGE and then transferred to a PVDF membrane (Immobilon-P, Millipore). Western blot analysis for the detection of bound Ran was performed using a mouse anti-Ran monoclonal antibody (BD Bioscience) (1:2,000 dilution in Can Get Signal Immunoreaction Enhancer Solution 1; Toyobo). The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG antibody (GAM-AP; Bio Rad) (1:2,000 dilution in Can Get Signal Immunoreaction Enhancer Solution 2; Toyobo). Immunoreactive bands were detected using an Alkaline Phosphatase Conjugate Substrate Kit (Bio Rad).

Western blot analysis and far-western blot analysis

Tissues were dissected from fresh scallop and homogenized in buffer J (20 mM imidazole, pH 7.0, 1 mM EGTA, 14 mM 2-mercaptoethanol, 1 mM benzamidine-HCl, 0.1 mM phenylmethanesulfonyl fluoride). Each homogenate was centrifuged at 500,000 × *g* for 20 min. The resulting supernatants were subjected to SDS-PAGE and the protein bands were transferred onto a nitrocellulose membrane.

Western blot analysis for the detection of Ran, CaNBP75, CaN and α-tubulin was performed using mouse anti-Ran monoclonal antibody (BD Bioscience, 1:2,000 dilution), rabbit anti-scallop CaNBP75 antiserum (FS0024, 1:5,000 dilution) or rabbit anti-scallop CaN antiserum (Moriya et al., 1995, 1:2,000 dilution), mouse anti-α-tubulin monoclonal antibody (T5168, Sigma, 1:20,000 dilution) as the primary antibody, and alkaline phosphatase-conjugated anti-mouse IgG (GAM-AP,

Bio Rad, 1:2,000 dilution for Ran, 1:3,000 dilution for α-tubulin) or anti-rabbit IgG (7151-1, New England Biolabs, 1:3,000 dilution) as the secondary antibody. Immunoreactive proteins were detected as described for the GST pull-down assay.

Far-western blot analysis for the detection of CaNBP75 was performed as described previously (Nakatomi and Yazawa, 2003). Briefly, transferred proteins on the membrane were preincubated in blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 5% skim milk), followed by incubation in blocking buffer supplemented with 0.25 μM CaN and 2 μM CaM. After washing, bound CaN was detected by western blot analysis.

Co-immunoprecipitation analysis

Scallop testis was homogenized in 10-fold volume of lysis buffer (50 mM Tris-HCl, pH7.5 at 4°C, 150 mM NaCl, 0.2 mM $CaCl_2$ or 1 mM EGTA, 0.2 mM $MgCl_2$, 0.1% Triton X-100, 1 × protease inhibitor cocktail (complete, EDTA-free, Roche Diagnostics), 1 × phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics)) and then subjected to centrifugation. In 0.5 ml of the supernatant, approximately 30 μg of purified normal rabbit IgG, purified anti-CaNBP75 polyclonal antibody (FS0025) or purified CaN polyclonal antibody was added and the supernatant was incubated with gentle agitation for 2 h at 4°C. In each sample, 6–7 × 10^7 of Dynabeads M-280 Sheep anti-Rabbit IgG beads (Dynal Biotech) that had been preincubated with 3% BSA/PBS and then equilibrated with IP-wash-buffer (lysis buffer without protease inhibitor and phosphatase inhibitor) was added and incubated with gentle agitation for 2 h at 4°C. After washing with IP-wash-buffer, beads were boiled with 2 × SDS-PAGE sample buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.02% bromophenol blue) to elute bound proteins. Eluted protein were separated by SDS-PAGE and then transferred to a PVDF membrane (Immobilon-P, Millipore). Western blot analysis for detection of the bound proteins was performed using a mouse anti-CaN monoclonal antibody (BD Bioscience) (1:1,000 dilution), a mouse anti-Ran monoclonal antibody (BD Bioscience) (1:5,000 dilution) as primary antibodies, and HRP-conjugated anti-mouse IgG antibody (BioRad) (1:50,000 dilution) as a secondary antibody, or HRP-conjugated anti-CaNBP75 polyclonal antibody (1:50 dilution). For

dilution of antibodies, Can Get Signal Immunoreaction Enhancer (Solution 1 for direct-conjugated antibody, primary antibodies and Solution 2 for the secondary antibody; Toyobo) was used. Immunoreactive signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the LAS-3000 imaging system (GE Healthcare).

Protein characterization

Protein concentration was determined using a Protein Assay (Bio Rad) and bovine gamma globulin as a standard.

To confirm the identity of purified scallop Ran, mass spectrometry was performed as previously described (Nozawa et al., 2010). Briefly, purified Ran was separated by SDS-PAGE. After trypsinization, the protein was analyzed by liquid chromatography/tandem mass spectrometry. The raw data files were analyzed using Mascot (Matrix Science) and searched against the protein database in which scallop Ran sequence determined from cDNA cloning was integrated.

Results

Isolation of scallop Ran cDNA

Scallop testis expresses a gonad-specific CaN-binding protein, CaNBP75, which contains a region of homology to the Ran-binding domain (Nakatomi and Yazawa, 2003). Ran has been involved in spermiogenesis (Coutavas et al., 1994; Kierszenbaum et al., 2002; López-Casas et al., 2003), but the details of its role have yet to be fully elucidated. To investigate whether Ran regulated CaN through or with CaNBP75 in scallop testis, we first isolated a scallop Ran cDNA from testis.

Degenerate PCR was performed using degenerate primers (F1, F2, and R1) designed based on the amino acid sequence of a highly conserved region of Ran (see Fig. 2). First-round PCR was performed using scallop testis ss cDNA as the template and the F1 and R1 primers; second-round PCR was performed using the product of the first round as the template, and the primers F2 and R1. A DNA fragment of about 400 bp (nucleotides 133–474; Fig. 1) was isolated and sequenced. The deduced amino acid sequence (amino acid residues 45–158; Fig. 1) was highly similar to the central region of Ran from various species (Fig. 2). Next, 5'-RACE was performed using the primer R2 (Fig. 1) to obtain the 5'-end of the scallop Ran cDNA fragment containing the initiation codon (nucleotides −50 to +170). To obtain the rest of the coding region, a scallop testis cDNA library (4×10^5 clones) was screened, yielding six positive clones. Considering regions of overlap, one nucleotide sequence of scallop Ran (nucleotides −34 to +885) was determined.

