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Immunofluorescence Localization of a 23-kDa *Tetrahymena* Calcium-Binding Protein, TCBP-23, in the Cell Cortex

Kazuko Hanyu, Osamu Numata, Mihoko Takahashi,[1] and Yoshio Watanabe[2]

Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305

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Previously, we succeeded in cloning a cDNA of the 23-kDa *Tetrahymena* Ca^{2+}-binding protein, designated TCBP-23. Analysis of the deduced amino acid sequences showed that TCBP-23 is a member of the EF-hand family of Ca^{2+}-binding proteins. However, its physiological function was not elucidated. In the studies reported here, recombinant TCBP-23 was expressed in *Escherichia coli* and purified. Since recombinant TCBP-23 binds Ca^{2+} *in vitro*, Ca^{2+}-binding domains of the protein are likely to be functional *in vivo*. Rabbit antibodies against TCBP-23 were raised and used to determine the intracellular localization of the protein in *Tetrahymena* cells by indirect immunofluorescence. The antibodies strongly stained the whole cell cortex except for the oral apparatus and around the basal bodies. TCBP-23 remained in detergent-extracted cells, suggesting that it is associated with the epiplasm, the membrane skeleton of *Tetrahymena*. These results suggest that TCBP-23 may mediate Ca^{2+}-regulated processes in the cell cortex.

Key words: Ca^{2+}-binding protein, cell cortex, EF-hand, localization, *Tetrahymena*.

Ca^{2+} is the most common second messenger in cells. In ciliated protozoa, some physiological phenomena such as ciliary beating, cell division, endocytosis, and exocytosis are known to be Ca^{2+}-dependent. But molecular dissection of these Ca^{2+}-dependent phenomena has not made much progress. As an intracellular Ca^{2+}-binding protein that transmits the Ca^{2+} signal in cellular responses, calmodulin was isolated in our laboratory from *Tetrahymena* for the first time in protozoa (*1, 2*). Calmodulin is a ubiquitous Ca^{2+}-binding protein playing an important role in many Ca^{2+}-dependent processes through interaction with other proteins (*3*). In our previous papers, *Tetrahymena* calmodulin has been suggested to play important roles in ciliary reversal, endocytosis and exocytosis (*4-6*). In *Paramecium*, calmodulin regulates swimming behavior through regulating the functions of Ca^{2+}-dependent channels (*7, 8*), and is necessary for membrane linking that enables membrane fusion during trichocyst exocytosis (*9*).

On the other hand, other putative Ca^{2+}-binding proteins are known to be present in ciliated protozoa. Spasmin, which was found from *Vorticellid*, is thought to be a Ca^{2+}-binding protein that is involved in Ca^{2+}-dependent contraction of spasmoneme (*10, 11*). In *Isotricha* and *Polyplastron*, it was shown that a 22-kDa putative Ca^{2+}-binding protein may be involved in the control of microfilament ordering (*12*).

We attempted to detect Ca^{2+}-binding protein(s) other than calmodulin to explain the complicated mechanism of the Ca^{2+}-dependent phenomena (*13*). The existence of two Ca^{2+}-binding proteins other than calmodulin was demonstrated in *Tetrahymena*. We cloned the gene for the second Ca^{2+}-binding protein, *Tetrahymena* Ca^{2+}-binding protein of 25 kDa (designated TCBP-25) (*14*). This protein was first reported as TCBP-10 (*15*), a proteolytic C-terminal fragment of TCBP-25. We also cloned the gene for the third Ca^{2+}-binding protein of 23 kDa (designated TCBP-23) (*16*). Both TCBP-25 and TCBP-23 contain four EF-hand Ca^{2+}-binding loops, as first described by Kretsinger for parvalbumin (*17*). Therefore, TCBP-25 and TCBP-23 are members of the calmodulin family.

