Immunocytochemical Studies on the EF-Hand Calcium-Binding Proteins in *Tetrahymena thermophila*

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ABBREVIATIONS

GST	glutathione *S*-transferase
IPTG	isopropyl-ß-D-thiogalactopyranoside
PBS	phosphate-buffered saline
PB	sodium-phosphate buffer
NP-40	Nonidet P-40
PMSF	phenylmethylsulfonyl fluoride
DAPI	4',6-diamidino-2-phenylindole dihydrochloride

ABSTRACT

Ca^{2+} is a key second messenger in eukaryotic cells. The Ca^{2+} signals are transduced into intracellular responses, in part by Ca^{2+}-binding proteins. One class of these proteins shares a common Ca^{2+}-binding motif, the EF-hand. Most EF-hand Ca^{2+}-binding proteins are proposed to function as Ca^{2+}-sensors and thereby to be critical for triggering the cellular responses.

In ciliated protozoa *Tetrahymena*, the genes for *Tetrahymena* Ca^{2+}-binding protein of 25 kDa (TCBP-25) and *Tetrahymena* Ca^{2+}-binding protein of 23 kDa (TCBP-23) were identified. Analysis of the deduced amimo acid sequences showed that TCBP-25 and TCBP-23 are members of the EF-hand Ca^{2+}-binding proteins, and these proteins were expected to play important roles in Ca^{2+}-dependent phenomena. Though their physiological functions were not elucidated.

In this study, TCBP-25 and TCBP-23 were expressed in *Escherichia coli* as glutathione *S*-transferase fusion proteins and then purified. Since universal stop codons, TAA and TAG, are used as glutamine codons in *Tetrahymena*, TCBP-25 and TCBP-23 genes carrying TAA and TAG cannot be expressed in *E. coli*. To express these proteins in *E. coli*, I have changed TAA and TAG codons in these genes to universal glutamine codons, CAA and CAG, by site-directed mutagenesis. Since recombinant proteins bind Ca^{2+} *in vitro*, Ca^{2+}-binding domains of these proteins are likely to be functional *in vivo*. Rabbit antibodies against TCBP-25 and TCBP-23 were raised and used to determine the intracellular localization of these proteins in *Tetrahymena* cells. Localization of TCBP-25 and

TCBP-23 was examined by indirect immunofluorescence microscopy. Using both antibodies, strong staining was observed in the whole cell cortex except for the oral apparatus and the areas around the basal bodies. These proteins remained in detergent-extracted cells, suggesting that they are associated with the epiplasm, the membrane skeleton of *Tetrahymena*. Epiplasmic localization of TCBP-25 was confirmed by immunoelectron microscopy. Furthermore, TCBP-25 is associated with both the migratory and stationary gametic pronuclei at the pronuclear exchange stage during conjugation. Therefore, I consider that TCBP-25 and TCBP-23 may play crucial roles in Ca^{2+}-regulated processes in the cell cortex and that TCBP-25 may be involved in a Ca^{2+}-dependent pronuclear exchange process during conjugation.

INTRODUCTION

Ca^{2+} is the most common second messenger important in eliciting cellular responses to external stimuli in eukaryotic cells. An increase in the cytosolic concentration of Ca^{2+} caused by hormonal or electrical signals can trigger a wide variety of cellular responses, such as contraction, cell proliferation, secretion, metabolic adjustments and change in gene expression. Transmission of the Ca^{2+}-signal requires intracellular Ca^{2+} sensors to trigger the cellular responses.

In 1965, Ebashi and Ebashi isolated troponin as regulator for the contraction of muscle fibers. Troponin C, one of the components of troponin, is the first Ca^{2+}-binding protein that triggers physiological phenomena in Ca^{2+}-dependent manner. In 1970, two groups working independently at the Ca^{2+}-activation of phosphodiesterase reported the isolation of a Ca^{2+}-related activation factor (Kakiuchi and Yamazaki, 1970; Cheung, 1970). This factor was found to be an acidic protein of 17 kDa with the ability to bind Ca^{2+}, and was named calmodulin. At the present time, calmodulin is considered a ubiquitous high affinity Ca^{2+}-binding protein that is involved in regulation of many cellular processes controlled by Ca^{2+}-dependent signaling pathways (Cohen and Klee, 1988).

The crystal structure of calmodulin in the Ca^{2+}-bound form shows a dumbbell-shaped molecule with two globular domains that are connected by a long extended central helix (Babu *et al.*, 1988). Each globular domain consists of two EF-hand Ca^{2+}-binding loops as first described by Kretsinger for parvalbumin (Kretsinger and

Nockolds, 1973). The EF-hand loop consists of two perpendicularly placed α-helixes and an interherical loop of 12 amino acids, which together form a single Ca^{2+}-binding site. Calmodulin has a Ca^{2+} affinity of 10^{-6} M and thus acts as a sensor for the concentration of free Ca^{2+} in the cytoplasm, which rises transiently from a resting value of approximately 10^{-7} M to 10^{-5} M. Upon binding of Ca^{2+}, calmodulin changes its conformation, then binds to its target proteins. Ca^{2+}/calmodulin is known to activate various enzymes including phosphodiesterases, protein kinases and phosphatases, and also react with structural proteins including microtubules and ion channels (Cohen and Klee, 1988).

The consensus amino acid sequence for the EF-hand loop has allowed the identification of numerous Ca^{2+}-binding proteins from the primary structures. There are over 200 proteins containing from two to eight of these common structural motifs, and these proteins can be classified into about 32 distinct subfamilies (Kawasaki and Kretsinger, 1994). Most of these EF-hand proteins are considered to function as intracellular Ca^{2+} receptors that transmit the Ca^{2+} signal in cellular responses. The physiological functions of some of them, such as calmodulin and troponin C, are well studied. However, the roles of many new members are unknown.

In ciliated protozoa, some physiological phenomena such as ciliary reversal, cell division, endocytosis and exocytosis are known to be Ca^{2+}-dependent. The involvement of Ca^{2+} has been best characterized in ciliary beating of *Paramecium*. This free-swimming organism generates a Ca^{2+}-based action potential, which results in the transient increase in intracellular Ca^{2+} that causes the cell to reverse its ciliary motion, stop, turn, and then resume swimming in a

new direction (Ekert and Brehm, 1979; Kung and Saimi, 1982). The exocytosis of the secretory vesicles of *Paramecium*, is also known to be Ca^{2+} influx dependent (Kerboeuf and Cohen, 1990), but an additional intracellular store may be involved (Erxleben and Plattner, 1994). Not much is studied in molecular dissection of these Ca^{2+}-dependent phenomena.

As an intracellular Ca^{2+}-binding protein, calmodulin was isolated from *Tetrahymena* for the first time in protozoa (Suzuki *et al.*, 1979, 1981; Jamieson *et al.*, 1979; Kumagai *et al.*, 1980). The amino acid sequence of *Tetrahymena* calmodulin was determined, and it shares 92% homology with vertebrate calmodulin (Yazawa *et al.*, 1981). It has ordinary properties, but has an exceptional feature of Ca^{2+}-dependent enhancement of guanylate cyclase activity in the place of adenylate cyclase activity (Nagao *et al.*, 1979; Suzuki *et al.*, 1981). Subcellular distribution of calmodulin was examined by fractionation of the cells (Nagao *et al.*, 1981). Approximately 93% of the total amounts of calmodulin was contained in the ciliary supernatants and postmicrosomal supernatants. From the immunofluorescence studies using an antibody against rat testis calmodulin, Satir *et al.* (1980) showed that calmodulin is localized in the cilia, a rim along the oral groove and basal bodies in *Tetrahymena*. Using an antibody against *Tetrahymena* calmodulin, Suzuki *et al.* (1982) corroborated these observations: calmodulin is localized in the oral apparatus, cilia, basal bodies, the anterior end of the cell and the contractile vacuole pores. In addition, they observed that anti-calmodulin drug, trifluoperazine, suppresses food vacuole formation and excretion of contractile vacuole contents and affects the ciliary motion. From these observations, they suggested

possible roles of calmodulin in ciliary reversal, endocytosis and exocytosis. Other evidences also suggested involvement of calmodulin in the control of ciliary movement: (i) Calmodulin is localized along the longitudinal axis of outer-doublet microtubules in a cilium (Ohnishi *et al.*, 1982). (ii) Calmodulin binding proteins are detected in cilia (Hirano and Watanabe, 1985; Hirano-Ohnishi and Watanabe, 1988). (iii) ß-tubulin, one of the main components of microtubules, is exclusively phosphorylated by Ca^{2+}/calmodulin dependent protein kinases (Hirano-Ohnishi and Watanabe, 1989). Recently, Kurasawa *et al.* (1996) showed that Ca^{2+}/calmodulin regulated F-actin bundling activity of *Tetrahymena* elongation factor 1α, and suggested that Ca^{2+}/calmodulin plays a role in the dynamics of the actin-based cytoskeletal system in addition to the microtubules system.