The isolated scallop Ran cDNA was 935 bp and contained a putative open reading frame (ORF) of 669 bp, which encoded a protein of 222 amino acids and a calculated mass of 25,058 Da (Fig. 1). The cDNA also contained a 5'-untranslated region (UTR) of 50 bp and a 3'-UTR of 216 bp (Fig. 1). There was no poly$(A)^+$ tail in the 3'-UTR; therefore, the scallop Ran mRNA in testis is likely more than 0.9 kb. In the 5'-UTR, an in-frame stop codon (tga) was present three codons upstream of the initiation codon (Fig. 1). In addition, recombinant scallop Ran expressed in *E. coli* from the isolated cDNA exhibited the same mobility (approximately 30 kDa) as native scallop Ran by SDS-PAGE and was recognized by an anti-Ran antibody (data not shown). The molecular mass estimated by SDS-PAGE was greater than the calculated mass (25 kDa) of scallop Ran, perhaps reflecting a high

percentage (25%) of charged residues in the protein. Native scallop Ran purified from testis was analyzed by mass spectrometry and the obtained amino acid sequence matched the predicted sequence derived from the isolated scallop Ran cDNA over 99% of the full-length sequence. These results indicated that the isolated cDNA encoded a protein that was identical to native scallop testis Ran, and that the position of the initiation codon and the ORF of the cDNA were correct.

Northern blot analysis of scallop Ran

A testis-specific isoform of Ran has been identified in mouse and rat (Coutavas et al., 1994; Kierszenbaum et al., 2002). Northern blot analysis was used to explore a similar possibility of other Ran mRNA species in scallop testis (Fig. 3). A single mRNA species of approximately 2.6 kb was detected, which, because it was larger than the isolated cDNA sequence, was most likely the full-length scallop Ran mRNA. These results indicated that there is only one Ran mRNA species expressed in scallop testis.

Sequence identity of scallop Ran and Ran homologs from other species

The deduced amino acid sequence of scallop testis Ran exhibited a high degree of sequence identity to Ran from various other species (Fig. 2 and Table 1). Notably, multiple regions important for Ran function were highly conserved, including the P-loop that interacts with the guanine-nucleotide; switch I and switch II, which undergo large conformational changes from the GDP to GTP-bound form of Ran; and the basic patch and C-terminal acidic region, which are indispensable for the interaction of Ran with heterologous Ran-binding domains in other proteins (Fig. 2; Milburn et al., 1990; Scheffzek et al., 1995; Vetter et al., 1999). Meanwhile, the N-terminal and C-terminal regions of scallop Ran that corresponded to regions of lower identity between phyla exhibited lower sequence identity to other species (Fig. 2). These regions are considered to be low homology regions of Ran across species. These results provided further evidence that the isolated cDNA sequence encoded scallop Ran, and indicated that the high degree of sequence identity among Ran proteins from various species extends to scallop Ran as well.

A single cDNA corresponding to scallop Ran was isolated in the initial screen of the scallop testis cDNA library. In a comparison with mouse Ran, the amino acid sequence of scallop testis Ran was 82.9% identical to somatic Ran, and more similar to somatic Ran than to testis-specific Ran (77.0% identity; Table 1). In mouse, there are 13 amino acid differences between somatic and testis-specific Ran (Fig. 2; Coutavas et al., 1994). In scallop Ran, the corresponding 13 amino acids are all of the somatic type (Fig. 2). In addition, it has been reported that Ser135 of vertebrate Ran is phosphorylated, and that phosphorylation may play an important role in mitosis (Bompard et al., 2010). The corresponding site in testis-specific mouse Ran is a proline, whereas this phosphorylation site is preserved in scallop (Ser140 of scallop Ran corresponds to the Ser135 of vertebrate Ran). Thus, scallop Ran appeared to be more similar to mouse somatic Ran than to testis-specific Ran.

Ran-binding capacity of CaNBP75

As reported previously, there is a high degree of similarity between the C-terminal region of CaNBP75 and that of RanBP3, a human Ran-binding protein (Nakatomi and Yazawa, 2003). Since the C-terminal region of RanBP3 contains a Ran-binding domain, we investigated the ability of CaNBP75 to bind to scallop Ran in a GST pull-down assay (Fig. 4). Initial attempts to prepare recombinant scallop Ran using the isolated cDNA were unsuccessful, as recombinant Ran expressed in *E. coli* was insoluble. Therefore, native Ran was purified from scallop testis and used for the binding assay. GST or GST-CaNBP75 immobilized to glutathione resin was incubated with purified Ran pre-loaded with GTPγS, and then bound proteins were analyzed by western blot. As shown in Fig. 4B, full length CaNBP75 fused to GST (GST-full) bound to RanGTPγS, whereas neither a truncated mutant lacking the Ran-binding domain homology region fused to GST (GST-ΔC) nor a GST alone (GST) did. These results indicated that CaNBP75 is a Ran-binding protein, and that the Ran-binding domain homology region of CaNBP75 is important for the interaction with Ran.

Tissue distribution of Ran, CaNBP75 and CaN

Since CaNBP75 can bind to both CaN and Ran, it may serve to link CaN-mediated calcium

signal transduction pathways with Ran-regulated cellular functions. Analysis of the expression profiles of these three proteins in scallop tissues by western blot analysis revealed that Ran is prominent in testis, and relatively high levels were also present in ovary (Fig. 5). In gill, mantle and kidney, Ran was weakly expressed. CaNBP75 distribution was detected by far-western blot analysis using CaN as a probe, as described previously (Nakatomi and Yazawa, 2003), and also by direct western blot analysis using an anti-CaNBP75 antiserum (Fig. 5). CaNBP75 expression was detected in the tissues where Ran was expressed, and the levels of CaNBP75 correlated with those of Ran in these tissues. CaN was also expressed abundantly in testis and ovary (Fig. 5). Interestingly, CaN expression was particularly high in adductor muscles consisting of striated muscle and smooth muscle (translucent and opaque portions), while Ran and CaNBP75 were barely detectable in these tissues. In digestive gland, Ran, CaNBP75 and CaN were not detected at all. These findings showed that Ran, CaNBP75 and CaN are highly expressed together in gonad tissue, and indicated that these three proteins may function cooperatively in the gonad. They also suggested that the function of CaN in muscle might differ from gonad and that there may be important muscle-specific functions of CaN that are independent of Ran and CaNBP75 (Chin et al., 1998).