Thus, *Tetrahymena* has at least three calmodulin family proteins: calmodulin, TCBP-25 and TCBP-23. These proteins are expected to play important roles in Ca^{2+}-dependent phenomena. Recently, we showed that TCBP-25 was localized in the cell cortex except around the basal bodies during every growth phase of *Tetrahymena*, and also around the migratory and stationary pronuclei at the stage of nuclear exchange during conjugation. These lines of evidence suggest that TCBP-25 plays crucial roles in the Ca^{2+}-mediated signaling process in the cell cortex and in the Ca^{2+}-dependent process of pronuclear exchange during conjugation (*18*).

Of these three *Tetrahymena* Ca^{2+}-binding proteins, possible biological functions have been suggested for calmodulin and TCBP-25. No attempt, however, has been made to reveal the biological function of TCBP-23. To understand the function and physiological roles of TCBP-23, we tried to express TCBP-23 in *Escherichia coli* and investigated its intracellular localization in *Tetrahymena*. In the present paper, we describe evidence that TCBP-23 is localized in the cell cortex except around the basal bodies in every growth phase of *Tetrahymena*. Based on the results obtained, we will discuss possible roles of TCBP-23 in the cortex.

[1] To whom correspondence should be addressed. Fax: +81-298-53-6614, E-mail: mihoko@biol.tsukuba.ac.jp

[2] Present address: Joubu University, Isesaki, Gumma 372.

Abbreviations: GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NP-40, Nonidet P-40; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Cell Culture—*Tetrahymena thermophila* strain B was kindly provided by Dr. T. Sugai (Ibaraki University). The cells were grown in 0.25% proteose peptone, 0.25% yeast extract, and 3.5% glucose at 26°C until early stationary phase.

Site-Directed Mutagenesis—Site-directed mutagenesis was carried out using the Mutan-G site-directed mutagenesis system (Takara), according to the manufacturer's instructions. The TCBP-23 cDNA (*16*) was subcloned into the *Bam*HI site of M13tv19. The following oligonucleotides were used to change TAA and TAG codons for the indicated amino acids to CAA and CAG codons: Gln 8, 5′-CAAATCATCACCCAAAACGTCTACGCT-3′; Gln 28, 5′-AAGCTTTTTGCTCAGTTTGATTCTAAC-3′; Gln 138, 5′-GATAAGAGTGGTCAATTAGAAGAAAAG-3′. The altered nucleotides are underlined. Mutations were confirmed by single-strand DNA sequencing.

Expression and Purification of Recombinant TCBP-23—The TCBP-23 cDNA with two CAAs and one CAG changed from TAAs and TAG, respectively, was amplified by PCR using oligonucleotides: 5′-GGGGATCCATGGAACACCAAATCATC-3′ (corresponding to nucleotides 1-18) and 3′-GGGGATCCATCAAGCTTGAGTGAATTCC-5′ (corresponding to nucleotides 606-625), which included the *Bam*HI site. The resulting product was cloned in-frame into the expression vector pGEX-2T (Pharmacia) using the *Bam*HI site. Expression and purification of recombinant protein were performed essentially as described by Smith and Johnson (*19*). The GST-TCBP-23 fusion protein was expressed in *E. coli*, strain JM109, and purified on glutathione-Sepharose 4B (Pharmacia). To purify free TCBP-23, GST-TCBP-23-bound beads were incubated in PBS containing thrombin (Pharmacia, 50 cleavage units/ml beads). The resultant supernatant including TCBP-23 was recovered by centrifugation. Cleavage of the fusion protein with thrombin releases TCBP-23 with a two-residue N-terminal extension.

Antibodies—Rabbit polyclonal antisera were raised against recombinant TCBP-23. Recombinant protein was subjected to two-dimensional polyacrylamide gel electrophoresis (*20*) and sliced from the gel. After dialysis against PBS, the gel slices containing recombinant protein were emulsified with Freund's complete adjuvant and injected into rabbits. Booster injections using Freund's incomplete adjuvant were carried out at 10-day intervals. Anti-TCBP-23 antibodies were affinity purified against recombinant TCBP-23 immobilized on Immobilon membrane filters (Millipore) (*21*). Guinea pig antiserum against *Tetrahymena* tubulin has been previously prepared and characterized (*22*).