In *Paramecium*, Satir and co-workers first reported the presence of calmodulin, and reported its localization in cilia, basal bodies, and cytoplasmic vacuolar inclusions (Satir *et al.*, 1980; Maihle *et al.*, 1981). Some biochemical studies revealed that calmodulin regulates dynein-ATPase activity (Blum *et al.*, 1980) and membrane-bound Ca^{2+}-dependent guanylate cyclase activity (Klumpp *et al.*, 1983) in cilia. From these studies, calmodulin was proposed to have some regulatory functions in ciliary activities. Moreover, *in vivo* studies on calmodulin have progressed. Analysis of behavioral mutants affected in their swimming response to various ionic stimuli permitted the identification of mutation for calmodulin (Hinrichsen *et al.*, 1986; Schaefer *et al.*, 1987; Lukas *et al.*, 1989; Kink *et al.*, 1990; Ling *et al.*, 1994). The mutants can be grouped according to whether they underreact or overreact behaviorally to certain stimuli,

reflecting their loss of either a Ca^{2+}-dependent Na^{+} current or a Ca^{2+}-dependent K^{+} current. All underreactors have amino acid substitutions in the N-terminal lobe of the calmodulin, whereas all overreactors have substitutions in the C-terminal lobe. Thus, calmodulin regulates swimming behavior through regulation of the functions of Ca^{2+}-dependent ion channels. The role of calmodulin in exocytosis of the trichocysts has also been suggested: (i) Membranes of trichocysts were labeled with an antibody against calmodulin in ultra-thin sections (Momayezi *et al.*, 1986). (ii) Secretion of trichocysts was inhibited by anti-calmodulin drugs (Garofalo *et al.*, 1983). (iii) Some biochemical evidences revealed that calmodulin activated an ATPase (Tiggemann and Plattner, 1982) and a calcineurin-like phosphatase (Momayezi *et al.*, 1987) which were thought to be involved in membrane fusion. (iv) By analysis of calmodulin mutants, it was demonstrated that calmodulin was necessary for the assembly of links between trichocysts and plasma membranes (Kerboeuf *et al.*, 1993).

On the other hand, the existence of other putative Ca^{2+}-binding proteins has been known in ciliated protozoa. Spasmins are major components of glycerinated spasmonemes of *Vorticellid*, and their apparent molecular masses were estimated to be 20 kDa (Amos *et al.*, 1975; Yamada and Asai, 1982). They are thought to be involved in Ca^{2+}-dependent contraction of spasmoneme. In *Isotricha* and *Polyplastron*, a 22 kDa putative Ca^{2+}-binding protein was detected (Vigues *et al.*, 1984; Vigues and Groliere, 1985). This protein is associated with a contractile filamentous layer of the cortex (ectoplasmic-endoplasmic boundary, EEB) of both ciliates and may be involved in the control of microfilament ordering. An antibody

raised against this 22 kDa protein of *Isotricha* cross-reacted with 23-24 kDa proteins of *Paramecium* (Garreau de Loubresse *et al.*, 1988, 1991). These 23-24 kDa proteins are major components of the innermost cortical cytoskeletal network (the infraciliary lattice), and they are also Ca^{2+}-binding proteins. Contraction of the infraciliary lattice occurs upon increase of internal Ca^{2+} concentration, and these Ca^{2+}-binding proteins are thought to be involved in the dynamics of the network.

In *Tetrahymena*, to explain the complicated mechanism of the Ca^{2+}-dependent phenomena, detection of Ca^{2+}-binding protein(s) other than calmodulin was attempted. Ohnishi and Watanabe (1983) detected a new Ca^{2+}-binding protein in the cilium and cell body, and purified this protein. This protein was designated to be *Tetrahymena* Ca^{2+}-binding protein of 10 kDa (TCBP-10), because its apparent molecular mass was estimated to be 10 kDa. Kobayashi *et al.* (1988) determined its complete amino acid sequence, and showed that it possesses two EF-hand Ca^{2+}-binding loops. An antiserum against TCBP-10 did not react with a 10 kDa protein, but it reacted with a 25 kDa protein in the *Tetrahymena* whole cell extracts. Therefore, it was suggested that TCBP-10 is a degradated product of a 25 kDa protein. Takemasa *et al.* (1989) cloned the gene for the 25 kDa protein and showed that the amino acid sequence of the C-terminal half of this protein coincided with the amino acid sequence of TCBP-10. Therefore, they corrected the name of this protein from TCBP-10 to TCBP-25 (*Tetrahymena* Ca^{2+}-binding protein of 25 kDa). TCBP-25 is composed of 218 amino acids residues, and its molecular weight is estimated to be 24,702. TCBP-25 is predicted to contain four EF-hand Ca^{2+}-binding loops.

During the purification of TCBP-10, a copurified Ca^{2+}-binding protein was detected. The gene for this protein was cloned, and it was shown that this protein composed of 207 amino acids residues, and that its molecular weight is estimated to be 23,413 (Takemasa *et al.*, 1990). Based on its deduced amino acid sequence, this protein also contains four EF-hand Ca^{2+}-binding loops. This protein was designated to be *Tetrahymena* Ca^{2+}-binding protein of 23 kDa (TCBP-23). Although TCBP-23 shows 35% homology with TCBP-25, it is clearly different from TCBP-25.

Thus, at least three different EF-hand Ca^{2+} binding proteins, calmodulin, TCBP-25 and TCBP-23 are present in *Tetrahymena*. These proteins are expected to play important roles in the Ca^{2+}-dependent phenomena. However, the functions of TCBP-25 and TCBP-23 have yet to be elucidated. To understand the functions and physiological roles of TCBP-25 and TCBP-23, I have tried to express these proteins in *E. coli* and also investigated their intracellular localizations in *Tetrahymena*. I will describe the evidence that TCBP-25 and TCBP-23 associate with membrane skeleton in every growth phase of *Tetrahymena*, and that TCBP-25 is also associated with the migratory and stationary pronuclei at the nuclear exchange stage during conjugation. Based on the results obtained, I will discuss the possible roles of these proteins in vegetative growth phase and in conjugation.

MATERIALS AND METHODS

Cell Culture

Tetrahymena thermophila cells, strain B2194 (the 21st generation of inbreeding of the strain B, established in 1994), were used in this study. The strains were kindly provided by Dr. T. Sugai (Ibaraki University). Conjugation was induced between strain B21942 (mating type II) and B21943 (mating type III). Some crosses were carried out between A* (A-star, mating type III) strain and B21942. A* strain was kindly provided by Dr. J. Orias (University of California, Santa Barbara, CA). Cells were grown axenically in 0.25% proteose peptone, 0.25% yeast extract and 3.5% glucose at 26°C until early stationary phase. In some experiments, synchronous conjugation was induced essentially according to the method of Sugai and Hiwatashi (1974). To prepare the cells for conjugation, cells of complementary mating types were grown separately. Each was washed, resuspended at a density of 5×10^5 cells/ml and starved overnight in 0.1% NaCl, 0.004% KCl and 0.006% $CaCl_2$ at 26°C. Conjugation was induced by mixing equal volumes of the cell suspension of complementary mating types at 26°C.

Site-Directed Mutagenesis

Site-directed mutagenesis was carried out as described by Kramer *et al.* (1984) using the Mutan-G site-directed mutagenesis system (Takara). The TCBP-25 cDNA (Takemasa *et al.*, 1989) and the TCBP-23 cDNA (Takemasa *et al.*, 1990) were 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: TCBP-25-Gln 89, 5'-ATTGTTACTTACCAAGATATTG-AAGAA-3'; TCBP-25-Gln 105, 5'-GGTACCACTGTCCAAAGAACTATC-GTC-3'; TCBP-25-Gln 147, 5'-GACGGTTCTGGTCAATTACAAGATGA-C-3'; TCBP-25-Gln 181, 5'-AAGATCTGGTTACAAATGGCTGACACC-3'; TCBP-25-Gln 214, 5'-AGAGTCGAAAAGCAATCCTTAGTTTTC-3'; TCBP-23-Gln 8, 5'-CAAATCATCACCCAAAACGTCTACGCT-3'; TCBP-23-Gln 28, 5'-AAGCTTTTTGCTCAGTTTGATTCTAAC-3'; TCBP-23-Gln 138, 5'-GATAAGAGTGGTCAATTAGAAGAAAAG-3'. The altered nucleotides are underlined. Mutations were confirmed by single-strand DNA sequencing.