Expression levels of Ran, CaNBP75 and CaN mRNA during scallop testis maturation

Previous analysis of mRNA expression in scallop testis showed that the expression of CaN fluctuates during the maturation cycle, with peak levels seen during spermiogenesis (Uryu et al., 2000). We analyzed the expression patterns of Ran, CaNBP75 and CaN during the maturation cycle of the scallop testis. The maturation cycle progresses throughout a period of 1 year, and spermatogenesis proceeds synchronously throughout the entire testis (Maru, 1976). In the vicinity of Hokkaido, in the northern part of Japan, scallop testis grows rapidly in the low temperature conditions of the sea, and meiosis and spermiogenesis occur actively from December to April. After April, with increasing sea temperatures, spermatozoa are released and testis weight decreases rapidly (Maru, 1976). To analyze Ran and CaNBP75 expression during maturation, scallops were sampled every month for 1 year, and total RNA was prepared from testes (Uryu et al., 2000). Northern blot

analysis was carried out using RNA probes specific for Ran or CaNBP75 (Fig. 6B, C). The mRNA expression profiles of Ran and CaNBP75 were then compared with that of CaN (Fig. 6D). As a control, the overall level of rRNA was constant for 1 year (Fig. 6A). Meanwhile, expression of Ran fluctuated during testis maturation, increasing during January and February and peaking in March (Fig. 6B, D). Peak expression of Ran occurred approximately 1 month earlier than the peak of testis maturation in April (Fig. 6D). The expression level of the 10-kb transcript of CaNBP75 exhibited similar fluctuations during testis maturation (Fig. 6C, D); by contrast, while the 5.2-kb transcript also fluctuated during maturation, the expression levels did not parallel the 10-kb transcript (Fig. 6C). These results suggested that there is differential processing of CaNBP75 mRNA during testis maturation. The large peak of CaNBP75 mRNA expression coincided with that of Ran, and the fluctuations of these two mRNA expressions were synchronous those of CaN (Fig. 6D; Uryu et al., 2000). These results indicated that Ran, CaNBP75 and CaN are co-expressed during spermatogenesis, and may cooperate during this stage of maturation.

Interaction of Ran, CaNBP75 and CaN in scallop testis during maturation

As shown above and previously, CaNBP75 interacted with Ran or CaN *in vitro* (Fig. 4 and Nakatomi and Yazawa, 2003). To confirm these interactions in scallop testis during maturation, co-immunoprecipitation analysis was performed using lysate of scallop testis harvested in February with anti-CaN polyclonal antibody and anti-CaNBP75 polyclonal antibody (Fig. 7). CaNBP75 co-immunoprecipitated with CaN in the presence of Ca^{2+} (Fig. 7A), and CaN co-immunoprecipitated with CaNBP75 in the presence of Ca^{2+} (Fig. 7B), indicating that CaNBP75 interacts with CaN in a Ca^{2+} dependent manner. In addition, compared with negative control, Ran also seemed to co-immunoprecipitate with CaN in the presence of Ca^{2+} (Fig. 7A, lane 7 compared with lanes 3 and 5), suggesting that Ran also interacts with CaN/CaNBP75 complex though the interaction is weak. On the other hand, Ran did not co-immunoprecipitate with CaNBP75 (Fig. 7B), possibly because anti-CaNBP75 antibody may compete with Ran and may disrupt the interaction between Ran and CaNBP75. Unfortunately, anti-Ran monoclonal antibody used in this study could not be used in

immunoprecipitation (data not shown). These results indicate that CaNBP75 and CaN interact in scallop testis during maturation and also suggest that Ran associates with CaN probably via CaNBP75.

Discussion

A scallop Ran cDNA was isolated that encoded a protein of 222 amino acids, and northern blot analysis revealed the presence of a corresponding scallop Ran mRNA of 2.6 kb. A single nucleotide sequence was obtained from degenerate PCR and screening of a scallop cDNA library, and only one mRNA signal was detected upon northern blot analysis, suggesting that there is a single species of Ran mRNA expressed in scallop testis.

The deduced amino acid sequence of scallop Ran showed a high degree of similarity to Ran homologs from various other species and was more similar to the somatic Ran isoform than to the testis-specific Ran isoform in mouse. Given that there may be only one species of Ran in scallop testis, these results suggest that it has somatic functions as well as testis-specific roles.

Analysis of the tissue distribution of Ran, CaNBP75 and CaN showed that these proteins are highly expressed in the gonad and that expression levels of all three mRNA expressions fluctuate synchronously during maturation of the scallop testis. Furthermore, co-immunoprecipitation analysis showed that CaNBP75 and CaN interacts in the scallop testis during maturation. Ran clearly interacts with CaNBP75 *in vitro* (Fig. 4), and *in vivo* co-immunoprecipitation experiments suggest the interaction of Ran and CaNBP75/CaN (Fig. 7). The relative weak interaction observed *in vivo* may due to the temporal interaction. These findings suggest that Ran, CaNBP75 and CaN may work cooperatively in the gonad. Interestingly, expression levels peaked from February to March, a period of active spermiogenesis during which spermatids develop into spermatozoa with flagella (Maru, 1976). In previous reports, CaN has been shown to localize to the nuclei of round spermatids in mouse testis and the postacrosomal region of dog sperm (Moriya et al., 1995; Tash et al., 1988). In other reports, Ran has been shown to localize to the nucleus of the round spermatid, with subsequent relocalization to the microtubules of the manchette in the elongating spermatid, after which it localizes to the centrosomal region of the elongated or condensed spermatid, the manchette of which disappears in rats (Kierszenbaum et al., 2002). These results have led to the suggestion that Ran and CaN colocalize in the spermatid and sperm for spermiogenesis and are involved in flagellum formation. As a Ran-binding protein that also interacts with CaN, CaNBP75 may serve to link the

functions of Ran and CaN in the process of spermiogenesis.

In testis, substrates of CaN have yet to be identified. This is in contrast to other tissues, in which endogenous substrates of CaN are known, including nuclear factor of activated T-cell (NFAT) in T-cell, muscle and other tissues (Feske et al., 2003; Chin et al., 1998), and inhibitor 1 in brain and heart (Mulkey et al., 1994; El-Armouche et al., 2006). Previously, Donella-Deana et al. (1994) characterized the phosphatase specificity of CaN using various phosphopeptide substrates. The properties of substrates of CaN include the following: (1) a basic residue located at position −3 relative to the phosphorylation site, and (2) a non-acidic residue N-terminal or C-terminal to the phosphorylation site. Furthermore, it has been argued that the specificity of CaN is determined by a variety of primary and higher-order structural features rather than a given consensus sequence. In scallop Ran, the sequence around Ser140, which corresponds to the phosphorylation site in vertebrate Ran, has the two characteristics of a CaN substrate described above. Thus, Ran may be a CaN substrate in scallop testis, with CaNBP75 involved in recruitment of Ran to CaN. Previously, Bompard et al. (2010) reported that the phosphorylation of Ser135 of vertebrate Ran is involved in the progression of mitosis. If Ran is a substrate of CaN, these proteins, along with CaNBP75, may be involved in meiosis in addition to spermiogenesis in scallop testis. In scallop testis, expression of Ran, CaNBP75 and CaN increased from January to February, which corresponds to a period of active meiosis, consistent with a role for these proteins in meiosis. Other CaN substrates may also be involved in spermatogenesis; however, the identity of these substrates remains to be determined by future studies.

