Expression of the exocytotic protein syntaxin in mouse oocytes

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Syntaxin is an integral membrane protein that is involved in membrane fusion. The exocytosis of the contents of cortical granules, secretory vesicles located in the cortex of an egg, modify the extracellular environment to block additional spermatozoa from penetrating the newly fertilized egg. The aim of this study was to characterize syntaxin expression in mouse oocytes, and to determine the specific isoform that is expressed. Syntaxin was demonstrated in the mouse ovary and in mouse oocytes by both western blot and reverse transcription–polymerase chain reaction analyses. Syntaxin 4 was specifically expressed in metaphase II oocytes. Syntaxin was also immunolocalized within meta-

phase II oocytes and one-cell embryos with pronuclei using laser scanning confocal microscopy. In metaphase II oocytes, syntaxin was located on the plasma membrane and in the cortex, where cortical granules are present, but was not seen at sites free of cortical granules. In one-cell embryos, no cytoplasmic region was free of syntaxin immunoreactivity. Immunoelectron microscopy detected syntaxin on both the plasma membrane and the vesicle membranes in mouse metaphase II oocytes. In conclusion the results indicate that syntaxin 4 co-localizes with cortical granules and participates in membrane fusion and exocytosis during the cortical reaction.

Introduction

Cortical granules (CGs) are membrane-bound secretory granules, derived from the Golgi apparatus, that are located immediately beneath the plasma membrane in mammalian eggs (Schuel, 1978; Gulyas, 1980; Guraya, 1982; Connors *et al.*, 1998). CG exocytosis (cortical reaction) is one of the earliest events that take place at fertilization (Braden *et al.*, 1954), and this exocytotic event acts to prevent polyspermy (Cran and Esper, 1990; Cherr and Ducibella, 1990; Hoodbhoy and Talbot, 1994). However, the molecular mechanism of CG exocytosis remains to be elucidated in mammalian eggs.

Vesicular trafficking and exocytosis have been investigated intensively, especially with respect to synaptic vesicles in neuronal cells. Integral membrane proteins termed SNAREs (soluble NSF attachment protein receptors) are present in the vesicle membrane (v-SNAREs) and in the target membrane (t-SNAREs) (Söllner *et al.*, 1993a). It has been proposed that SNAREs act in key steps of vesicle targeting, docking or fusion (Rothman and Warren, 1994; Scheller, 1995; Südhof, 1995; Jahn and Hanson, 1998). Identified v-SNAREs include vesicle-associated membrane proteins (VAMP) 1 and 2 as well

as cellubrevin (Trimble *et al.*, 1988; Baumert *et al.*, 1989; Elferink *et al.*, 1989; McMahon *et al.*, 1993). The t-SNAREs include a 25 kDa synaptosome-associated protein (SNAP-25) and its isoform (SNAP-23) as well as syntaxins, a family of t-SNAREs with multiple isoforms (Linial, 1997). Each t-SNARE binds to only a limited member of v-SNAREs (Pevsner *et al.*, 1994), leading to a 'SNARE hypothesis' postulating a proofreading mechanism in which SNAREs contribute to the specificity of vesicle targeting, docking or fusion (Bennett *et al.*, 1993; Rothman, 1994). VAMP, SNAP-25 and syntaxin assemble spontaneously to form ternary complexes (Wilson and Rothman, 1992; Söllner *et al.*, 1993b), and the complexes are disassembled by N-ethylmaleimide-sensitive fusion protein (NSF). This disassembly allows SNAREs to be recycled, and membrane fusion processes require such cyclic assembly and disassembly of complexes (Jahn and Hanson, 1998; Littleton *et al.*, 2001).