Expression and Purification of Recombinant TCBPs

The TCBP-25 cDNA and the TCBP-23 cDNA with CAAs and CAG changed from TAAs and TAG respectively, and *T. pyriformis* calmodulin cDNA (Takemasa *et al.*, 1992) were amplified by PCR using oligonucleotides, which included the *Bam*HI site. The oligonucleotide pair 5'-GGGGATCCATGGCTGATCAATTAACC-3' (corresponding to nucleotides 1-18) and 3'-GGGGATCCAATTTGTTC-ACTTAGCC-5' (corresponding to nucleotides 441-457) was used to amplify the calmodulin cDNA. The oligonucleotide pair 5'-GGGGAT-CCATGGCTCAATACTCTC-3' (corresponding to nucleotides 1-16) and 3'-GGGGATCCTGCTCAGAAAACTAAGG-5' (corresponding to nucleotides 644-660) was used for the TCBP-25 cDNA. The oligonucleotide pair 5'-GGGGATCCATGGAACACCAAATCATC-3' (corresponding to nucleotides 1-18) and 3'-GGGGATCCATCAAGCTT-GAGTGAATTCC-5' (corresponding to nucleotides 606-625) was used for the TCBP-23 cDNA. The resulting products were cloned in-

frame into the expression vector pGEX-2T (Pharmacia) using the *Bam*HI site.

Expression and purification of recombinant TCBPs were performed essentially as described by Smith and Johnson (1988). Three plasmids encoding TCBPs as GST fusion proteins were transformed into *E. coli*, strain JM109. The transformed cells were grown in 2 x TY-G medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl and 2% glucose) containing 100 µg/ml ampicillin to OD_{600} ~1.0 at 37°C. Protein expressions were induced by the addition of IPTG to a final concentration of 0.1 mM. At 4 to 6 hr after induction, the cells were centrifuged at 4,500*g* for 10 min and the pellets were resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.3). The cells were disrupted by sonication using Ultrasonic Disrupter (Tomy Seiko) and were lysed by addition of Triton X-100 to the sonicates at a final concentration of 1%. The lysates were centrifuged at 12,000*g* for 10 min to remove debris and supernatants were directly applied to glutathione-Sepharose 4B (Pharmacia). After washing with PBS, GST fusion protein-bound beads were incubated in PBS containing thrombin (Pharmacia, 50 cleavage units/ml beads) for 12 hr at room temperature. The resultant supernatants including TCBPs were recovered by centrifugation. Cleavage of the fusion proteins with thrombin releases TCBPs with a two-residue N-terminal extension.

Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). *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. Electrophoresis on alkaline glycerol gels was performed as described (Perrie and Perry, 1970). The gel was made up with 10% polyacrylamide (W/V) in 25 mM Tris, 80 mM glycine (pH 8.6) including 40% glycerol (V/V). Samples were dissolved in 8 M urea containing 5.5% 2-mercaptoethanol and 0.02% bromophenol blue and then incubated in the presence of either 2 mM $CaCl_2$ or 2 mM EGTA in the same solution. Proteins were stained with Coomassie brilliant blue R-250.

Ca^{2+}-Binding

The binding of $^{45}Ca^{2+}$ was performed as described by Maruyama *et al.* (1984). The recombinant TCBPs were resolved by SDS-PAGE and transferred onto Immobilon membrane filters (Millipore), using a semi-dry blotting apparatus (Sartorius). The membrane was washed in a buffer containing 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazol-HCl (pH 6.8) before incubation for 20 min with a 1 mCi/ml $^{45}CaCl_2$ in the same buffer at room temperature. The membrane was washed with 67% (V/V) ethanol and was completely dried. The dried membrane was autoradiographed using Kodak XAR-5 x-ray film. $^{45}CaCl_2$ was obtained from Amersham.

Polyclonal Antibodies

Rabbit polyclonal antisera were raised against TCBP-25 and TCBP-23. The recombinant proteins were subjected to alkaline glycerol gel electrophoresis or two-dimensional polyacrylamide gel electrophoresis (Hirabayashi, 1981) and sliced from the gels. After

dialysis against PBS, the gel slices containing TCBP-25 or TCBP-23 were emulsified with Freund's complete adjuvant and injected into male rabbits. Booster injections using Freund's incomplete adjuvant were carried out at 10-day intervals. Following booster injections, anti-TCBP-25 antisera and anti-TCBP-23 antisera were produced in four rabbits each. Anti-TCBP-23 antibodies were affinity purified against recombinant TCBP-23 immobilized on Immobilon membrane filters (Talian *et al.*, 1983). A guinea pig antiserum against *Tetrahymena* tubulin has been previously prepared and characterized (Takagi *et al.*, 1991).

Immunoblotting

Proteins resolved by SDS-PAGE were transferred onto Immobilon membrane filters, using a semi-dry blotting apparatus. Filters were blocked with 3% non-fat dry milk in PBS containing 0.05% Tween 20 before incubation with primary antibodies. The immunoblots were visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:2000 dilution, Tago) using a BCIP/NTB phosphatase substrate system (KPL).

Indirect Immunofluorescence

Tetrahymena cells were prepared by three different methods. Method (A): cells were fixed with 4% paraformaldehyde in 0.05 M PB (pH 7.3) for 30 min at 4°C. After washing with PB, the cells were incubated with 0.05 M NH_4Cl in PB for 30 min at 4°C to reduce free aldehydes and then washed with PB. The fixed cells were air-dried on poly-L-lysine coated slides and were permeabilized with 0.1% Triton X-100 in PB for 1 min at 4°C. Method (B): cells were

permeabilized with 0.5% NP-40 according to the method of Goodenough (1983) except that 1 mM PMSF was used instead of aprotinin. The permeabilized cells were fixed with 2.5% paraformaldehyde in PB for 30 min at 4°C, treated with 0.05 M NH_4Cl and then air-dried on slides. Method (C): cells were deciliated using the method of Rosenbaum and Carlson (1969) as modified for starved cells according to Guttman and Gorovsky (1979). The deciliated cells were fixed with 4% paraformaldehyde in PB, treated with 0.05 M NH_4Cl and air-dried on slides. The cells prepared by these three methods were washed in PBS and preincubated in PBS containing 3% non-fat dry milk for 30 min to block nonspecific binding of antibodies. The cells were incubated with primary antibodies diluted with PBS containing 2% BSA for 1 hr at room temperature. After six washings with PBS, the cells were incubated with secondary antibodies diluted with PBS containing 2% BSA for 1 hr at room temperature and then washed six times with PBS. For single immunofluorescence staining, anti-TCBP-25 antiserum (1:100-200 dilution), anti-TCBP-23 antibody (7 µg/ml) or preimmune serum (1:100-200 dilution) was used as the primary antibody. Fluorescein-conjugated goat anti-rabbit IgG (1:100-200 dilution, Tago) was used as the secondary antibody. For double immunofluorescence staining, a mixture of rabbit anti-TCBP-25 antiserum and guinea pig anti-tubulin antiserum (1:100-200 dilution) or a mixture of polyclonal rabbit anti-TCBP-23 antibody and guinea pig anti-tubulin antiserum was used as the primary antibody. Fluorescein-conjugated goat anti-rabbit IgG and rhodamin-conjugated anti-guinea pig IgG (1:100-200 dilution, Tago) were used as the secondary antibodies. The cells were mounted in 50%

glycerol in PBS containing 1 µg/ml DAPI. Cells were examined on a Nikon fluorescence microscope and were photographed on color transparencies 400 ASA Fuji chrome film or Tri-X pan 400 film.

Immunoelectron Microscopy

Tetrahymena cells were fixed with 4% paraformaldehyde in PB for 30 min at 4°C. After three washings with PB, the cells were incubated with 0.05 M NH_4Cl in PB and then washed with PB. The cells were embedded in 1% agar. The agar blocks were dehydrated through a graded series of acetone at 4°C, and embedded in Lowicryl HM20 at –50°C for 48 hr. Ultrathin sections were cut with a diamond knife using a Reichert Ultracut S ultramicrotome and collected on carbon coated 150 mesh nickel grids. The sections were washed with PBS and incubated with PBS containing 1% BSA for 30 min at room temperature. The sections were incubated with anti-TCBP-25 antiserum (diluted 1:400 with PBS containing 0.1% BSA) for 2 hr followed by six times washings with PBS. The sections were then incubated with 5 nm colloidal gold-conjugated goat anti-rabbit IgG (diluted 1:500 with PBS containing 0.1% BSA, British Bio Cell) for 1 hr. The sections were then washed six times with PBS and twice with 0.1 M sodium cacodylate (pH 7.4), and postfixed with 0.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 5min. After washing with DW, the sections were contrasted with 2% uranyl acetate for 30 min and Reynolds' lead citrate for 5 min. The sections were examined on a JEOL 2000EXII electron microscope.

