

# Members of the SNARE Hypothesis Are Associated With Cortical Granule Exocytosis in the Sea Urchin Egg

SEAN CONNER,<sup>1</sup> DAVID LEAF,<sup>2</sup> AND GARY WESSEL<sup>1\*</sup>

<sup>1</sup>*Department of Molecular and Cell Biology & Biochemistry, Brown University, Providence, Rhode Island*

<sup>2</sup>*Department of Biology, Western Washington University, Bellingham, Washington*

**ABSTRACT** Cortical granule exocytosis is important for the block to polyspermy at fertilization in the eggs of most vertebrates and many invertebrates. Cortical granules are poised at the cell surface and exocytose in response to sperm stimulation. Following exocytosis, the cortical granule contents modify the extracellular environment of the egg, the major result of which is to block additional sperm binding. Here we show that proteins homologous to members of the SNARE hypothesis—a molecular model designed to explain the trafficking, docking, and exocytosis of vesicles in the secretory compartment—are present in eggs at the right time and place to be involved in the regulation of cortical granule exocytosis. Using polymerase chain reaction (PCR) screens we have found homologues of synaptobrevin/VAMP, syntaxin, synaptotagmin, and rab3. Antibodies generated to fusion proteins or to synthetic peptides encoded by the cloned cDNAs were used in an immunofluorescence assay to show that each of the cognate proteins are present in the cortex of the egg. A synaptobrevin/VAMP homologue appears to be specifically associated with the membrane of cortical granules before fertilization and, following cortical granule exocytosis, is incorporated into the plasma membrane of the zygote. A rab3 homologue is also associated with cortical granules specifically but, following fertilization, the protein reassociates with different, yet undefined, vesicles throughout the cytoplasm of the zygote. Homologues of synaptotagmin and syntaxin are also present at the egg cortex but, in contrast to rab3 and VAMP, appear to be associated with the plasma membrane. Following fertilization, syntaxin and tagmin remain associated with the plasma membrane and are more readily immunolabeled, presumably due to an increased accessibility of the antibodies to the target protein domains. We also show by immunoblotting experiments that the cognate proteins are of the sizes predicted for these homologues. These results suggest that at least some steps in the biology of cortical granules may be mediated by SNARE homologues, and this finding, along with the unique biology of cortical granules, should facilitate examination of specific events of the fertilization reaction and the mechanism of stimulus-

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**Key Words:** fertilization; VAMP; syntaxin; synaptobrevin; synaptotagmin, rab

## INTRODUCTION

The fertilization reaction consists of a series of specific membrane fusions in both sperm and eggs, and the final set of membrane fusions—the fusion of cortical granules with the egg plasma membrane—is important for the block to polyspermy. Cortical granules are oocyte-specific organelles that accumulate in the cytoplasm during oogenesis, reaching approximately 8,000 in mice and 15,000 in sea urchins (Ducibella et al., 1994; Laidlaw and Wessel, 1994). Some time late in oogenesis, the cortical granules translocate to the cell cortex, attach to the plasma membrane, and remain poised for secretion until the egg is activated by sperm. In some animals like sea urchins, the cortical granules form a perfect monolayer at the cell surface and remain for weeks without spontaneous exocytosis. Within seconds of sperm contact, however, the cortical granules exocytose their contents which modifies the egg's cell surface and its extracellular layers. These modifications permanently block additional sperm–egg interactions. Some cortical granule contents are evolutionarily conserved including a protease activity found in the eggs of mice (Moller and Wasserman, 1989; Hoodbhoy and Talbot, 1994), frogs (Lindsay and Hedrick, 1995), and sea urchins (reviewed in Schuel 1985; Wessel, 1996).