In addition to testis, Ran, CaNBP75 and CaN were highly expressed in the ovary. CaN is involved in the progression of meiosis II in the vertebrate oocyte (Mochida and Hunt, 2007; Nishiyama et al., 2007). Our results suggest that Ran, CaNBP75 and CaN may also be involved in meiosis in the scallop oocyte as well as in the spermatocyte.

Due to the homology between CaNBP75 and RanBP3 in the C-terminal regions of the proteins, CaNBP75 is considered a homolog of human RanBP3. In human, RanBP3 is highly expressed in testis and heart, where expression of CaN is also abundant (Mueller et al., 1998, Su et al., 1995). In

addition, in mouse, while expression of Ran is much higher in testis, it is expressed in most somatic tissues (Coutavas et al., 1994). The fact that these proteins are highly expressed in mammalian testis suggests that the putative regulatory axis involving Ran, CaN-binding protein(s) and CaN is conserved among mammals.

The cDNA sequence of a Ran homolog from abalone, a marine gastropod, was recently reported (Wu et al., 2011). The amino acid sequence of abalone Ran (abRan) shows a high level of sequence identity (80.6%) to scallop Ran. Notably, multiple regions important for Ran function are highly conserved in abRan. Interestingly, the N-terminal region of abRan does not resemble that of scallop, as is observed for other Ran homologs; the length of this region, however, is similar to that of scallop Ran. In line with the current results, abRan appears to have a high degree of sequence identity with scallop Ran and other Ran homologs from other species.

In summary, a cDNA encoding scallop Ran was isolated from testis tissue. Analysis of the binding properties *in vitro* and *in vivo* and expression patterns of scallop Ran suggested that Ran collaborates with CaN in the regulation of spermatogenesis through binding to CaNBP75. This is the first proposal of the possibility of collaboration of Ran-dependent pathway and CaN-dependent pathway, and the proposed collaboration might be very important for regulation of spermatogenesis. Further studies of the details of CaN function in testis, including the identification of CaN substrates, are in progress.

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Figure legends

Fig. 1 Nucleotide sequence of scallop Ran cDNA and deduced amino acid sequence.

The partial nucleotide sequence of the scallop Ran cDNA (top, small letters) and the deduced amino acid sequence (bottom, capital letters) are shown. Asterisks indicate stop codons. The double underline indicates an in-frame stop codon in the 5'-UTR. The numbers in the left and right margins indicate nucleotide and amino acid positions, respectively. The box indicates the position of the reverse primer R2 used for 5'-RACE. The underline indicates the sequence of the degenerate PCR product. The nucleotide sequence data reported will appear in the DDBJ/EMBL/GenBank databases under the accession number AB646685 for scallop Ran.

Fig. 2 Alignment of Ran amino acid sequences.

The deduced amino acid sequence of scallop Ran was aligned with other known Ran homologs. Species and type are indicated in the left margin as follows: mouse somatic Ran (Mouse(s)) (AAA64247); mouse testis-specific Ran (Mouse(t)) (NP_033054); xenopus (NP_001128547); fruit fly (NP_651969), and yeast (*S. cerevisiae* Gsp1p) (NP_013396). Gaps are indicated by hyphens. The numbers in the right margin indicate amino acid positions. Amino acids that are identical in more than four of the six molecular species were defined as conserved residues and are shown in white letters against a black background. The residues indicated in italics in the N-terminal region represent weakly conserved residues across species. The boxed sequences indicate regions that are important for Ran function (Milburn et al., 1990, Scheffzek et al., 1995, Vetter et al., 1999). The sequences indicated by arrows were used to design the degenerate primers for PCR. The asterisks indicate positions of substituted residues between mouse somatic and testis-specific Ran.

Fig. 3 Northern blot analysis of scallop testis Ran.

Five micrograms of total RNA from scallop testis were separated on a 1% agarose formaldehyde gel and then visualized by ethidium bromide staining (lane 1). RNA was transferred to a nylon membrane and hybridized with a Ran RNA probe (lane 2). Size markers are indicated by arrowheads

in the left margin. A transcript of approximately 2.6 kb indicated by an arrow was detected.

Fig. 4 Ran-binding capacity of CaNBP75.

(A) Schematic representation of GST-CaNBP75 and the truncated variant of CaNBP75 used in the GST pull-down assay. The RanBP3 homology region (light gray) and the putative Ran-binding domain (dark gray) are indicated. Amino acid positions are indicated at the top of the diagram. (B) GST-tag only or GST-fusion proteins immobilized to glutathione resin were incubated with RanGTPγS. Bound proteins were separated by SDS-PAGE, transferred to a membrane and then visualized by amido black staining (top), or detected by western blot (WB) analysis using an anti-Ran antibody (bottom). Molecular weights are indicated in the left margin, and the positions of the GST-fusion proteins (top) or Ran (bottom) are indicated by open arrowheads. The asterisk indicates GST alone, with which the secondary antibody reacts nonspecifically.

Fig. 5 Tissue distributions of Ran, CaNBP75 and CaN detected by western blot or far-western blot analysis.

Various tissue extracts (30 μg protein/lane) prepared from fresh scallop were analyzed by western blot (WB) using anti-Ran antibody, anti-CaNBP75, anti-CaN antisera or anti-α-tubulin antibody. Far-western blot (Far-WB) analysis was performed using CaN as a probe in the presence of CaM, followed by detection using an anti-CaN antiserum, as indicated in the left margin. Tissues are indicated at the top of the panel. Abbreviation: sm, smooth.