RESULTS

Expression and Purification of TCBPs in E. coli

Since *Tetrahymena* uses 'universal' stop codons, TAA and TAG, as glutamine codons (Horowits and Gorovsky, 1985; Kuchino *et al.*, 1985; Hanyu *et al.*, 1986), *Tetrahymena* genes carrying TAA and TAG in their open reading frames cannot be expressed in *E. coli*. As shown in Fig. 1, five TAA codons which encode glutamines are present in TCBP-25 cDNA, and as shown in Fig. 2, two TAA and one TAG codons which encode glutamines are also present in TCBP-23 cDNA. To express TCBP-25 and TCBP-23 in *E. coli*, I have changed the TAA and TAG codons to glutamine codons, CAA and CAG, by site-directed mutagenesis. To express calmodulin, *T. pyriformis* calmodulin gene is used. It encodes the exact same amino acid sequence as that of calmodulin gene from *T. thermophila*, and does not carry TAA and TAG in its open reading frame (Takemasa *et al.*, 1992). TCBP-25 and TCBP-23 genes with CAAs and CAG changed from TAAs and TAG, and calmodulin genes were subcloned into the bacterial expression vector pGEX-2T.

Results of expression and purification of calmodulin (Fig. 3A), TCBP-25 (Fig. 3B) and TCBP-23 (Fig. 3C) are shown. Bacteria transformed with pGEX-2T recombinants were taken before (lanes 1) and after (lanes 2) IPTG induction and lysed in SDS-containing buffer. The lysates of bacteria were resolved by SDS-PAGE and stained with Coomassie brilliant blue. GST fusion proteins, which had apparent molecular weight consistent with the size of calmodulin plus the 26 kDa GST fragment, TCBP-25 plus the GST fragment and TCBP-23 plus the GST fragment were expressed in

bacteria (marked by the open arrowheads). Bacterial sonicates were prepared and incubated with glutathione-Sepharose 4B. The bound proteins were eluted by boiling in an SDS-containing buffer and resolved by SDS-PAGE (lanes 3). GST-calmodulin fusion protein, GST-TCBP-25 fusion protein and GST-TCBP-23 fusion protein were found in the soluble fractions of total bacterial sonicates and bound to glutathione-Sepharose 4B. To remove the GST carrier from the fusion proteins, GST fusion protein-bound beads were incubated with thrombin. As a result, purified calmodulin, TCBP-25 and TCBP-23 were recovered in the supernatants (lanes 4, marked by the closed arrowheads). The apparent molecular weight of the recombinant TCBP-23 estimated by gel electrophoresis using the method of Laemmli is slightly larger than the molecular weight estimated from its deduced amino acid sequence. About 10 mg of calmodulin, 1.5 mg of TCBP-25 and 5 mg of TCBP-23 were produced in 1 liter of the bacterial culture.

TCBPs Bind Ca^{2+} In Vitro

To determine whether TCBP-25 and TCBP-23 are functional Ca^{2+}-binding proteins, I have tested their abilities 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 (Head *et al.*, 1979). TCBP-25 and TCBP-23 were incubated either with $CaCl_2$ or the Ca^{2+} chelator EGTA and electrophoresed on an alkaline glycerol gel, in the absence of SDS. Fig. 4 shows that the Ca^{2+}-bound forms of TCBP-25 (Fig. 4B) and TCBP-23 (Fig. 4C) migrated at slower rates than

those in the Ca^{2+}-free forms. In the same gel, calmodulin (Fig. 4A) also displayed a Ca^{2+}-dependent shift in electrophoretic mobility as in authentic *Tetrahymena* calmodulin. Therefore, TCBP-25 and TCBP-23 undergo conformational changes upon Ca^{2+}-binding.

The abilities of TCBP-25 and TCBP-23 to bind Ca^{2+} were examined directly by incubation with $^{45}Ca^{2+}$. TCBPs were resolved by SDS-PAGE, immobilized onto a membrane, incubated with $^{45}Ca^{2+}$ and an autoradiograph of the membrane was made. Fig. 5 shows that TCBP-25 and TCBP-23 were labeled with $^{45}Ca^{2+}$ (Fig. 5B, lanes 3 and 4). As a positive control, calmodulin was labeled with $^{45}Ca^{2+}$ (Fig. 5B, lane 2). No protein in the molecular weight standards gave a positive signal (Fig. 5B, lane 1).

These results clearly show that recombinant TCBPs have the abilities to bind Ca^{2+} *in vitro*, as expected from their deduced amino acid sequences.

Characterization of Antibodies against TCBP-25 and TCBP-23

To investigate the intracellular localization of TCBP-25 and TCBP-23, I have prepared four rabbit polyclonal antibodies against TCBP-25 and TCBP-23 each.

The antibodies were analyzed for specificity by immunoblotting. Fig. 6 shows that an antiserum against TCBP-25 specifically reacted to a single protein in *Tetrahymena* cell extracts (Fig. 6B, lane 2). This protein had the same migration behavior as that of the purified TCBP-25 (Fig. 6B, lane 3). The antiserum did not recognize the purified TCBP-23 (Fig. 6B, lane 4). The other three antisera against TCBP-25 showed almost the same properties.

An antibody against TCBP-23 recognized a single protein (Fig.

6C, lane 2) not present in the preimmune sera (Fig. 6C, lane 1) in *Tetrahymena* cell extracts. This protein had the same migration behavior as that of the purified TCBP-23 (Fig. 6C, lane 4). The antibody did not recognize the purified TCBP-25 (Fig. 6C, lane 3). The three other antibodies against TCBP-23 showed almost the same properties.

Localization of TCBP-25

First, I examined the localization of TCBP-25 in the vegetative growth phase of *Tetrahymena*. Fig. 7 shows the indirect immunofluorescence localization of TCBP-25. TCBP-25 was localized in the whole cell cortex except for a linear punctuate array in paraformaldehyde fixed cells (Fig. 7A). To investigate whether this punctuate array corresponded to the areas around the basal bodies, double-staining immunofluorescence with anti-TCBP-25 and anti-tubulin antisera was performed. For selective visualization of basal body microtubules, deciliated cells were used and permiabilization of the cells with Triton X-100 was omitted. Immunofluorescence images demonstrated that this array corresponded to the areas around the basal bodies as revealed by immunofluorescence for tubulin (Fig. 7B). No appreciable immunofluorescence for TCBP-25 was observed inside the cytoplasm (data not shown). To determine whether TCBP-25 exists in a soluble form, localization of TCBP-25 was examined after the soluble proteins in the cytoplasm had been extracted with 0.5% NP-40. Strong fluorescence remained in the cell cortex (Fig. 7C), as observed in directly fixed cells without NP-40 extraction. This result shows that most TCBP-25 exists in a NP-40-insoluble form in the cell

cortex. Judging from the fluorescence localization, TCBP-25 seems to be localized in the epiplasm.

Furthermore, cortical localization of TCBP-25 was confirmed by immunoelectron microscopy. The cortical organization of *Tetrahymena* has been well characterized, and its principal components are the plasma membrane, the alveoli and the epiplasm (Pitelka, 1961; Allen, 1967; Collins *et al.*, 1980; Satir and Wissig, 1982). The alveoli are composed of a single layer of membrane vesicles and are located close to the inner surface of the plasma membrane. Beneath the inner surface of the alveoli is a thin filamentous layer designating the epiplasm. Post-embedding immunoelectron microscopy using the anti-TCBP-25 antiserum was carried out. Fig. 8 shows that the immunogold labeling specifically accumulates in the epiplasm.