SDS-PAGE and Immunoblot Analysis—SDS-PAGE was performed as described (*23*). *Tetrahymena* cells were solubilized with 8 M guanidine HCl containing 10% 2-mercaptoethanol and 0.1 M Tris-HCl (pH 7.5), and dialyzed against 7 M urea. Molecular weight markers were purchased from Bio-Rad. Proteins were stained with Coomassie Brilliant Blue R-250. For immunoblot analysis, proteins resolved by SDS-PAGE were transferred onto Immobilon membrane filters (Millipore), using a semi-dry blotting apparatus (Sartorius). Filters were blocked with 3% non-fat dried milk in PBS containing 0.05% Tween 20 before incubation with primary antibody. The immunoblots were visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Tago, 1:2,000 dilution) using a BCIP/NTB phosphatase substrate system (KPL).

Ca^{2+}-Binding—Electrophoresis on alkaline glycerol gels was performed as described (*24*). The binding of $^{45}Ca^{2+}$ was performed as described by Maruyama *et al.* (*25*). $^{45}CaCl_2$ was obtained from Amersham.

Indirect Immunofluorescence—Immunofluorescence staining was carried out as previously described (*18*). NP-40 extracted cells were prepared as described by Goodenough (*26*). Deciliation was induced by the Ca^{2+}-shock method (*27*). For single immunofluorescence staining, polyclonal anti-TCBP-23 antibody was used as the primary antibody. Fluorescein-conjugated goat anti-rabbit IgG (Tago) was used as the secondary antibody. For double immunofluorescence staining, polyclonal rabbit anti-TCBP-23 antibody and guinea pig anti-tubulin antiserum were used as primary antibodies. Fluorescein-conjugated goat anti-rabbit IgG (Tago) and rhodamin-conjugated anti-guinea pig IgG (Tago) were used as secondary antibodies. Cells were examined under a Nikon fluorescence microscope and were photographed on Tri-X Pan 400 film.

RESULTS

Expression and Purification of Recombinant TCBP-23 in E. coli—Since *Tetrahymena* uses "universal" stop codons, TAA and TAG, as glutamine codons (*28-30*), *Tetrahymena* genes carrying TAA and TAG in their open reading frames cannot be expressed in *E. coli*. To express TCBP-23 gene in *E. coli*, we changed the two TAA and one TAG codons in this gene to glutamine codons, CAA and CAG, by site-directed mutagenesis. The TCBP-23 gene with CAAs and CAG changed from TAAs and TAG was subcloned into the expression vector pGEX-2T.

Results of expression and purification of TCBP-23 are shown in Fig. 1. Whole-cell lysates of bacteria transformed with pGEX-2T recombinant before (lane 1) and after IPTG induction (lane 2) were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. GST-TCBP-23 fusion protein, which had an apparent molecular weight consistent with the size of the TCBP-23 plus the 26-kDa GST fragment, was expressed in bacteria (marked by the open arrowhead in Fig. 1). A bacterial sonicate was prepared and incubated with glutathione-Sepharose 4B. The bound proteins were eluted by boiling in an SDS-containing buffer and resolved by SDS-PAGE (lane 3). GST-TCBP-23 fusion protein was found in the soluble fraction of a total bacterial sonicate and bound to glutathione-Sepharose 4B. To remove the GST carrier from the fusion protein, GST-TCBP-23-bound beads were incubated with thrombin. As a result, purified TCBP-23 was recovered in the supernatant (lane 4). The apparent molecular weight of the recombinant protein estimated by gel electrophoresis using the method of Laemmli is slightly larger than the molecular weight estimated from its deduced amino acid sequence. About 6 mg of TCBP-23 was produced in 1 liter of bacterial culture.

TCBP-23 Binds Ca^{2+} In Vitro—To determine whether TCBP-23 is a functional Ca^{2+}-binding protein, we tested its ability to bind Ca^{2+} *in vitro*.