An increase of intracellular free calcium is an imperative step for cortical granule exocytosis. Although the signal transduction pathway leading to calcium release

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\*Correspondence to: Gary Wessel, Box G, 69 Brown Street, Department of Molecular and Cell Biology and Biochemistry, Brown University, Providence, RI 02912; E-mail: rhet@brown.edu

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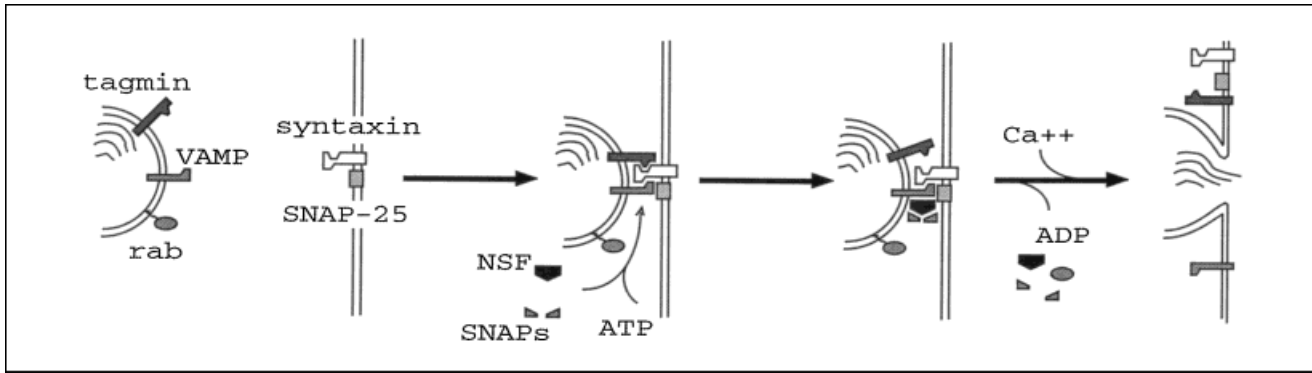


Fig. 1. Representation of the SNARE hypothesis modeled for cortical granules. (Adapted from Bennett, 1995 and Söllner et al., 1993.)

is not completely understood, it is initiated by the binding of sperm to the sperm receptor on the egg plasma membrane (reviewed in Foltz, 1995), and results in a release of calcium from stores within the endoplasmic reticulum. Cytoplasmic calcium concentrations reach  $1.5\text{--}2.5\ \mu\text{M}$  within 15 sec following sperm mediated egg activation (Whitaker and Swan, 1993; Shen, 1995) and exocytosis of the cortical granules rapidly follows. This exocytosis begins from the point of sperm contact and extends in a wave around the circumference of the egg. Since cortical granules are attached tightly to the plasma membrane in sea urchin eggs, exocytosis does not require vesicle transport, and the entire population of cortical granules exocytose within 60 sec in an egg with a diameter of between  $80\text{--}100\ \mu\text{m}$ . The mechanism of cortical granule interaction with the plasma membrane is unknown as is the calcium sensor for exocytosis.

A convergence of studies in synaptic vesicle secretion (Bennett and Scheller, 1994), in the genetic studies of secretion in yeast (Ferro-Novick and Jahn, 1994), and in *in vitro* analysis of intra-Golgi traffic (Rothman and Orci, 1992) has resulted in the identification of a population of proteins that appear to mediate membrane interactions in vesicular trafficking and membrane fusion (Söllner et al., 1993; Bennett, 1995). Key to membrane fusion is an *n*-ethylmaleimide sensitive fusion protein (NSF) that is found in the cytoplasm (Rothman and Warren, 1994). Before NSF can participate in membrane fusions, however, it must be recruited to the site of interaction between the vesicle membrane and the target membrane by a "receptor complex." This complex includes soluble NSF attachment proteins, or SNAPS, and membrane-bound proteins from both the vesicle and the plasma membrane, the latter of which form the *snare* receptors, or SNAREs. The membrane bound proteins important for the SNARE complex include SNARE elements on the vesicle, or v-SNAREs, and SNARE elements on the target membrane, or t-SNAREs. Examples of v-SNAREs include synaptobrevin and cellubrevin, and of t-SNAREs include syntaxin and SNAP-25, the latter of

which is not related to the SNAPS. The binding of a v-SNARE to the appropriate t-SNARE is hypothesized to be responsible for the specificity of vesicle and target membrane binding and is thought to be a required step for the recruitment of the NSF/SNAP complex (Söllner et al., 1993). Following ATP hydrolysis by the recruited NSF, the vesicles exocytose in response to free calcium. Synaptotagmin has been suggested to act as a calcium clamp rendering the SNARE fusion complex sensitive to calcium (Südhof, 1995; Chapman et al., 1996).