Fig. 6 Expression analysis of Ran, CaNBP75 and CaN mRNA during scallop testis maturation cycle

(A–C) Fifteen micrograms (for rRNA and Ran) or 20 μg (for CaNBP75) of total RNA prepared from scallop testis harvested monthly were separated by electrophoresis and then subjected to ethidium bromide (EtBr) staining to visualize the rRNA (A), or analyzed by northern blot using a Ran (B) or CaNBP75 (C) RNA probe. (D) Quantitative analysis of the mRNA expression levels of

CaNA (black bar), CaNB (white bar), CaNBP75 (light gray bar) and Ran (dark gray bar) during the maturation cycle of the scallop testis. For Ran and CaNBP75, three different testes were analyzed each month, and average values were plotted. The maximal expression level of each mRNA was set at 100; standard deviations are shown by error bars. For CaNBP75, only the 10-kb mRNA species was quantified. Open circles with lines indicate changes of gonad index, an estimate of the developmental stage of the gonad. Gonad index = (weight of testis/body weight without shell) × 100. The data for CaNA and CaNB were drawn from Uryu et al. (2000). *, not determined.

Fig. 7 Co-immunoprecipitation analysis of Ran, CaNBP75 and CaN in scallop testis.

Scallop testis lysates were immunoprecipitated using no antibody (-), normal rabbit IgG (IgG) and anti-CaN polyclonal antibody (A) or anti-CaNBP75 polyclonal antibody (FS0025) (B) as indicated on top of panels under Ca^{2+} (Ca) or EGTA (E) condition. The precipitates were subjected to western blot (WB) analysis using anti-CaNBP75, anti-CaN, anti-Ran antibodies as indicated on left of the panels. The lane numbers are indicated on bottom of the panels. On the input lane, 1/200 of the input was loaded for detection of CaNBP75 and CaN, and 1/5,000 of the input was loaded for detection of Ran. On the immunoprecipitate (IP) lane, 1/8 of the precipitate was loaded.

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Figure 1 Hino et al.

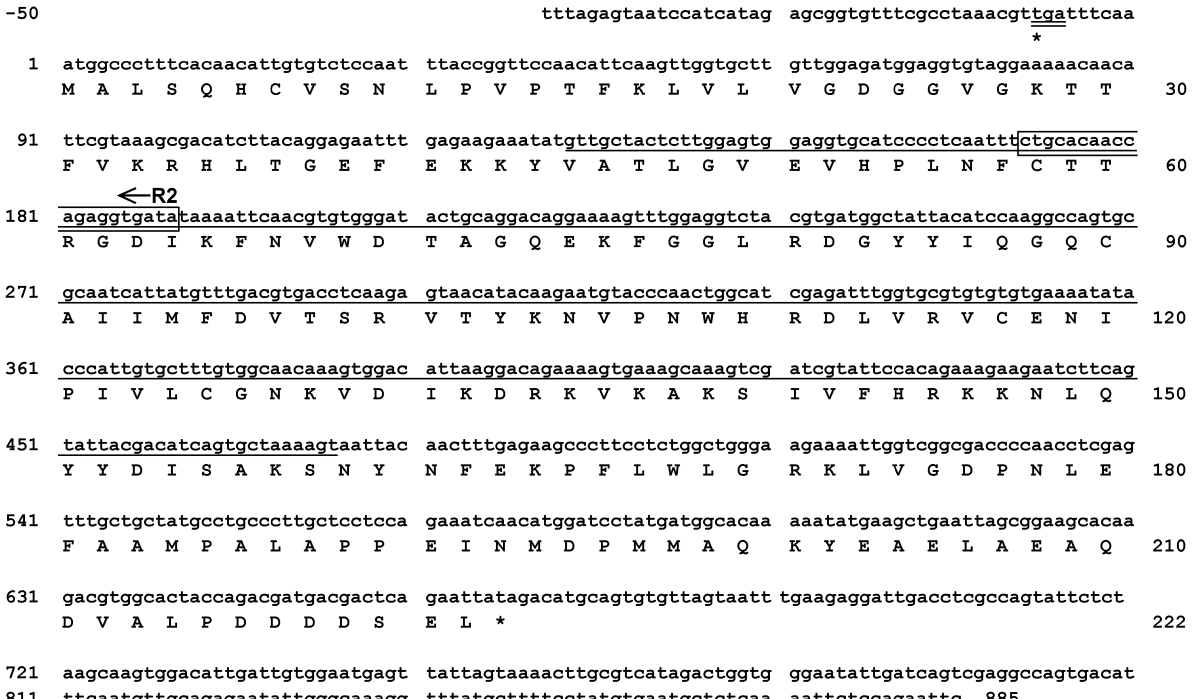

Figure 2 Hino et al.