Calmodulin and other members of the EF-hand Ca^{2+}-

binding protein family are known to show a characteristic Ca^{2+}-dependent shift in electrophoretic mobility (*31*). Recombinant TCBP-23 was incubated either with $CaCl_2$ or the Ca^{2+} chelator EGTA and electrophoresed on an alkaline glycerol gel, in the absence of urea and SDS. Figure 2A shows that the Ca^{2+}-bound form of TCBP-23 migrated at a slower rate than the Ca^{2+}-free form. Therefore, TCBP-23 undergoes a conformational change upon Ca^{2+}-binding.

The ability of TCBP-23 to bind Ca^{2+} was examined directly by incubation with $^{45}Ca^{2+}$. Recombinant TCBP-23 was immobilized onto a membrane, incubated with $^{45}Ca^{2+}$ and an autoradiograph of the membrane was made. Figure 2B shows that TCBP-23 was labeled with $^{45}Ca^{2+}$ (lane 2′). No protein in the molecular weight standards gave a positive signal (lane 1′).

These results clearly show that recombinant TCBP-23 has the ability to bind Ca^{2+} *in vitro*, as expected from its deduced amino acid sequence.

Characterization of Antibodies against TCBP-23—To investigate the intracellular localization of TCBP-23, we prepared four rabbit polyclonal antibodies against recombinant TCBP-23. When the antibodies were analyzed for specificity by immunoblotting, they showed almost the same properties. Figure 3 shows that an antibody recognized a single protein (lane 2′) not present in the preimmune sera (lane 1′) in *Tetrahymena* cell extracts. This protein had the same migration behavior as purified TCBP-23 (lane 3′). The antibody did not recognize purified TCBP-25 (lane 4′).

Localization of TCBP-23—Figure 4 shows the indirect immunofluorescence localization of TCBP-23. In vegetative cells, the antibody against TCBP-23 stained the whole cell cortex except for a linear punctuate array in paraformaldehyde-fixed cells (Fig. 4B). The preimmune serum did not stain this structure (Fig. 4A). Double-staining immunofluorescence images with anti-TCBP-23 antibody and anti-tubulin antiserum demonstrated that this array corresponded to the areas around the basal bodies as revealed by immunofluorescence for tubulin (Fig. 4C). No appreciable immunofluorescence for TCBP-23 was observed inside the

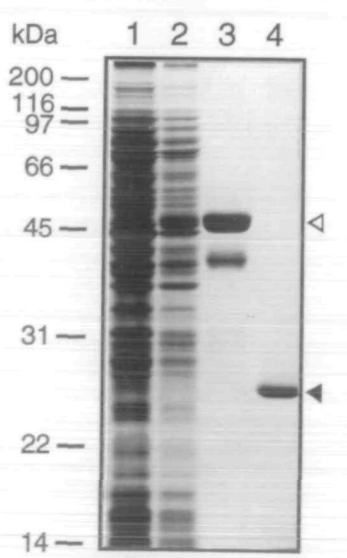

Fig. 1. **Expression and purification of recombinant TCBP-23.** Bacteria transformed with pGEX-2T-TCBP-23 were solubilized before (lane 1) and after IPTG induction (lane 2) A sonicate of IPTG-induced cells was incubated with glutathione-Sepharose 4B and the bound proteins were eluted by boiling (lane 3) The fusion protein purified on glutathione-Sepharose 4B was incubated with thrombin and TCBP-23 was recovered by centrifugation (lane 4). Proteins were resolved by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue. The open arrowhead indicates the position of GST-TCBP-23 fusion protein and the closed arrowhead indicates the position of purified TCBP-23.