Next, I examined the localization of TCBP-25 during conjugation. Conjugation is the sexual phase in the ciliate life cycle. In *Tetrahymena*, it occurs when two starved cells of different mating types pair and partially fuse anteriorly. Pairing triggers a pathway of nuclear events, which are well characterized (Nanney, 1953; Elliott and Hayes, 1953; Ray, 1956; Orias, 1983). These events are diagrammed in Fig. 9. At the onset of the conjugation, the micronucleus leaves its normal position within a 'pocket' on the macronucleus surface and migrates towards the anterior of the cell (Fig. 9A). The micronucleus then undergoes meiotic divisions. A remarkable elongation (Fig. 9B) and compaction (Fig 9C) of the micronucleus is observed during the prophase of the first meiotic division. The first and second meiotic divisions are completed, and four haploid nuclei are generated (Fig. 9D). One of them is

selected and comes to be located near the conjugation junction. The other three migrate to the posterior of the cell and gradually degenerate. The selected nucleus undergoes one mitotic division (the third prezygotic division), giving rise to two gametic pronuclei (Fig. 9E). One product, called a migratory pronucleus remains near the conjugation junction, while the other, called a stationary pronucleus, is located more posteriorly. The migratory pronucleus is transferred into its mate (Fig. 9F) and moves towards the stationary pronucleus, and then the two pronuclei fuse to generate a diploid fertilization nucleus (Fig. 9G and 9H). The fertilization nucleus undergoes two mitotic postzygotic divisions (Fig. 9I). During the second postzygotic division, the separation spindles are very long and longitudinally oriented; as a consequence, two of the mitotic division products are located at the anterior pole of the cell, and the other two at the opposite end. The two anterior nuclei are determined as the macronuclear anlagen and will differentiate into the macronulei while one of the posterior nuclei becomes the new micronucleus. During the nuclear differentiation, macronuclear anlagen and micronuclei move towards the center of the cell, while the old macronucleus moves towards the posterior pole and begins to disintegrate (Fig. 9J). The cells of the pair then separate. The destruction of the old macronucleus is completed, and one of the new micronuclei is destroyed in the exconjugants. During the first postzygotic cell cycle, the new surviving micronucleus divides, while the macronucleus does not divide. Products of the division (karyonides) receive one macro- and micronucleus.

Indirect immunofluorescence analysis was performed to determine the localization of TCBP-25 during each stage of

conjugation. Fig. 10 shows the localization of TCBP-25 in paraformaldehyde fixed conjugating cells. At 6.5 hr after mixing complementary mating-type cells, the third prezygotic division to give rise to the migratory and stationary gametic pronuclei occurred. Until this stage, immunofluorescence was observed only in the cell cortex, and not in the conjugation junction (Fig. 10A). During migratory pronuclear transfer across the conjugation junction, strong fluorescence was observed around the migratory and stationary gametic pronuclei (Fig. 10B). At 7.5 hr after mixing, the gametic pronuclei fused to form a fertilization nucleus, and at this stage fluorescence around the nucleus disappeared, but some fluorescence persisted in the conjugation junction (Fig. 10C). After these periods, immunofluorescence for TCBP-25 was observed only in the cell cortex (data not shown). In detergent-extracted cells, immunofluorescence for TCBP-25 was observed around the migratory and stationary gametic pronuclei, and was the same as that of directly fixed cells (Fig. 11). This suggests that most TCBP-25 around the gametic pronuclei exists in an insoluble form.

The migratory pronucleus is associated with a meshwork of microtubules, the fertilization basket (Orias *et al.*, 1983). The basket appears to perform an essential function in the migratory pronucleus transfer (Hamilton and Suhr-Jessen, 1980; Orias *et al.*, 1983). I have then studied the localization of TCBP-25 in relation to microtubules. To clarify the migratory pronuclear position in the cell, conjugation was induced between the normal fertile strain and the star strain which has a very small micronucleus and produces no gametic pronuclei. Fig. 12 shows the double immunofluorescence images of TCBP-25 and tubulin in conjugating cells. Distribution of

intracytoplasmic microtubules during conjugation has been well studied, using the indirect immunofluorescence method (Takagi *et al.*, 1991; Gaertig and Fleury, 1992). At the third prezygotic division, the mitotic spindle was detected in the normal cell partner, while TCBP-25 was associated only with the cell cortex (Fig. 12A). After the division, the migratory and the stationary gametic pronuclei in the normal partner were surrounded by microtubules. Just prior to the exchange, the migratory pronucleus took on the form of a lens shape, since it was tightly sandwiched between the microtubular basket and the cell junction. At this time, immunofluorescence for TCBP-25 first appeared in the peripheries of the migratory and stationary gametic pronuclei in the normal partner (Fig. 12B). Once the junction became deformed, the migratory pronucleus reacquired its spherical shape, and was quickly transferred into the star partner. The microtubular basket became more densely packed. Strong immunofluorescence for TCBP-25 was observed around the migratory and the stationary pronuclei (Fig. 12C). After unilateral transfer, the migratory pronucleus was out of the microtubular basket, but the basket remained at the conjugation junction. The stationary pronuclei was still surrounded by microtubules. Immunofluorescence for TCBP-25 was detected both at the conjugation junction and around the stationary pronucleus (Fig. 12D). When the microtubular basket began to disrupt, immunofluorescence for TCBP-25 at the conjugation junction also began to disappear. At this stage, the migratory and stationary pronuclei were surrounded by the microtubules, while TCBP-25 never associated with these nuclei (Fig. 12E). The immunofluorescence patterns for TCBP-25 match with the

distribution of microtubules in the fertilization basket and at the peripheries of the stationary pronucleus.

Localization of TCBP-23

Fig. 13 shows the indirect immunofluorescence localization of TCBP-23. In vegetative cells, the antibody against TCBP-23 recognized the whole cell cortex except for a linear punctuate array in paraformaldehyde fixed cells (Fig. 13B). The preimmune serum did not recognize this structure (Fig. 13A). 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. 13C). No appreciable immunofluorescence for TCBP-23 was observed inside the 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. 13D). This result shows that most 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. This TCBP-23 localization coincides with the TCBP-25 localization.

In conjugating cells, however, no appreciable immunofluorescence for TCBP-23 was observed around the gametic pronuclei at the pronuclear exchange stage, unlike TCBP-25 (Fig. 14). TCBP-23 was localized only in the cell cortex during all stages of conjugation (data not shown), as well as at the pronuclear

exchange stage.

DISCUSSION

In this study, I succeeded in expressing TCBP-25 and TCBP-23 genes in *E. coli* by changing the TAA and TAG codons in these genes to CAAs and CAG, respectively, and in subsequent purification of the proteins. The purified TCBP-25 and TCBP-23 showed Ca^{2+}-dependent shifts in electrophoretic mobility and abilities to bind $^{45}Ca^{2+}$, which are special properties shared by EF-hand-type Ca^{2+}-binding proteins. The characterization of TCBP-25 and TCBP-23 was so far hindered by degradation of the proteins during purification from *Tetrahymena* cells. Expression of TCBP-25 and TCBP-23 genes in *E. coli* has resolved this problem.

TCBP-25 and TCBP-23 in the Cell Cortex

I prepared polyclonal antibodies raised against TCBP-25 and TCBP-23, and used these to examine the intracellular localization of TCBP-25 and TCBP-23 by immunocytochemical studies. TCBP-25 was localized in the cell cortex except for the oral apparatus and the areas around the basal bodies and remained in detergent-extracted cells. Immunoelectron microscopic observations revealed that TCBP-25 was associated with the epiplasm. Based on immunofluorescence studies, distribution of TCBP-23 was similar with that of TCBP-25. Thus, I suppose that TCBP-23 is also associated with the epiplasm.

The epiplasm is a dense submembranous layer which exists in almost all ciliates and some other protists. In most ciliates with the alveoli, the epiplasm is located beneath the inner alveolar membrane. On the other hand, in a few ciliates and other protists

without the alveoli, the epiplasm is located beneath the plasma membrane. The epiplasm is thought to be a membrane skeleton which contributes to the organization and maintenance of cell shape (Grain, 1986). Moreover, it seems to be involved in regulating the regional distribution of surface components such as basal bodies, trichocysts and mitochondria (Peck, 1977; Williams *et al.*, 1979; Aufderheide, 1983).

Molecular analysis of the epiplasm is in progress. In *Tetrahymena*, three high molecular weight proteins, called band A (235 kDa in *T. pyriformis*), band B (135 kDa in *T. pyriformis*), and band C (125 kDa in *T. pyriformis*), are the major components of the epiplasm (Vaudaux, 1976; Williams *et al.*, 1979, 1987, 1995). Immunofluorescence localizations of TCBP-25 and TCBP-23 seems to overlap with those of the three epiplasmic proteins (Williams *et al.*, 1987, 1995).

The major high molecular weight proteins of the the epiplasm are thought to be functionally equivalent with the spectrin superfamily proteins (Grain, 1986). In this class of proteins, spectrin is well characterized as the major structural protein of the erythrocyte membrane skeleton (Bennett, 1990). Spectrin has an actin-binding domain, and the spectrin tetramers from large α (240 kDa) and ß (260 kDa) subunits are interconnected by short actin filaments to form a dense two-dimensional scaffolding. The spectrin-based network is essential for the maintenance of the cell shape and membrane stability. In erythrocyte membrane skeleton, Ca^{2+}/calmodulin binds to adducin which promotes binding of spectrin to actin (Mische *et al.*, 1987). Therefore, Ca^{2+}/calmodulin is thought to play a role in the assembly and maintenance of the

spectrin-based network. The major epiplasmic proteins of the flagellate *Euglena gracilis* (Marrs and Bouck, 1992) and of the ciliate *Pseudomicrothorax dubius* (Huttenlauch *et al.*, 1995) are characterized by internal tandem repeats, a feature common to spectrin superfamily proteins. In *Tetrahymena*, although it is unknown whether band A, B and C are structurally and functionally equivalent with spectrin, there is a possibility that TCBP-25 and/or TCBP-23 interact to these major epiplasmic proteins and regulate their organization, as calmodulin regulates the spectrin-based cytoskeletal network in erythrocytes.