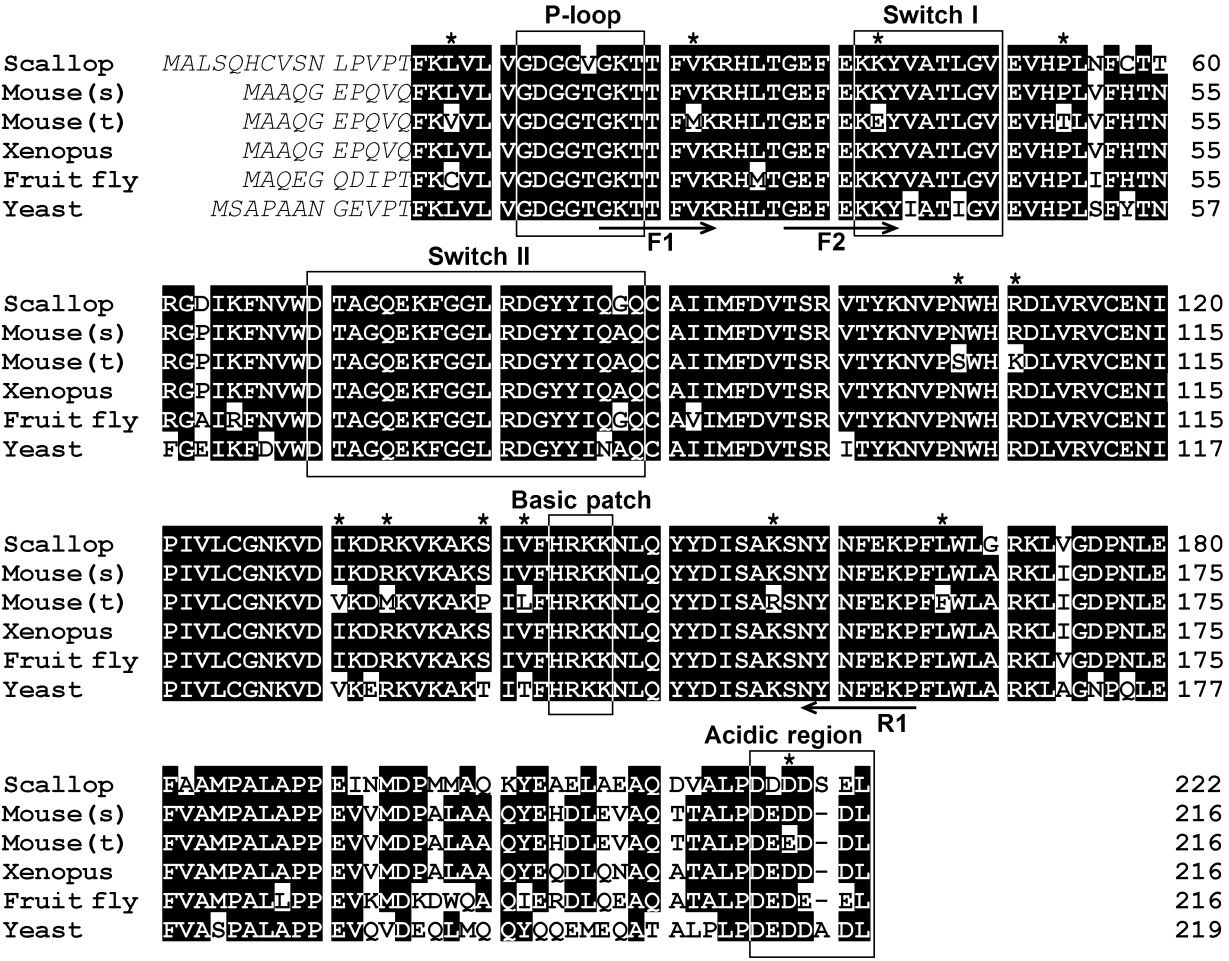

Figure 3

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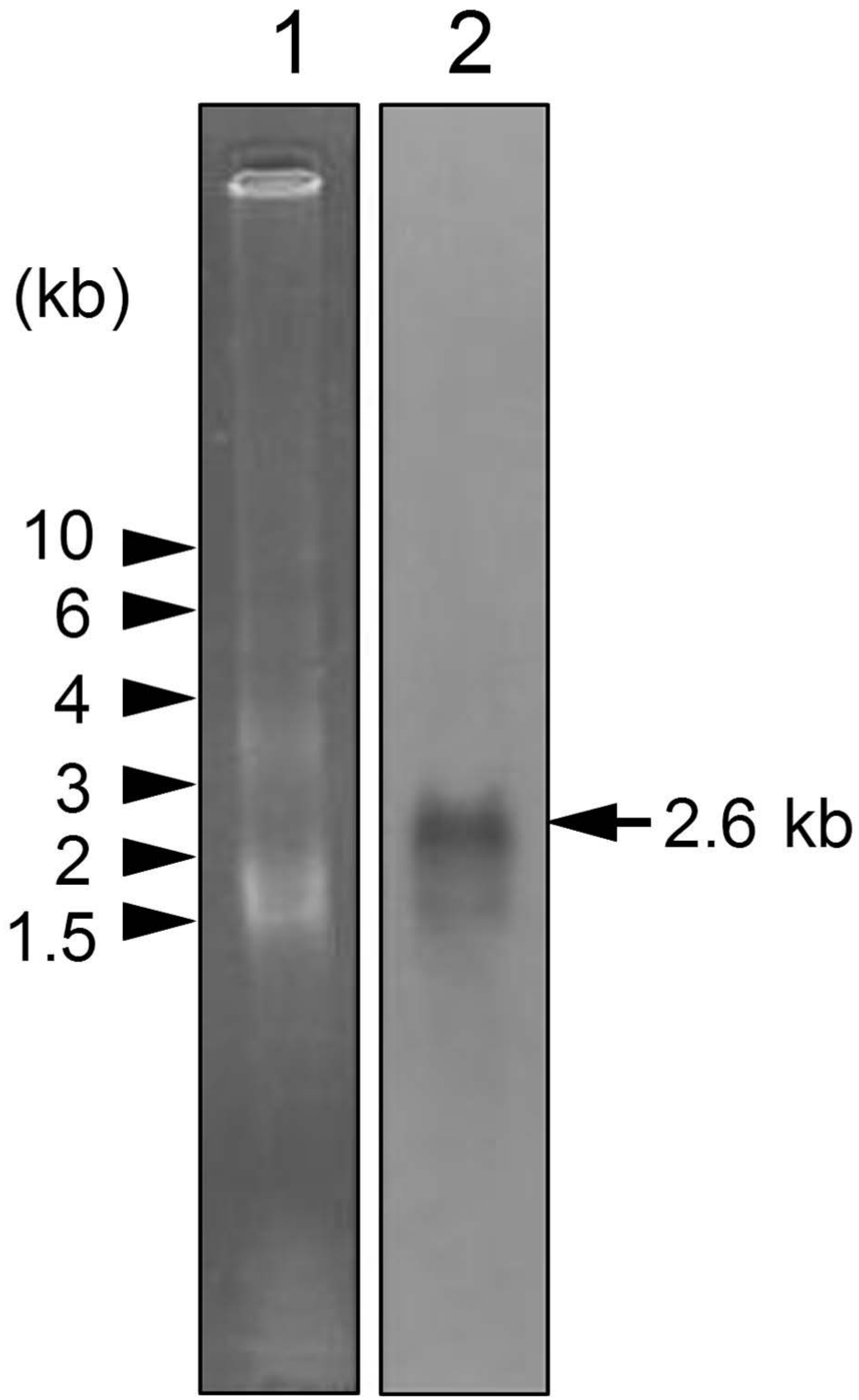