Among the many SNARE proteins, SNAP-25 and HPC-1/syntaxin 1A as well as VAMP-2 play central roles in the exocytosis of synaptic vesicles (Inoue *et al.*, 1992; Söllner *et al.*, 1993a; Calakos and Scheller, 1996). SNAREs are also present and function in various non-neuronal tissues (adipocytes, skeletal muscles, renal cells and platelets), including the sea urchin egg and spermatozoa (Conner *et al.*, 1997; Mandon *et al.*, 1997; Schulz *et al.*, 1997; Foster *et al.*, 1998; Rea *et al.*, 1998; Tahara *et al.*, 1998;

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Fig. 1. Anti-syntaxin polyclonal antibody was raised in rabbits against recombinant rat syntaxin 1B (amino acids 22–180). Syntaxin recombinants (1A, 1B and 4) were solubilized in lysis buffer and SDS-PAGE was carried out. The peptides were transferred on to nitrocellulose membranes. (a) The gels were stained with Coomassie brilliant blue to detect the peptides. (b) The immunostained band in each lane represents syntaxin 1A (lane 1), 1B (lane 2) and 4 (lane 3).

Flaumenhaft *et al.*, 1999; Yang *et al.*, 2001). In addition, VAMP and syntaxin 1 are present in mammalian spermatozoa (Ramalho-Santos *et al.*, 2000); SNAP-25 may be involved in CG exocytosis in mouse eggs (Ikebuchi *et al.*, 1998).

In the present study the expression of syntaxin in mouse oocytes was investigated.

Materials and Methods

Media

Media used in these experiments included M2 medium (94.7 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.71 mmol CaCl₂ l⁻¹, 1.19 mmol KH₂PO₄ l⁻¹, 1.19 mmol MgSO₄ l⁻¹, 4.15 mmol NaHCO₃ l⁻¹, 20.9 mmol Hepes l⁻¹, 23.3 mmol sodium lactate l⁻¹, 0.33 mmol sodium pyruvate l⁻¹, 5.56 mmol glucose l⁻¹, 0.05 mg streptomycin ml⁻¹, 100 iu penicillin G ml⁻¹, 0.001% (w/v) phenol red and 4 mg BSA ml⁻¹, pH 7.4) and M16 medium (94.7 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.71 mmol CaCl₂ l⁻¹, 1.19 mmol KH₂PO₄ l⁻¹, 1.19 mmol MgSO₄ l⁻¹, 25.0 mmol NaHCO₃ l⁻¹, 23.3 mmol sodium lactate l⁻¹, 0.33 mmol sodium pyruvate l⁻¹, 5.56 mmol glucose l⁻¹, 0.05 mg streptomycin ml⁻¹, 100 iu penicillin G ml⁻¹, 0.001% phenol red and 4 mg BSA ml⁻¹, pH 7.4). All chemicals were purchased from Sigma Chemical Co. (St Louis, MO). All media were filtered through 0.22 mm filters (Millipore, Bedford, MA) and equilibrated in an atmosphere of 5% CO₂ at 37°C.

Ovary, oocyte and one-cell embryo preparation

Ovaries were removed surgically from female ICR mice of 8–10 weeks of age from a closed colony (SLC,

Tokyo). Metaphase II (MII) oocytes were collected from superovulated ICR females at 14–15 h after administration of hCG. Cumulus-enclosed oocytes were collected in M2 medium. Cumulus cells were removed using 0.3% (w/v) hyaluronidase in M16 medium for 5 min. One-cell embryos with pronuclei were flushed from the oviducts of superovulated, mated ICR females at 20–22 h after hCG administration. Oocytes and one-cell embryos were washed in M16 medium. Only mature oocytes of normal appearance with an ejected first polar body and normal, one-cell embryos with pronuclei were used in these experiments.