The epiplasm of most ciliates is in close contact with the alveoli. On the basis of their ultrastructural morphology and especially their close apposition to the plasma membrane, the alveoli in *Paramecium* (Allen and Eckert, 1969) and in *Tetrahymena* (Satir and Wissig, 1982) 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* (Stelly *et al.*, 1991) and to be distributed in the pellicle of *Paramecium* in high concentration (Stelly *et al.*, 1995). Therefore, the alveoli of *Paramecium* and *Tetrahymena* may play an important role in regulating the intracellular Ca^{2+} concentration, and may be related to Ca^{2+}-regulated processes that take place in the immediate vicinity of the alveoli (Stelly *et al.*, 1991). Thus, I infer that TCBP-25 and TCBP-23 may interact with the membrane cytoskeleton protein(s) and regulate the cytoskeletal elements dynamics in a Ca^{2+}-dependent manner through binding to Ca^{2+} that is released from the alveoli.

Immunofluorescences for TCBP-25 and TCBP-23 were absent

around the basal bodies and at the conjugation junction. These regions appear to be continuous with the cortical epiplasm but are structurally distinct from the typical epiplasm (Wolfe, 1985; Williams *et al*, 1987, 1990). Wolfe (1982, 1985) suggested that changes in the structure of the submembrane layer plays a significant role in the regulation of formation of the mature conjugation junction. Absence of TCBP-25 and TCBP-23 confirms these ultrastructural data describing a regional differentiation of the epiplasm. Regulation of the cytoskeletal organization by TCBP-25 and TCBP-23 may be involved in the establishment of the regional differentiation.

TCBP-25 Surrounding the Migratory and Stationary Pronuclei

TCBP-25 is also associated with the circumference of the migratory and stationary gametic pronuclei during conjugation, though the correlation between two localized regions of the cell cortex and the gametic pronuclei is unsettled. During conjugation of *Tetrahymena*, the micronucleus undergoes sequential events, including meiosis, gametogenesis, fertilization and differentiation of the new macro- and micronuclei. Immunofluorescence of TCBP-25 around the migratory pronuclei first appeared when the migratory pronucleus took on the form of a lens shape, and persisted through the pronuclear exchange process. After pronuclear exchange, TCBP-25 remained at the cell junction but gradually disappeared. On the other hand, TCBP-25 surrounds the stationary pronuclei just after the entry of the migratory pronuclei into the partner cells but before pronuclear fusion. These distributions of TCBP-25 requires the presence of the functional pronuclei, because immunofluorescence around the migratory and stationary gametic

pronuclei did not appear in the star partner in which the pronuclei was never formed, even though the gametic pronuclei were formed and were surrounded by TCBP-25 in the normal partner. I infer that TCBP-25 plays an important role in the migratory pronuclear exchange and/or in the distinction between the two functional gametic pronuclei and the other three degenerative nuclei at the pronuclear exchange stage.

In *Paramecium*, Ca^{2+} is present in high concentration at the periphery of the migratory pronucleus at the pronuclear exchange stage (N. Haga, Ishinomaki Sensyu University, personal communication). The Ca^{2+}-dependent regulation for the transfer of the gametic pronuclei may be a common mechanism in *Tetrahymena* and *Paramecium*. Thus, I suggest that TCBP-25 is involved in the migratory pronuclear exchange which is Ca^{2+}-dependent process.

Distribution of TCBP-25 coincides with that of tubulin during the pronuclear exchange stage. Microtubule assembly and disassembly seems to perform an essential function in the pronuclear exchange. Genetic and autoradiographic evidence revealed that the exchange of the pronulei is blocked by vinblastin or other anti-microtubule agents, and self-fertilization is induced (Hamilton and Suhr-Jessen, 1980). Based on the ultrastructural studies, Orias *et al.* (1983) showed that a meshwork of microtubule called 'the fertilization basket' surrounds each migratory pronucleus during their transfer across the conjugation junction, and that the conjugating cells treated with vinblastin are devoid of the basket. Immunofluorescence studies using anti-tubulin antibody confirm these ultrastructural data (Takagi *et al.*, 1991; Gartig and Fleury, 1992). Though it is not yet clear how the microtubules generate the

motive force required to transfer the pronuclei, the fertilization basket is the most obvious candidate for the source of the motive force. TCBP-25 coexists with the fertilization basket and may regulate microtubule organization which is involved in the generation of the motive force in a Ca^{2+}-dependent manner. In addition to the transfer of the pronuclei, microtubules appear to have other important roles during conjugation. Indirect immunofluorescence studies revealed that various microtubular structures are assembled in the cytoplasm during conjugation, such as the fertilization basket, the microtubules surrounding the stationary pronuclei, meiotic and mitotic spindles, microtubules surrounding the nucleus which undergoes the third prezygotic division, and microtubular bundles interconnecting the pronuclei and the fertilization nucleus with the conjugation junction (Gartig and Fleury, 1992). Nocodazole blocks both meiosis and selection of one nuclei, which undergoes the third prezygotic division (Kaczanowski *et al.*, 1985). Another microtubule affecting drug, taxol, blocks meiosis (Gartig and Fleury, 1992). Vinblastin induces failure of pronuclear fusion (Hamilton and Suhr-Jessen, 1980; Hamilton et al., 1988). These results suggest that microtubules are involved in meiosis, in the selection of the nucleus from the four meiotic products, in the third prezygotic division, in the pronuclear fusion, and in the postzygotic division. Immunofluorescence studies suggest that TCBP-25 is not required in these events in which microtubules are involved, but is only required in the pronuclear exchange. It seems that TCBP-25 causes microtubules to acquire some special functions.

So far, two proteins have been inferred to be associated with

the gametic pronuclei from the immunofluorescence studies. A 14-nm filament protein (49 kDa) has been shown to resemble intermediate filament proteins (Numata and Watanabe, 1982), and to have citrate synthase activity (Numata *et al.*, 1991). This protein associates with the periphery of the migratory pronuclei, with a pattern that partially matches the distribution of microtubules in the fertilization basket, and may have a crucial role in the transfer of the gametic pronuclei (Numata *et al.*, 1985; Takagi *et al.*, 1991). However, this protein is not associated with the periphery of the stationary pronucleus when the pronuclear exchange occurs. This protein also appears to be involved in some nuclear events during conjugation; the selection of one nucleus from four nuclei generated by meiosis, the third prezygotic division, and the fusion of the gametic pronuclei. In these respects, the supposed biological function of the 14-nm filament protein differs from that of TCBP-25. On the other hand, fenestrin (64 kDa) associates with the migratory and stationary pronuclei at the pronuclear exchange stage (Nelsen *et al.*, 1994). Fenestrin associates with the pronuclei in a pattern similar to the distribution of TCBP-25, and appears to be involved in the pronuclear exchange process. However, the association of fenestrin with the conjugation junction zone does not match the distribution of TCBP-25. Though relationships among 14-nm filament protein, fenestrin and TCBP-25 are unknown, these proteins may be transiently cooperated in the pronuclear exchange process.

Three EF-hand Ca^{2+}-binding protein family proteins, calmodulin, TCBP-25 and TCBP-23 are present in *Tetrahymena*. In addition, J. Frankel and co-workers showed that an antibody against

Chlamydomonas centrin, another member of this Ca^{2+}-binding protein family, recognized the basal bodies in *Tetrahymena* (personal communication). Based on immunofluorescence studies, the intracellular localizations of TCBP-25 and TCBP-23 are quite different from those of calmodulin (Satir *et al.*, 1980; Suzuki *et al.*, 1982) and centrin, implying that TCBP-25 and TCBP-23 may have functions different from calmodulin and centrin. On the other hand, TCBP-25 and TCBP-23 are colocalized in the vegetative cells and may function cooperatively in membrane cytoskeleton. In conjugating cells, TCBP-25 has a unique distribution and may have a unique role together with microtubules, whereas TCBP-23 does not show such a unique distribution. Though little is known on the molecular basis of Ca^{2+}-dependent phenomena in *Tetrahymena*, the EF-hand Ca^{2+}-binding proteins mentioned here must mediate these processes as Ca^{2+} sensors. Some times they might cooperate and other times they might perform their own functions.