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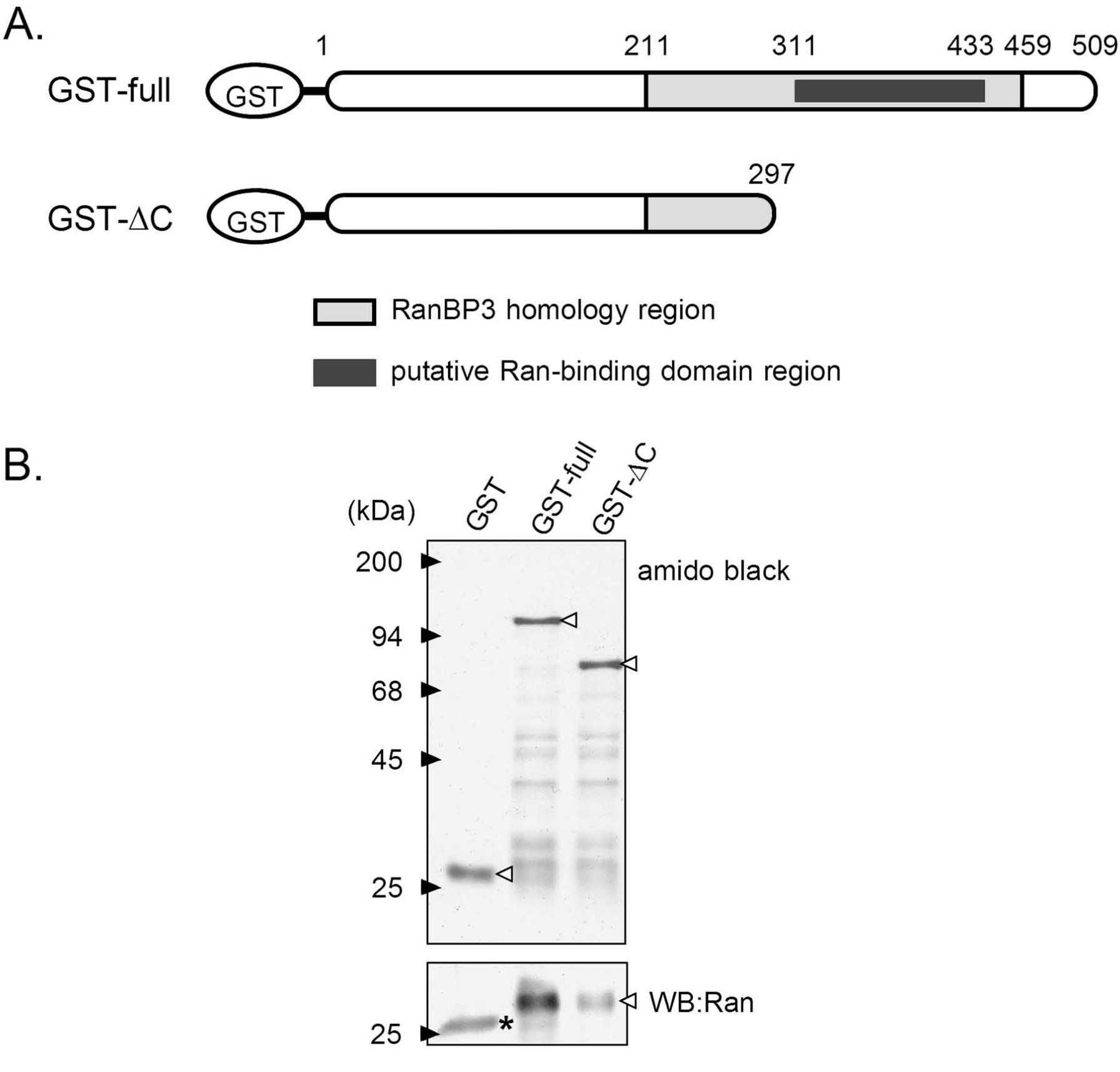

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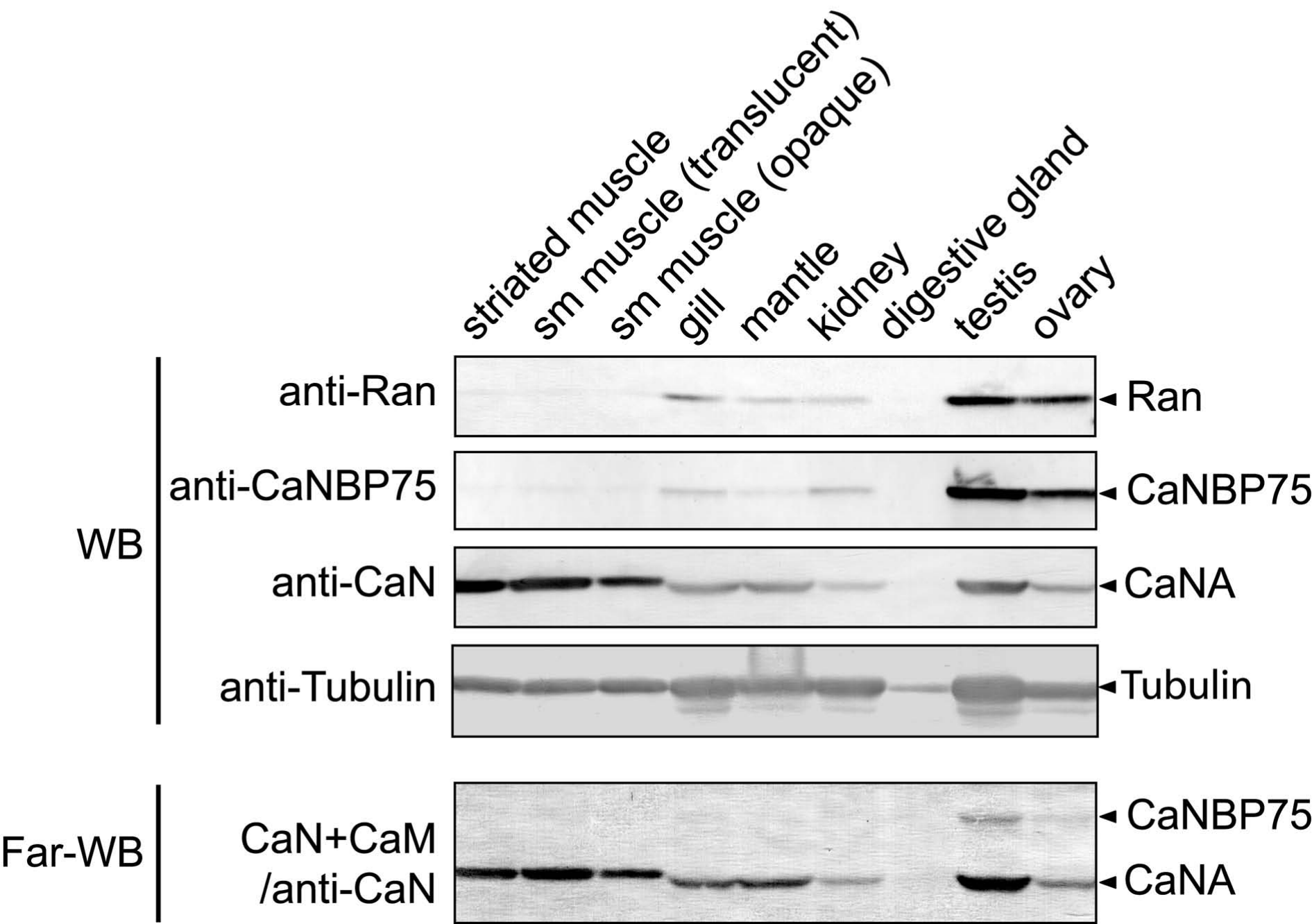