Do all exocytic fusion events employ members of the SNARE hypothesis? SNARE components do appear to participate in the exocytosis of several vesicle types in somatic cells, e.g., in synaptic vesicles of neurons, in zymogen granules of pancreatic acinar cells, and in chromaffin granules of the adrenal gland. However, it was unclear whether eggs would share such mechanisms, since some cells do not employ SNAREs to mediate exocytosis, e.g., eosinophils (Lacy et al., 1995), and apical secretion in MDCK cells (Ikonen et al., 1995). Furthermore, cortical granules are distinct from other stimulus-dependent secretory vesicles, in that the entire population of cortical granules is primed for fusion, and they are not recycled following exocytosis. To determine whether eggs use a SNARE-mediated exocytotic mechanism at fertilization, we used PCR with primer sequences based on conserved protein domains of SNARE homologues to screen an ovary cDNA library. We identified homologues of syntaxin, synaptotagmin, synaptobrevin, and rab3, and this report describes that each of the cognate proteins is present at the appropriate time and place to participate in the exocytosis of cortical granules at fertilization.

## MATERIALS AND METHODS

### Animals and Reagents

Adult *Strongylocentrotus purpuratus* were obtained from Marinus (Long Beach, CA). Gametes were obtained and fertilized as described (McClay, 1986). Cell surface complex was isolated as described (Kinsey, 1986).

### PCR Primers and Conditions

**Syntaxin.** The design of the PCR primers used here was based on the conserved amino acid sequences of syntaxin 1A from rat (Bennett et al., 1992) and from *Drosophila* (Schulze et al., 1995).

#### External primers

*Sense:* 5'-CA(A/G)GTIGA(G/A)GA(G/A)AT(A/C/T)(A/C)GIGG-3', corresponding to amino acids 36–42 (QV-EEIRG) of rat syntaxin 1A

*antisense:* 5'-CAT(A/G/T)AT(C/T)TT(C/T)TTIC(T/G)IC(T/G)IGC-3', corresponding to amino acids 261–267 (ARRKKIM) of rat syntaxin 1A

#### Internal primers

*Sense:* 5'-AA(A/G)TT(T/C)GTIGA(G/A)GTIATGI(C/G)IGA(G/A)TA-3', corresponding to amino acids 126–134 (KFVEVMSEY) of rat syntaxin 1A

*Antisense:* 5'-GGCAT(G/A)TCCAT(A/G)AACAT(G/A)TC(G/A)TG-3', corresponding to amino acids 214–221 (HDMFMDMA) of rat syntaxin 1A

In each case, letters in parentheses indicate a degeneracy.

**Tagmin.** The design of the polymerase chain reaction (PCR) primers used here was based on the conserved amino acid sequences between rat synaptotagmins I and II (Perin et al., 1990; Geppert et al., 1991), *Drosophila* (Perin et al., 1991), squid (Bommert et al., 1993), and *C. elegans* (Nonet et al., 1993).

#### External

*Sense:* 5'-AA(A/G)AA(A/G)TT(C/T)GA(A/G)ACIAA(A/G)GTICA-3', corresponding to amino acids 253–259 (KFETKVVH) of rat synaptotagmin I

*Antisense:* 5'-GT(A/G)TGCCA(C/T)TGIGC(A/G/T)ATIGG-3', corresponding to amino acids 464–470 (PIAQWHT) of rat synaptotagmin I

#### Internal

*Sense:* 5'-AA(A/G)AA(A/G)ATGGA(C/T)GTIGGIGG-3', corresponding to amino acids 354–360 (KKMDVGG) of rat synaptotagmin I

*Antisense:* 5'-(C/T)TG(C/T)TC(A/G)AAIGGIAC(C/T)TC(A/G)-3', corresponding to amino acids 409–416 (FEVPFEQ) of rat synaptotagmin I

**VAMP.** The design of the PCR primers used here was based on the conserved amino acid sequences between rat cellubrevin, rat synaptobrevins I and II, and *Drosophila* synaptobrevin (McMahon et al., 1993).