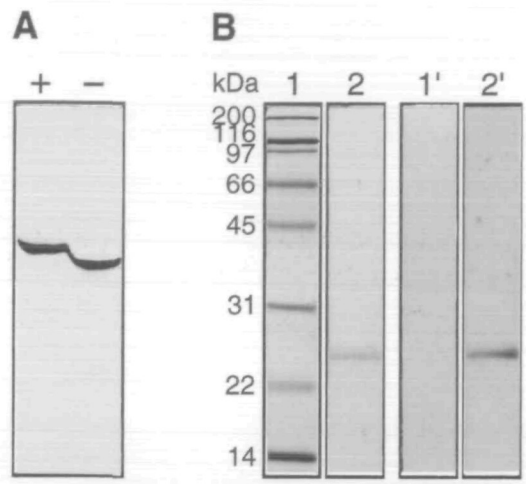

Fig. 2. **Binding of Ca^{2+} to TCBP-23.** (A) Recombinant TCBP-23 was electrophoresed on alkaline glycerol gel in the presence of 2 mM $CaCl_2$ (+) or 2 mM EGTA (−) and stained with Coomassie Brilliant Blue TCBP-23 that binds Ca^{2+} displays a slower mobility on the gel. (B) Molecular weight markers (lanes 1 and 1′) and recombinant TCBP-23 (lanes 2 and 2′) were resolved by 12.5% SDS-PAGE and either stained with Coomassie Brilliant Blue (lanes 1 and 2) or transferred to a membrane and incubated with $^{45}Ca^{2+}$ (lanes 1′ and 2′) Autoradiography of the membrane shows that TCBP-23 binds $^{45}Ca^{2+}$.

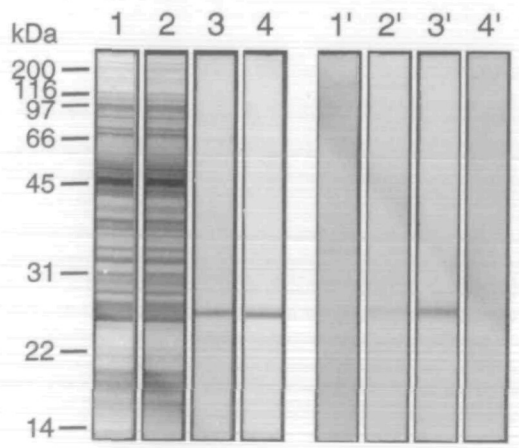

Fig. 3 **Specificity of an antibody against TCBP-23.** *Tetrahymena* cell extracts (lanes 1, 2, 1′, and 2′), purified TCBP-23 (lanes 3 and 3′) and purified TCBP-25 (lanes 4 and 4′) were resolved by 12.5% SDS-PAGE and either stained with Coomassie Brilliant Blue (lanes 1-4) or analyzed by immunoblotting (lanes 1′-4′) A preimmune serum (lane 1′) and an antibody against TCBP-23 (lanes 2′-4′) were used as primary antibodies, and the immunoreactive bands were detected with an alkaline phosphatase-conjugated secondary antibody. The antibody reacted to TCBP-23 in *Tetrahymena* cell extracts (lane 2′) and purified TCBP-23 (lane 3′) but did not react to TCBP-25 (lane 4′). A weak background band is recognized with both the antibody (lane 2′) and the preimmune serum (lane 1′).