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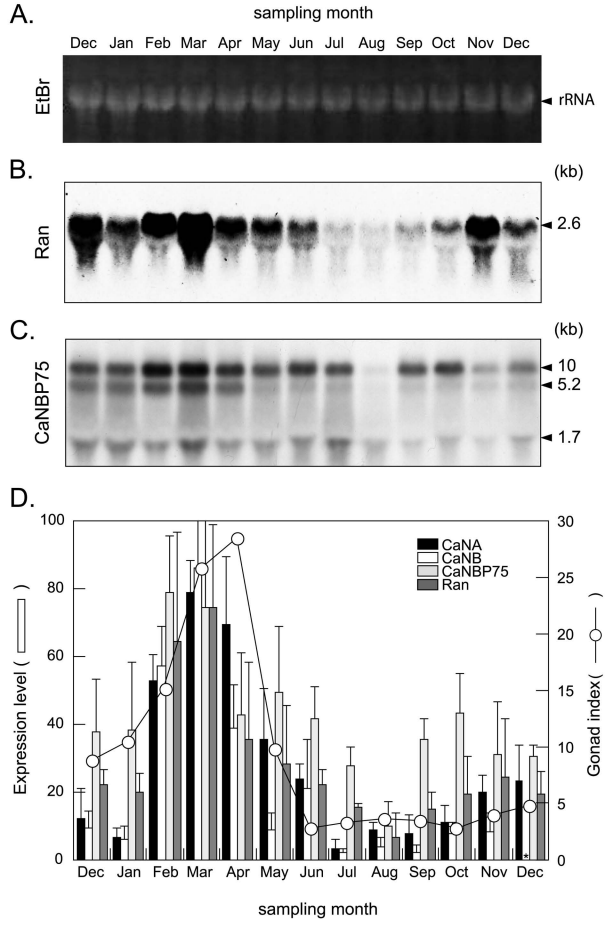

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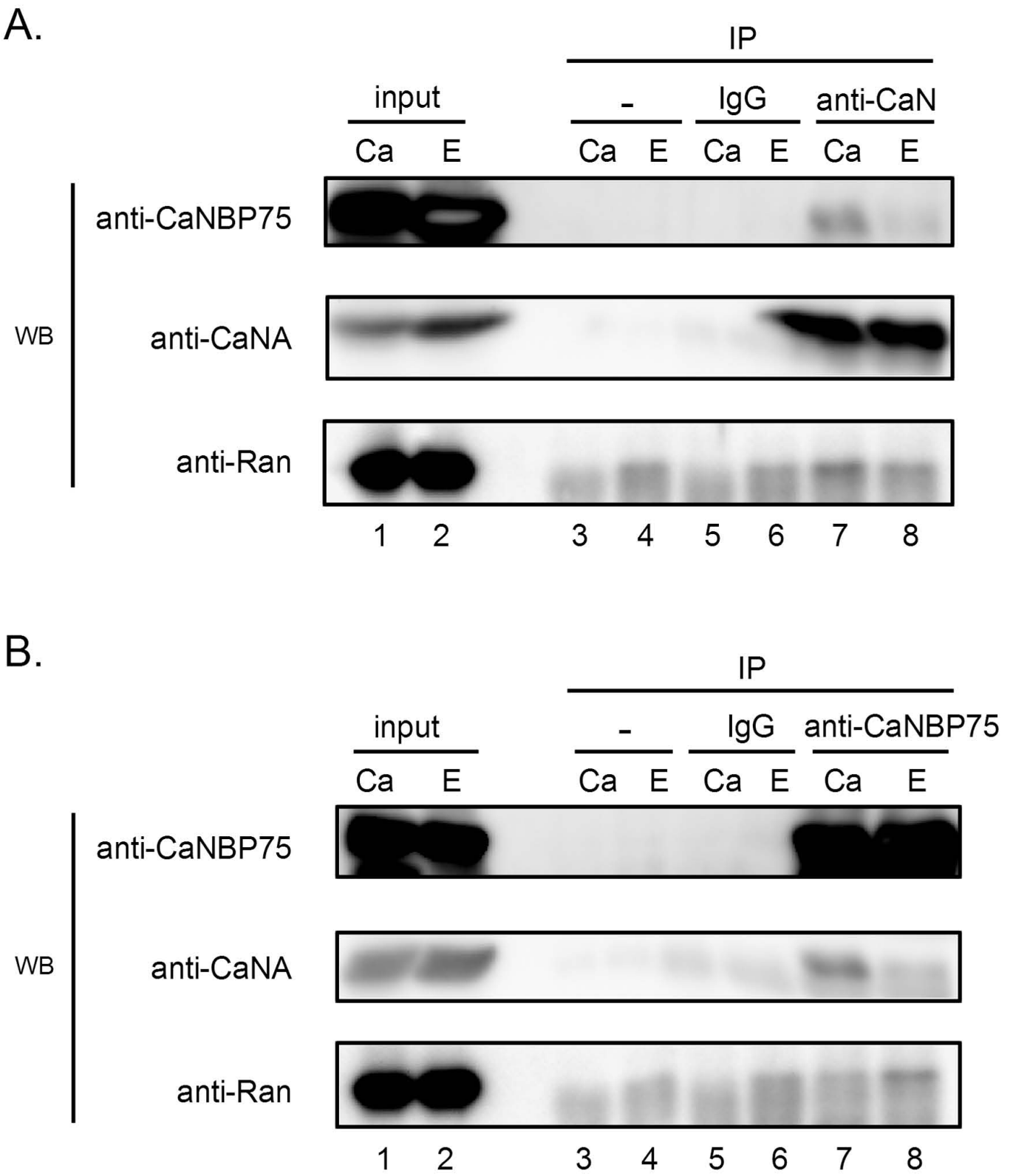

Table 1 Identity of the amino acid sequences of Ran proteins.

Species	Identity to scallop Ran [a)]
Mouse (somatic)	82.9%
Mouse (testis)	77.0%
Xenopus	82.9%
Fruit fly	81.1%
Yeast (*S. cerevisiae* Gsp1p)	76.1%

[a)] Identity is calculated with the number of total amino acid residues as 222. The deletions in the N-terminal region and the gap in the C-terminal region are counted as non-identical residues.