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FIGURES

Fig. 1. **Site-directed mutagenesis of TCBP-25 cDNA.** The nucleotide sequence and its deduced amino acid sequence are cited from the report of Takemasa *et al.* (1989). Black boxes indicate the TAA codons used as glutamine codons. Bars indicate the oligonucleotides used in site-directed mutagenesis to change 'T' in the TAA codons into 'C'. Each oligonucleotide corresponds to the sense sequense.


```
                                   -45    AATTC ATAAAATTCA AAAAACAACA AACAAACAAT  -11

     AAATAAATAA ATG GCT CAA TAC TCT CAA ACT CTC AGA TCT TCT GGT TTC ACT TCC ACT   48
                Met Ala Gln Tyr Ser Gln Thr Leu Arg Ser Ser Gly Phe Thr Ser Thr
                  1                                   10

GTT GGT CTC ACC GAT ATT GAA GGT GCT AAG ACT GTC GCT AGA AGA ATC TTC GAA AAC TAC  108
Val Gly Leu Thr Asp Ile Glu Gly Ala Lys Thr Val Ala Arg Arg Ile Phe Glu Asn Tyr
            20                                      30

GAC AAG GGC AGA AAG GGC AGA ATC GAA AAC ACC GAC TGT GTT CCC ATG ATT ACT GAA GCC  168
Asp Lys Gly Arg Lys Gly Arg Ile Gln Asn Thr Asp Cys Val Pro Met Ile Thr Glu Ala
            40                                      50

TAC AAG TCC TTC AAC TCC TTC TTT GCC CCC TCT TCT GAT GAC ATC AAG GCC TAC CAC AGA  228
Tyr Lys Ser Phe Asn Ser Phe Phe Ala Pro Ser Ser Asp Asp Ile Lys Ala Tyr His Arg
            60                                      70

GTC CTC GAC AGA AAC GGT GAC GGT ATT GTT ACT TAC TAA GAT ATT GAA GAA CTT TGC ATC  288
Val Leu Asp Arg Asn Gly Asp Gly Ile Val Thr Tyr Gln Asp Ile Glu Glu Leu Cys Ile
            80                                      90

AGA TAC CTC ACT GGT ACC ACT GTC TAA AGA ACT ATC GTC ACT GAA GAA AAG GTT AAG AAG  348
Arg Tyr Leu Thr Gly Thr Thr Val Gln Arg Thr Ile Val Thr Glu Glu Lys Val Lys Lys
            100                                     110

TCC AGC AAG CCC AAA TAC AAC AAT GAA GTT GAA GCT AAG CTC GAC GTC GCT AGA AGA CTC  408
Ser Ser Lys Pro Lys Tyr Asn Pro Glu Val Glu Ala Lys Leu Asp Val Ala Arg Arg Leu
            120                                     130

TTC AAG AGA TAC GAC AAG GAC GGT TCT GGT TAA TTA CAA GAT GAC GAA ATC GCT GGC TTA  468
Phe Lys Arg Tyr Asp Lys Asp Gly Ser Gly Gln Leu Gln Asp Asp Glu Ile Ala Gly Leu
            140                                     150

TTA AAG GAC ACC TAT GCT GAA ATG GGT ATG TCC AAC TTC ACC CCT ACT AAG GAA GAC GTT  528
Leu Lys Asp Thr Tyr Ala Glu Met Gly Met Ser Asn Phr Thr Pro Thr Lys Glu Asp Val
            160                                     170

AAG ATC TGG TTA TAA ATG GCT GAC ACC AAC TCT GAT GGT TCA GTC TCC CTT GAA GAA TAC  588
Lys Ile Trp Leu Gln Met Ala Asp Thr Asn Ser Asp Gly Ser Val Ser Leu Glu Glu Tyr
            180                                     190

GAA GAC CTC ATT ATC AAG TCT CTC CAA AAG GCT GGT ATT AGA GTC GAA AAG TAA TCC TTA  648
Glu Asp Leu Ile Ile Lys Ser Leu Gln Lys Ala Gly Ile Arg Val Glu Lys Gln Ser Leu
            200                                     210

GTT TTC TGA   GCAAAATAAT AACCAATTTC TTTAACAAAT CAATACACAC TAATATTAAT TTGTCCTAAC  717
Val Phe ***
    218

TAATATGAAA ATAACATATA ACACTGTACA GAGAAATTTT ATGTATGCTT TGTATTTGAG ACATTTTTTA     787

ACTTTTCTTT ATAAAATCAT TAAAAAAAAA                                                 817
```