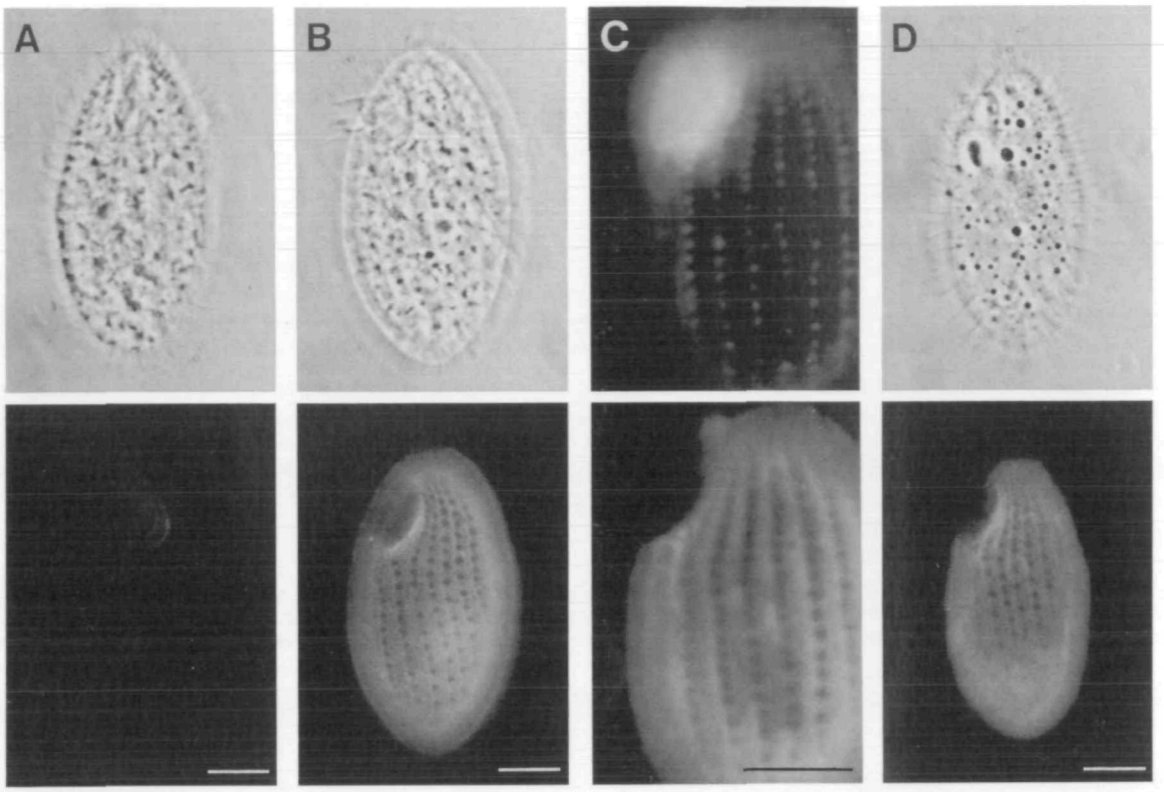

Fig 4. **Localization of TCBP-23 in *Tetrahymena* cells.** *Tetrahymena* cells were fixed directly with 4% paraformaldehyde (A and B), deciliated and fixed with 4% paraformaldehyde (C) or extracted with 0 5% NP-40 and fixed with 2 5% paraformaldehyde (D). The directly fixed cells and the extracted cells were stained with the preimmune serum (A) or the antibody against TCBP-23 (B and D) In (A), (B), and (D), upper panels are phase-contrast images and lower panels are the respective immunofluorescence images The deciliated cells were double stained with the antibody against TCBP-23 and the antiserum against tubulin (C) In (C), the upper panel is the immunofluorescence image for tubulin and the lower panel is the respective immunofluorescence image for TCBP-23. TCBP-23 is localized in the cell cortex except for the oral apparatus and around the basal bodies. Bars, 10 μm

cytoplasm (data not shown). To determine whether TCBP-23 existed in a soluble form, localization of TCBP-23 was examined after soluble proteins in the cytoplasm had been extracted with 0.5% NP-40. Immunofluorescence remained in the cell cortex, in the same way as observed in directly fixed cells without NP-40 extraction (Fig. 4D). This result shows that most of the TCBP-23 exists in a NP-40-insoluble form in the cell cortex. Judging from the fluorescence localization, TCBP-23 seems to be localized in the epiplasm lining the inner alveolar membranes. This TCBP-23 localization coincides with the TCBP-25 localization (*18*). In conjugating cells, however, no appreciable immunofluorescence for TCBP-23 was observed around gametic pronuclei, unlike for TCBP-25 (*18*)(data not shown).