*Sense:* 5'-GA(A/G)GTIGUIGA(C/T)ATIATG-3', corresponding to amino acids 40–46 (EVVDIMR) of rat cellubrevin

*Antisense:* 5'-CCACCAIT(G/A)(C/T)TTIC(G/T)(T/C)TT-3', corresponding to amino acids 72–77 (KRK(Y/Q)WW) of rat cellubrevin

**Rab 3.** The design of the primers used was based on the conserved GTP-binding domains found in Rabs.

*Sense:* 5'-5'CGAGCTGCAG(AG)TC(AG)CA(CT)T-T(AG)TT(ACGT)CC 3', corresponding to the second GTP binding domain (GNKCD) of small GTP-binding proteins (Yu et al., 1993)

*Antisense:* 5'-GAGGAATTCTGGGA(CT)AC(ACT)G-C(ACT)GG(ACGT)CA(AG)GA 3', corresponding to the third GTP binding domain (WDTAGQE) of small GTP-binding proteins (Yu et al., 1993; Leaf and Blum, 1996)

**Templates.** To screen for homologues of syntaxin, synaptotagmin, and synaptobrevin/VAMP, a  $\lambda$ ZAP cDNA library was used that was made from poly dT selected RNA isolated from ovaries of *S. purpuratus* (Laidlaw and Wessel, 1994). RT-PCR was employed to identify Rab homologues using RNA isolated from ovaries of *S. purpuratus* as described (Leaf and Blum, 1996). Two clones, referred to as nos. 17 and 73, were used for hybridization screens because of their similarity to rab3 sequences.

### cDNA Screening Procedure

For plaque hybridizations for additional cDNA sequence, PCR products were labeled by random priming (Feinberg and Vogelstein, 1983) and were used to screen a cDNA library. The library was constructed in  $\lambda$ ZAP (Stratagene, La Jolla, CA) using poly dT selected RNA isolated from *S. purpuratus* ovaries. Positive plaques were purified by repeated replating and hybridization and the recombinant cDNA of each phage was excised with helper phage R408 (Stratagene) and recovered as a Bluescript plasmid (Short et al., 1988). Additional cDNA clones encoding either full-length (syntaxin) or partial sequences (VAMP and rab3) were obtained by this method. We were unsuccessful in isolating additional cDNA sequence for tagmin using this technique.

### DNA Sequencing and Analysis

PCR products were cloned into pGEM-T (Promega, Madison, WI) for sequencing by the Sanger chain termination method (Sanger et al., 1977) using [<sup>35</sup>S]dATP (Dupont, Boston, MA) and Taq DNA polymerase (Promega Biotech, Madison, WI), and detected with BioMax MR film (Kodak, Rochester, NY). Sequence data were assembled and analyzed using the University of Wisconsin Genetic Computer Group (UWGCG) sequence analysis package (Devereux et al., 1984). The Pileup program was used for alignment of all sequences using a 3.00 gap weight and 0.10 gap length weight as defaults. The Distances program was then used to determine the evolutionary distance between the aligned sequences with Kimura protein distance correction. Finally, cladograms were generated with Grootree using the neighbor-joining method. Bootstrap values were generated using ClustalW (Higgins et al., 1991) with alignments generated in Pileup.

### Antibody Generation

The following protein sequences were used for generating rabbit polyclonal antiserum; VAMP and Syn-

taxin: amino acids 20–64 (numbered according to the human homologue) and 122–220 (numbered according to the sea urchin homologue, since this is a full-length cDNA clone), respectively, were fused to a truncated  $\beta$ -galactosidase of pWR 590; Rab3: the peptide CDKMSETIDTDQTLRPSTT from the hypervariable region of the Rab predicted protein sequence was synthesized and covalently conjugated to BSA; Tagmin: the peptides CGLSDPYVKISLYMNNKRMK and CRMKTKKTTIKKRTLNPYYN were synthesized and conjugated to BSA. Immunogens were resuspended in Freund's adjuvant, injected subcutaneously into New Zealand White rabbits every 3 weeks for 3 months. One week after the last boost, plasma was collected from the central ear artery (Harlow and Lane, 1988). Resultant immunoreactivity to  $\beta$ -galactosidase or to bovine serum albumin (BSA), used as a fusion partner or conjugate protein, respectively, showed no detectable crossreactivity in eggs or zygotes when used in immunoblot or immunolocalization assays.