Polyclonal antibody

Anti-syntaxin polyclonal antibody was obtained by using recombinant rat syntaxin 1B protein (His Tag fusion protein) as the antigen. The recombinant protein was produced in *Escherichia coli* by induction with 1 mmol isopropyl-β-D-thiogalactopyranoside (IPTG) l⁻¹ and was purified with an Ni-agarose column. Antiserum against rat syntaxin 1B was raised in Japanese white rabbits. An IgG fraction of this antiserum was purified on a protein A affinity column (Amersham, Arlington Heights, IL). This polyclonal antibody recognizes recombinant rat syntaxin 1A, 1B and 4 (Fig. 1). An IgG fraction of preimmune serum was purified on a Protein A affinity column (Amersham) for use as a negative control.

Western blot analysis

ICR mice were killed by decapitation, and their ovaries were removed immediately. Approximately 1 g of ovary was homogenized in ice-cold PBS using a tissue homogenizer. Homogenates were initially centrifuged at 800 *g* for 10 min to pellet incompletely homogenized fragments and nuclei. Supernatants then were centrifuged at 100 000 *g* for 1 h to obtain a crude membrane fraction. Pellets were resuspended in PBS. The protein was dissolved in sample buffer for SDS-PAGE and then boiled for 5 min. Cumulus-free oocytes were homogenized with SDS-PAGE sample buffer by repeated freezing and thawing, and then by boiling for 5 min. The protein was separated by SDS-PAGE using 12% (w/v) acrylamide gels and transferred to polyvinylidene difluoride membranes (PVDF; Millipore). The membranes were incubated with anti-syntaxin polyclonal antibody at a 1:500 dilution, and then the membranes were washed and incubated in blocking buffer containing horseradish peroxidase (HRP)-conjugated anti-rabbit IgG. Immunoreactive bands were visualized on radiographic film (Fuji, Tokyo) using an enhanced chemiluminescence (ECL) immunoblot kit (Amersham) according to the manufacturer's instructions.

RT-PCR

Total RNA was prepared from MII oocytes and from mouse brain tissue by a modification of the method of Chomczynski and Sacchi (1987). ICR mice were decapitated, and oocytes and brain samples were collected. About 500 MII oocytes were lysed in Trizol reagent (Life Technologies, Gaithersburg, MD) containing 15 µg of carrier tRNA. Brain tissue was homogenized in Trizol reagent. RNA was extracted according to the manufacturer's instructions. The RNA was suspended in a dilution buffer (10 mmol Tris l⁻¹, pH 7.5, 0.1 mmol EDTA l⁻¹, 2 mmol dithiothreitol l⁻¹, and 40 U ribonuclease inhibitor ml⁻¹).

RNA from 20 oocytes or 1 µg of tissue was reverse-transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia Biotech, Piscataway, NJ) for 60 min at 37°C. Pd(N)6 was used as a random primer. After completion of the reaction, first-strand cDNAs were analysed by PCR. The primer sequences and their corresponding locations in the nucleotide sequence of the syntaxin isoforms were as follows: syntaxin 1A, sense primer: 5'-TTGCCTCTGGGATCATCATGGAC-3' (530–552); antisense primer: 5'-TGCTCCCCTCTGCCTCCTGT-3' (1083–1102); syntaxin 1B, sense primer: 5'-ACTCGCAGATGACAAAGCAAGCC-3' (548–570); antisense primer: 5'-CTGGGTCTGTTTGGGAGTGAGC-3' (953–975); syntaxin 4, sense primer: 5'-CGACAGGACCCATGAGTTGAGGC-3' (6–28); antisense primer: 5'-GGTGATGCCAATGATGACAGCCAA-3' (859–882). These sequence data are available under GenBank accession numbers M95735, M95734 and L20821, respectively. The expected size of the syntaxin 4 PCR products was 816 bp. PCR was initiated by adding 50 µl of a mixture containing PCR buffer, Taq polymerase (Perkin-Elmer, Norwalk, CT), and corresponding sense and antisense primers. PCR analysis was conducted in a Perkin-Elmer Cetus DNA thermal cycler. One cycle of PCR consisted of denaturation at 95°C for 60 s, primer annealing at 58°C for 120 s, and primer extension at 72°C for 60 s. Amplification consisted of 40 cycles. PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gels and stained with ethidium bromide.