Fig. 2. **Site-directed mutagenesis of TCBP-23 cDNA.** The nucleotide sequence and its deduced amino acid sequence are cited from the report of Takemasa *et al.* (1990). Black boxes indicate the TAA and TAG codons used as glutamine codons. Bars indicate the oligonucleotides used in site-directed mutagenesis to change 'T' in the TAA and TAG codons into 'C'. Each oligonucleotide corresponds to the sense sequense.


```
-39   CAAAAAAAAAAAGTAGAAAGCAAACAAACACTAAAAGAA     ATG GAA CAC CAA ATC ATC ACC   21
                                                   Met Glu His Gln Ile Ile Thr
                                                     1

TAA AAC GTC TAC GCT CCT GAT ACT GAA GCC AAG CTT GAT GTT GCT AGA AAG CTT TTT GCT   81
Gln Asn Val Tyr Ala Pro Asp Thr Glu Ala Lys Leu Asp Val Ala Arg Lys Leu Phe Ala
        10                                      20

TAG TTT GAT TCT AAC AAG AAC GGT ACT TTA GAT CCT AGC GAA GTT GCT GGA CTC ATC AAG  141
Gln Phe Asp Ser Asn Lys Asn Gly Thr Leu Asp Pro Ser Glu Val Ala Gly Leu Ile Lys
        30                                      40

ACT ACT TTT GAA AAT ATG GGT GTT AAG GAC TAC AGC GTC ACT GCT GAT GAT GTC AAG CTT  201
Thr Thr Phe Glu Asn Met Gly Val Lys Asp Tyr Ser Val Thr Ala Asp Asp Val Lys Leu
        50                                      60

TAC ATG AAG AGT GTT GAT GTT GAT AAC AAC GGT CTT GTT TCC TAC TCT GAA TAC GAA GAA  261
Tyr Met Lys Ser Val Asp Val Asp Asn Asn Gly Leu Val Ser Tyr Ser Glu Tyr Glu Glu
        70                                      80

TAC GTC ATT GCT TGC CTC AAG AAA GCT GGC TTC GAC TGT GAA GTT AAG CAA AAG GTA AAA  321
Tyr Val Ile Ala Cys Leu Lys Lys Ala Gly Phe Asp Cys Glu Val Lys Glu Lys Val Lys
        90                                      100

AGA TCT GCC AAA AAG AGA GAC GCT GCT ACT GAA ATG AAG TTG GAC GTT GCC AGA AGA CTC  381
Arg Ser Ala Lys Lys Arg Asp Ala Ala Thr Glu Met Lys Leu Asp Val Ala Arg Arg Leu
        110                                     120

TTC GCT AAG TAC GAC TCT GAT AAG AGT GGT TAA TTA GAA GAA AAG GAA GTT TAT GGT GTC  441
Phe Ala Lys Tyr Asp Ser Asp Lys Ser Gly Gln Leu Glu Glu Lys Glu Val Tyr Gly Val
        130                                     140

ATT ACT GAA ACC TAT AAG CAA ATG GGT ATG GAT TAC AAG CCC ACT GAA GCT GAT GTT AAG  501
Ile Thr Glu Thr Tyr Lys Gln Met Gly Met Asp Tyr Lys Pro Thr Glu Ala Asp Val Lys
        150                                     160

CTT TGG ATG TCC ATG ACT GAT ACT GAT AAG AAT GGA ACT GTC TCT ATT GTT GAA TAT GAA  561
Leu Trp Met Ser Met Thr Asp Thr Asp Lys Asn Gly Thr Val Ser Ile Val Glu Tyr Glu
        170                                     180

GAT TTC GTC ATT TCT GGT CTT AAG AAG GCT GGT TTC ATG GTC AAG GAA TTC ACT CAA GCT  621
Asp Phe Val Ile Ser Gly Leu Lys Lys Ala Gly Phe Met Val Lys Glu Phe Thr Gln Ala
        190                                     200                         207

TGA TCTTATTTTAATAAACATATTTCAAATAAAAATTGAACTGTATTTGTGTATTTCATGTCATTTCTTAAAA      691
***

TTTTAAATGATTTTTTTAATATCGTTAAAA                                                   724
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Fig. 3. **Expression and purification of TCBPs.** Calmodulin (A), TCBP-25 (B) and TCBP-23 (C) were expressed in *E. coli* as GST fusion proteins. Bacteria transformed with pGEX-2T-calmodulin, pGEX-2T-TCBP-25, and pGEX-2T-TCBP-23 were solubilized before (lanes 1) and after IPTG induction (lanes 2). Sonicates of IPTG-induced cells were incubated with glutathione-Sepharose 4B and the bound proteins were eluted by boiling (lanes 3). The fusion proteins purified on glutathione-Sepharose 4B were incubated with thrombin and TCBPs alone were recovered by centrifugation (lanes 4). Proteins were resolved by 12.5% SDS-PAGE and stained with Coomassie brilliant blue. The open arrowheads indicate the position of GST-TCBPs fusion proteins and the closed arrowheads indicate the position of purified TCBPs.

Fig. 4. **Ca^{2+}-dependent electrophoretic mobility shift of TCBPs.** Recombinant calmodulin (A), TCBP-25 (B) and TCBP-23 (C) were electrophoresed on an alkaline glycerol gel in the presence of 2 mM $CaCl_2$ (+) and 2 mM EGTA (−), and stained with Coomassie brilliant blue. Each TCBP that binds Ca^{2+} displays a slower mobility on the gel.

Fig. 5. **Binding of $^{45}Ca^{2+}$ to TCBPs.** Molecular weight markers (lanes 1), recombinant calmodulin (lanes 2), TCBP-25 (lanes 3) and TCBP-23 (lanes 4) were resolved by 12.5% SDS-PAGE and either stained with Coomassie brilliant blue (A) or transferred to a membrane and incubated with $^{45}Ca^{2+}$ (B). Autoradiography of the membrane shows that calmodulin, TCBP-25 and TCBP-23 bind $^{45}Ca^{2+}$.

Fig. 6. **Characterization of antibodies against TCBP-25 and TCBP-23.** Extract of *Tetrahymena* cells (lanes 1 and 2), purified TCBP-25 (lanes 3) and TCBP-23 (lanes 4) were resolved by 12.5% SDS-PAGE and either stained with Coomassie brilliant blue (A) or analyzed by immunoblotting (B and C). An antiserum against TCBP-25 (B, lanes 2-4), an antibody against TCBP-23 (C, lanes 2-4), and respective preimmune sera (B and C, lanes 1) were used as primary antibodies, and the immunoreactive bands were detected with an alkaline phosphatase-conjugated secondary antibody. The antiserum against TCBP-25 only reacts to TCBP-25 in *Tetrahymena* cell extracts and purified TCBP-25 but not to TCBP-23. The antibody against TCBP-23 reacts to TCBP-23 in cell extracts and purified TCBP-23 but not to TCBP-25. A weak background band is recognized with both the antibody against TCBP-23 and the preimmune serum.

Fig. 7. **Localization of TCBP-25 in the vegetative growth phase.** *Tetrahymena* cells were fixed directly with 4% paraformaldehyde (A), deciliated and fixed with 4% paraformaldehyde (B) or extracted with 0.5% NP-40 and fixed with 2.5% paraformaldehyde (C). The fixed cells were stained with the antiserum against TCBP-25 (A and C), or double stained with the antiserum against TCBP-25 and antiserum against tubulin (B). In (A) and (C), the left panels show phase-contrast images and the right panels show the respective immunofluorescence images. In (B), the left panel shows the immunofluorescence image for tubulin and the right panel shows the respective immunofluorescence image for TCBP-25. TCBP-25 is localized in the cell cortex except for the oral apparatus and the areas around the basal bodies. Scale bars, 10 μm.

Fig. 8. **Ultrastructural localization of TCBP-25.**
Tetrahymena cells were fixed with 4% paraformaldehyde and embedded in Lowicryl HM20. Sections were stained with the antiserum against TCBP-25 followed by 5 nm colloidal gold-conjugated goat anti-rabbit IgG. The cortex of *Tetrahymena*, from outside to inside, consists of the plasma membrane (pm), the alveoli (alv) which are composed of a single layer of membrane vesicles, and the epiplasm (epi) which is a thin submembranous layer, although the plasma membrane and the alveoli are fragmented in this preparation. The cortically-located mitochondrion (m) is found. The gold particles are localized predominantly in the epiplasm. Scale bar, 100 nm.

Fig. 9. **A diagram of the nuclear events in conjugation.** (A) Two cells of different mating fuse anteriorly to form a pair. The micronucleus of each cell migrates towards the anterior of the cell. (B) The micronuclei undergo meiosis. During the prophase of the first meiotic division, the micronucleus forms a unique linear configuration called the crescent. (C) The crescent shortens, and metaphase follows in succession. (D) Meiosis is completed, and four haploid nuclei are present in each conjugant. (E) Three meiotic products migrate to the posterior of each conjugant and are gradually degenerated. The fourth product approaches the conjugation junction and divides mitotically, giving rise to two gametic pronuclei called the migratory and stationary pronuclei. (F) The migratory pronucleus moves across the conjugation junction. (G) The two gametic pronuclei in each cell fuse to form the zygotic pronucleus. (H) The zygotic nuclei undergo two mitotic divisions. (I) The two mitotic products are located at the anterior pole of each cell and the other two at the opposite end. (J) The anterior nuclei differentiate into macronuclear anlagen and the posterior nuclei become the new micronuclei. The old macronucleus moves to the posterior pole of each conjugant and is eventually degenerated.

Fig. 10. **Localization of TCBP-25 in conjugating cells.** Conjugating cells were fixed directly with 4% paraformaldehyde and stained with the antiserum against TCBP-25. After immunofluorescence staining, the cells were stained with DAPI to visualize DNA. The left panels show the immunofluorescence images and the right panels show respective fluorescent DNA images. (A) Early anaphase of the third prezygotic division. (B) Pronuclear exchange stage. Arrowheads indicate the migratory pronuclei and arrows indicate the stationary pronuclei. (C) Pronuclear fusion. TCBP-25 is localized around both the migratory and stationary pronuclei at the pronuclear exchange stage. Scale bar, 10 μm.

Fig. 11. **Localization of TCBP-25 in NP-40 extracted conjugating cells.** Conjugating cells were extracted with NP-40 and fixed with 2.5% paraformaldehyde. The cells were then stained with the antiserum against TCBP-25 (left) and co-stained with DAPI (right). A pair in which pronuclear exchange occurred is presented. Arrowheads indicate the migratory pronuclei and arrows indicate the stationary pronuclei. TCBP-25 remains around both the migratory and stationary pronuclei. Scale bar, 10 μm.

Fig. 12. **Localization of TCBP-25 and tubulin in conjugating cells.** Conjugation was induced between the normal strain (the left partners) and the star strain (the right partners). Conjugating cells were fixed directly with 4% paraformaldehyde, double stained with the antiserum against TCBP-25 and the antiserum against tubulin, and co-stained with DAPI. The middle panels show fluorescent DNA images, and the left and right panels show the immunofluorescence images for TCBP-25 and tubulin, respectively in the same pairs. (A) Anaphase of the third prezygotic division. (B) and (C) Unilateral pronuclear transfer from normal partner to defective partner. Arrowheads indicate the migratory pronuclei and arrows indicate the stationary pronuclei. (D) and (E) Posttransfer stage in defective conjugation. TCBP-25 and tubulin are colocalized in the fertilization basket and around the stationary pronucleus. No immunofluorescence for TCBP-25 is observed in defective partners. Scale bar, 10 μm.

Fig. 13. **Localization of TCBP-23 in the vegetative growth phase.** *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 fixed cells were stained with the antibody against TCBP-23 (B and D) or the preimmune serum from the same rabbit (A), or double stained with the antibody against TCBP-23 and the antiserum against tubulin (C). In (A), (B), and (D), the left panels show phase-contrast images and the right panels show the respective immunofluorescence images. In (C), the left panel shows the immunofluorescence image for tubulin and the right panel shows the respective immunofluorescence image for TCBP-23. TCBP-23 is localized in the cell cortex except for the oral apparatus and the areas around the basal bodies. Scale bars, 10 μm.

Fig. 14. **Localization of TCBP-23 in conjugating cells.** Conjugating cells were fixed with 4% paraformaldehyde, stained with the antibody against TCBP-23 (left) and co-stained with DAPI (right). A pair in which pronuclear exchange occurred is presented. TCBP-23 does not appear around the migratory and stationary pronuclei nor around the macronuleus and the relic nuclei. Scale bar, 10 μm.