DISCUSSION

In this study, we succeeded in expressing the TCBP-23 gene in *E. coli* by changing the two TAA and one TAG codons in this gene to CAAs and CAG, and in subsequent purification of the protein (Fig. 1). The purified TCBP-23 showed a Ca^{2+}-dependent shift in electrophoretic mobility and an ability to bind $^{45}Ca^{2+}$ (Fig. 2), which are special properties shared by EF-hand-type Ca^{2+}-binding proteins. Until recently, the characterization of TCBP-23 was hindered by degradation of the protein during purification from *Tetrahymena* cells. Expression of TCBP-23 gene in *E. coli* has resolved this problem. Detailed physical and chemical analysis of the Ca^{2+}-binding properties of the TCBP-23 is in progress.

We generated polyclonal antibodies raised against recombinant TCBP-23 (Fig. 3), and used these to examine the intracellular localization of TCBP-23 by immunofluorescence studies. TCBP-23 was localized in the cell cortex except for the oral apparatus and around the basal bodies and remained in detergent-extracted cells (Fig. 4). Thus, we suppose that TCBP-23 is associated with the epiplasm. Three major components of *Tetrahymena* epiplasm, called band A, B, and C, have been identified (*32, 33*). Localization of TCBP-23 seems to overlap with those of the three epiplasmic proteins (*33, 34*).

The cortical organization of *Tetrahymena* has been well characterized and, like that of most ciliates, consists of the plasma membrane, the alveoli, and the epiplasm (*35-38*). The membrane skeleton, epiplasm, forms an amorphous stiff layer lining the alveoli. The alveoli are composed of a continuous layer of membrane vesicles and are located close to the inner surface of the plasma membrane. On the basis of their ultrastructural morphology, especially their close apposition to the plasma membrane, the alveoli in *Paramecium* (*39*) and in *Tetrahymena* (*38*) have been proposed to constitute a Ca^{2+}-storage compartment that resembles the muscle sarcoplasmic reticulum. Indeed, Ca^{2+} is known to be actively sequestered inside the alveoli of

Paramecium (*40*) and to be distributed in the pellicle of *Paramecium* in high concentration (*41*). Therefore, the alveoli of ciliates such as *Paramecium* and *Tetrahymena* may play an important role in regulating intracellular Ca^{2+} concentration. TCBP-23 may mediate several Ca^{2+}-regulated responses of the cell cortex through binding Ca^{2+} that is released from the alveoli. Since it has been thought that the membrane skeleton is of a generating cell-type specific architecture, controlling the cell shape, and regulating the regional distributions and mobility of surface components, TCBP-23 may interact with the membrane cytoskeleton protein(s) and regulate the cytoskeletal elements dynamics in a Ca^{2+}-dependent manner. Alternatively, TCBP-23 may regulate protein(s) embedded in the inner alveolar membrane and be responsible for membrane functions. It may regulate the Ca^{2+}-pumping activity as calmodulin stimulates the plasma membrane Ca^{2+}-ATPase (*42*).

We have so far reported the existence of three calmodulin family proteins in *Tetrahymena*: calmodulin, TCBP-25 and TCBP-23. In addition, Frankel's group showed that an antibody against *Chlamydomonas* centrin, another member of the calmodulin family, recognized the basal bodies in *Tetrahymena* (personal communication). Based on immunofluorescence studies, the intracellular localization of TCBP-23 is quite different from those of calmodulin (*5, 43*) and centrin, implying that TCBP-23 may have functions different from these proteins. On the other hand, TCBP-23 is colocalized with TCBP-25 in vegetative cells (*18*) and may function cooperatively with TCBP-25. In conjugating cells, TCBP-25 has a unique distribution and may have unique roles (*18*), whereas TCBP-23 does not show such a unique distribution. To elucidate the biological functions of TCBP-23 and TCBP-25, more detailed observations on the localization of these proteins are necessary. We are studying the localizations of these proteins by immunoelectron microscopy. In addition, to ascertain whether these three calmodulin family proteins, calmodulin, TCBP-25, TCBP-23, function independently or cooperatively, we are trying to identify proteins which interact with them.

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