Immunofluorescence

MI I oocytes and one-cell embryos with pronuclei were washed, fixed with 4% (w/v) paraformaldehyde for 30 min at 37°C, blocked in 5% goat serum, and permeabilized with 0.2% (v/v) Triton X-100 in 5% goat serum for 15 min. The zona pellucida was removed from oocytes and one-cell embryos with Tyrode's solution. Rabbit polyclonal anti-syntaxin antibody was applied as a primary antibody (dilution; 1:500) for 24 h at 4°C. A negative control experiment was run with replacement of the primary antibody with preimmune rabbit IgG at the same concentration. After repeated washing in 5% goat

serum, goat anti-rabbit IgG conjugated with rhodamine was applied at a 1:600 dilution as a secondary antibody for 1 h at 37°C. To label the CGs additionally, the oocytes or one-cell embryos subsequently were washed and incubated for 10 min at 37°C in 10 µg of fluorescent lectin ml⁻¹ (fluorescein isothiocyanate (FITC) conjugated to *Lens culinaris* agglutinin (LCA; Sigma), to label the CG with LCA. Unbound lectin was removed by washing, and fluorescence from rhodamine and FITC was visualized by excitation at 488 nm, using an argon laser with a 570 nm filter and a BP 535 nm filter respectively, in a confocal microscope (LSM-GB200, Olympus, Tokyo).

Immunoelectron microscopy

The pre-embedding silver enhancement immunogold method was used for immunoelectron microscopy (Nakamura *et al.*, 2000) with slight modifications. Briefly, MII oocytes were fixed for 2 h using 4% paraformaldehyde in PBS (pH 7.4). The oocytes were frozen and thawed using 14% (v/v) glycerol and 35% (w/v) sucrose in liquid N₂, and were reacted with the anti-syntaxin polyclonal antibody (dilution; 1:500), and then incubated with secondary antibodies conjugated to colloidal gold (diameter 1.4 nm). The gold labelling was intensified using a silver enhancement kit, HQ silver (Nanoprobes, Yaphank, NY). The control experiment was performed with replacement of the primary antibody with preimmune rabbit IgG. Ten oocytes were examined in each condition and the experiment was repeated twice.

Results

Expression of syntaxin-like protein in ovaries and oocytes

Polyclonal antibodies were used to detect syntaxin-like protein in extracts from mouse ovaries and brain. The anti-syntaxin antibody recognized a single distinct 35 kDa band in crude membrane fractions (Fig. 2a) from brain (lane 1) and ovary (lanes 2 and 3), confirming that a syntaxin-like protein is present in the ovary.

Syntaxin-like protein was detected in MII oocytes by analysing extracts from oocytes by western blot analysis as above. An immunoreactive band was again detected in the total lysate from 500 MII oocytes (lane 2), similar to those seen for the brain and ovary (Fig. 2b). No other significant band was seen in the lane. This 35 kDa band in brain, ovary and MII oocytes was consistent with the expected size of syntaxin.

Expression of syntaxin mRNAs in metaphase II oocytes

As the anti-syntaxin antibody could detect several isoforms of recombinant syntaxin, RT-PCR was carried out using total RNA from MII oocytes and from brain

Fig. 2. Western blot analysis of syntaxin. Proteins were extracted and separated by gel electrophoresis. The gel was blotted on to a membrane, and deposited proteins were probed with anti-syntaxin antibody. (a) Proteins from the brain (0.25 μ g in lane 1) served as a positive control, two different aliquots of proteins from the ovary were also loaded on to the gel (25 μ g in lane 2; 50 μ g in lane 3).

Fig. 3. Syntaxin mRNA expression in metaphase II (MII) oocytes. About 500 MII oocytes were lysed in Trizol reagent with 15 μ g of carrier tRNA, and RNA was extracted. The RNAs were resuspended in RNA dilution buffer and an aliquot was subjected to RT-PCR. An ethidium bromide-stained agarose gel of the oocyte RT-PCR products using primers specific for syntaxin isoforms is shown. As a positive control, RNA isolated from the brain was subjected to RT-PCR using the same primers. RNA from 20 oocytes or 1 μ g of brain tissue was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase for 60 min at 37°C. Pd(N)6 was used as a random primer. The first-strand cDNAs were amplified by PCR. Each PCR cycle consisted of denaturation at 95°C for 60 s, primer annealing at 58°C for 120 s, and primer extension at 72°C for 60 s for a total of 40 cycles. Only the amplification product of the size predicted for syntaxin 4 was observed in extracts from MII oocytes.

to identify the specific isoform expressed (Fig. 3). Each primer set used in these experiments was specific for one of three syntaxin isoforms (rat syntaxin 1A, 1B and 4) (Bennett *et al.*, 1992, 1993; Mandon *et al.*, 1996). RT-PCR analysis revealed that only syntaxin 4 was expressed in MII oocytes, and only the predicted-size amplification product for syntaxin 4 was observed. Neither syntaxin 1A nor 1B was detectable in the MII oocytes. The syntaxin 4 RT-PCR product was cloned and sequenced, and was found to be identical to the reported sequence for mouse syntaxin 4 (Genbank accession no. U76832). Furthermore, RT-PCR carried out with primers for syntaxin 1A, 1B, 2, 3, 4 and 5 indicated that only the product for syntaxin 4 was detectable (data not shown).

The immunostained 35 kDa band in membrane fractions of both the brain and ovary had the expected molecular mass of syntaxin. (b) Proteins from the brain (0.5 μ g in lane 1) served as a positive control and proteins from oocytes were also loaded (extract from 500 oocytes in lane 2). Again, the immunostained 35 kDa band in both the brain membrane fraction and oocyte preparation had the expected molecular mass of syntaxin.

Fig. 4. (a,d,g) Immunofluorescence labelling of syntaxin and cortical granules (CGs). After fixation and permeabilization, CGs were stained with fluorescein isothiocyanate (FITC)-conjugated *Lens culinaris* agglutinin. (e,h) Syntaxin immunoreactivity was detected using an anti-syntaxin primary antibody and a rhodamine-conjugated secondary antibody. (f,i) The double-labelled oocytes and one-cell embryos show both syntaxin (red) and CG (green) immunoreactivity. (b,c) A negative control experiment was run in which the primary antibody was replaced with preimmune rabbit IgG. Confocal microscopy with filters specific for FITC, rhodamine and both were used to localize CG and syntaxin in metaphase II (MII) oocytes (a–f) and one-cell embryos (g–i). Syntaxin immunoreactivity (red) was seen predominantly on the plasma membrane and cortical region in MII oocytes (e). This staining co-localized with CG staining, also showing the absence of syntaxin immunofluorescence in the CG-free area (arrows with asterisks in d–f). After fertilization, syntaxin immunoreactivity too was detected on the plasma membrane and in the cortical region of one-cell embryos (g–i). The staining-free region of syntaxin observed in the MII oocytes was not detected (e,h). Ten oocytes were examined in each condition. The experiment was performed three times. A representative result of one experiment is shown. Scale bar represents 20 μm .

Fig. 5. Immunogold electron micrographs of mouse metaphase II (MII) oocytes using antibodies to syntaxin. MII oocytes were fixed and processed for pre-embedding immunoelectron microscopy. (a) Silver enhanced gold particles were detected on the membranes of CGs in the cortical region of MII oocytes (arrows) and on the plasma membrane (arrowheads). (b,c) As represented at higher magnification, gold particles can be seen on the membranes of CGs (arrows) and on the plasma membrane (arrowhead). Scale bars represent 1 μm .

Thus the immunoreactive band in oocytes was thought to represent syntaxin 4.

Localization of syntaxin on metaphase II oocytes and one-cell embryos with pronuclei

Syntaxin 4 was localized within MII oocytes and one-cell embryos by laser scanning confocal fluorescence microscopy. For a clear characterization of the intracellular location of syntaxin 4 with respect to CGs, the same MII oocyte and one-cell embryo was stained with two different fluorescent dyes. CGs were stained with FITC-LCA, and syntaxin immunostaining was detected with a rhodamine-conjugated secondary

antibody. Preimmune rabbit IgG was used as a negative control (Fig. 4a–c). Immunofluorescence was also not detected in non-permeabilized MII oocytes (data not shown). In MII oocytes syntaxin immunoreactivity resided predominantly on the plasma membrane and the cortex region. The syntaxin staining pattern in the cortex also indicated a region free of immunostaining that coincided with the cortical free area (arrows in Fig. 4d–f). Thus the distribution of syntaxin immunoreactivity was the same as that of the CGs detected with FITC-LCA staining. Although syntaxin immunoreactivity was also detected on the plasma membrane and in the cytoplasm in one-cell embryos, there was no prominent staining in the cortical region similar to that seen in MII oocytes (Fig. 4g–i).

Immunoelectron microscopy of syntaxin in the metaphase II oocytes

The detailed intracellular localization of syntaxin in MII oocytes was analysed using immunoelectron microscopy. Silver-enhanced gold particles were detected on the membranes of CGs (arrows in Fig. 5a and b). A few gold particles were also present on the plasma membrane (arrow heads in Fig. 5a and 5c), but no gold particles were detected in other organelles. Controls consisting of oocytes incubated with preimmune rabbit IgG instead of the primary antibody showed almost no gold particles.

Discussion

Some of the SNARE proteins, which form ternary complexes and mediate membrane fusion in several exocytotic cell types, have been identified in mammalian (Ikebuchi *et al.*, 1998) and non-mammalian (Conner *et al.*, 1997; Tahara *et al.*, 1998) oocytes. In mouse oocytes, SNAP-25 has been shown to be involved in Ca^{2+} -dependent CG exocytosis, and sperm-induced CG exocytosis is inhibited in botulinum neurotoxin A-treated eggs (Ikebuchi *et al.*, 1998). These studies have led to a hypothesis that the mechanism of CG exocytosis in eggs is similar to that of synaptic vesicles in neurones as well as to that in other non-neuronal tissues. Western blot analysis revealed syntaxin-like protein expression in mature murine ovary and oocytes. The findings provide strong support for the proposal of a common mechanism, as syntaxin is thought to be an essential component of the fusion machinery.

The syntaxin isoforms 1A, 1B and 4 have been shown previously to be at least partially located in the plasma membrane of neurones and some non-neuronal cells, and to function in the exocytotic pathway by forming ternary complexes with VAMP and SNAP-25 (Inoue *et al.*, 1992; Söllner *et al.*, 1993a; Calakos and Scheller, 1996; Mandon *et al.*, 1996; Foster *et al.*, 1998; Rea *et al.*, 1998; Flaumenhaft *et al.*, 1999). Whereas syntaxin 1A and 1B are involved in the docking or fusion reaction between neurotransmitter-containing vesicles and the plasma membrane in neuronal cells, syntaxin 4 has been found in a variety of tissues including brain, lung, spleen, kidney and fat (Bennett *et al.*, 1993). Syntaxin 4 has been shown to be involved in the regulation of vesicle trafficking, docking, or fusion events in non-neuronal cells as a complex with VAMP-2 and SNAP-23 or -25. The RT-PCR results in the present study using primers for syntaxin 1A, 1B, 2, 3, 4 and 5 indicated that only the product for syntaxin 4 was detectable (data not shown). This is consistent with the conclusion that the single immunoreactive band in oocytes is syntaxin 4. Since functional SNAP-25 is present in mouse oocytes (Ikebuchi *et al.*, 1998), syntaxin 4 in mouse oocytes may form ternary complexes with VAMP-2 and SNAP-25

to mediate the CG exocytosis as in other non-neuronal cells.

We have subsequently localized syntaxin 4 within mouse oocytes by using laser scanning confocal microscopy and immunoelectron microscopy. These immunohistochemical findings provide strong evidence that syntaxin 4 is expressed on the CG membrane as well as on the plasma membrane. In sea urchin oocytes Conner *et al.* (1997) detected a syntaxin-like molecule not only in the plasma membrane fraction but also in the CG fraction. Although syntaxin was originally described as an integral plasma membrane protein, evidence arose indicating that it was present too on the neuronal synaptic vesicle membrane (Nichols *et al.*, 1997; Ungermann *et al.*, 1998). Highly purified synaptic vesicles have been shown to contain syntaxin and SNAP-25, members of the t-SNARE family, as well as VAMP, a neuronal v-SNARE (Walch-Solimena *et al.*, 1995). The immunohistochemical localization of syntaxin 4 shown in the present study coincided well with the location of CGs in mouse MII oocytes, and the results of immunoelectron microscopy showed that syntaxin is present mainly on the vesicle membrane in mouse oocytes.

Oocyte maturation is accompanied by formation of a CG-free domain (CGFD) overlying the metaphase II spindle (Ducibella *et al.*, 1988a). It was also observed that this CGFD is found in MII mouse oocytes (this study). No FITC-LCA fluorescence was detected immediately adjacent to the first polar body, where the meiotic spindle was present. The absence of syntaxin immunoreactivity in the CGFD also indicates that this molecule is located on the CG membrane.

In MII oocytes, immunoreactivity for syntaxin 4 was observed mainly on the plasma membrane and in the cortex, with a small portion located deep in the cytoplasm. These results can be explained in one of two ways. The first possibility is that these molecules may be undergoing transfer from their site of origin, the Golgi apparatus, to their ultimate destination, the plasma membrane (Kasai and Akagawa, 2001). The second possibility is that deep cytoplasmic immunoreactivity represents CG located deep in the cytoplasm. In general CGs are membrane-bound vesicles that originate from the Golgi apparatus and migrate toward the cortex during oocyte maturation. Although most CGs are located peripherally by the end of oocyte maturation, as in GV stage oocytes and MII stage oocytes, some CGs still reside in the interior of the mature oocyte (Ducibella *et al.*, 1988b; Abbott *et al.*, 2001). These reports support our suggestion of a small portion of immunoreactivity in the deep cytoplasm.

After fertilization, faint fluorescence of syntaxin was observed on the plasma membrane but a syntaxin immunoreactivity-free area, corresponding to the CG distribution seen in MII oocytes, was not seen. FITC-LCA fluorescence of residual CG products was observed

on the plasma membrane in one-cell embryos. During exocytotic membrane fusion, the SNARE proteins of the membrane (t-SNARE) interact with those of the vesicle (v-SNARE) to form ternary complexes, and are thought to change the molecular configuration of SNARE proteins. The formation of ternary complexes could alter access by an antibody, or the configurational change of the syntaxin molecule could affect antibody affinity for syntaxin on the plasma membrane. This speculation might explain the decrease of syntaxin immunoreactivity on the oocyte plasma membrane after fertilization.

Although our results show the expression of syntaxin 4 in mouse oocytes, the exact functional roles remain to be elucidated. Investigation of the effects of micro-injection experiments using syntaxin antagonists (botulinum neurotoxin or anti-syntaxin antibody) would provide further understanding of the functional roles of this molecule during cortical reaction.

In conclusion, the present study demonstrates that syntaxin 4 mRNA is detected in MII oocytes and that syntaxin 4 expression may be distributed in a different manner in MII oocytes from that in one-cell embryos. These results indicate that syntaxin 4 co-localizes with CGs in the cortex, where it is likely to function in membrane fusion during CG exocytosis.

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