#### Electrophoresis and Immunoblot Analysis

Eggs, embryos and cell fractions were subjected to SDS-PAGE and immunoblot analysis essentially as described (Towbin et al., 1979). Samples for analysis were pelleted, resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 10 mM DTT and a protease inhibitor cocktail (consisting of a final concentration per ml of aprotinin, 1 TIU; benzamidine, 10  $\mu$ g; soybean trypsin inhibitor, 10  $\mu$ g; antipain, 1  $\mu$ g; leupeptin, 1  $\mu$ g; bestatin, 0.5  $\mu$ g; E-64, 1  $\mu$ g; phosphoramidon, 1  $\mu$ g; phenylmethylsulfonyl fluoride, 10  $\mu$ g; chymostatin, 1  $\mu$ g; pepstatin, 1  $\mu$ g), and denatured for 3 minutes at 100°C. The proteins were resolved on an acrylamide gel and either stained with Coomassie Blue or transferred to nitrocellulose for immunolabeling as described (Towbin et al., 1979). For immunolabeling, blots were washed twice for a total of 1 hr in blotto buffer (50 mM Tris pH 7.5, 0.9% NaCl, 0.05% Tween-20, and 3% nonfat dry milk) and then incubated for 1 hr in blotto containing diluted antibody (see Immunolocalization Assays *in situ*, below). The blots were then washed three times in blotto over 30 min and incubated in blotto with goat antirabbit antibodies conjugated to alkaline phosphatase (Organon Teknika, Durham, NC) diluted  $\times 32,000$ . Blots were washed in blotto three more times over 30 min, then washed in blotto without milk. Immunolabel signals were detected by BCIP/NBT colorimetric development as described (Harlow and Lane, 1988) (Promega). Controls used in this experiment included blots incubated with preimmune antisera, and secondary antibody alone. Each of these immunoblots showed no signal (data not shown).

#### Immunolocalization Assays *In Situ*

Immunofluorescence localization was performed in whole mounts and on sections of embryos that were fixed and processed as previously described (Laidlaw and Wessel, 1994). Primary antibodies were diluted between 1/50 and 1/10,000 and the secondary antibody

(Cy3-conjugated affinity-purified goat anti-mouse IgG; Kirkegaard & Perry Labs, Gaithersburg, MD) was diluted 1 : 100. Signals were recorded either by epifluorescence with a Zeiss Axioplan or by a laser scanning microscopy with a Zeiss LSM 410.

#### Antibody Competition Assays

Antibodies were preabsorbed with the protein or peptide used for antibody generation for 1 hr at 22°C. Rab3 (1:50) was preabsorbed with CDKMSETIDTDQTLRPSTT-BSA conjugate, syntaxin (1:10,000) with lysate from XL1-Blue expressing a syntaxin- $\beta$ -galactosidase fusion protein, and VAMP (1:200) with lysate from XL1-Blue expressing VAMP- $\beta$ -galactosidase fusion protein. Preabsorbed antibody was then used as described above.

### RESULTS

This study tests the possibility that components of the SNARE hypothesis are present in eggs, at the correct time and place, to be involved in cortical granule exocytosis at fertilization. Figure 1 presents a schematic diagram of the SNARE model adapted to cortical granules. We used PCR to search for homologues of syntaxin, synaptotagmin, synaptobrevin/VAMP, and rab3, in an ovary cDNA library using primers designed to conserved regions within each protein from human, rodent and *Drosophila*. We identified single members for each of these families, and following the generation of antibodies to these oocyte-encoded sequences, we found that each of these proteins are present at the appropriate time and place to participate in exocytosis of cortical granules. We present the evidence below for each homologue that includes the cDNA sequence, as well as immunological assays to characterize the cognate proteins.

#### VAMP

Synaptobrevin/VAMP is a transmembrane vesicle-associated protein (Trimble et al., 1988). As a v-SNARE, it is postulated to interact with cognate t-SNAREs in the target membrane (syntaxin and SNAP-25) and participate in vesicle docking and fusion by recruiting SNAPs and NSF to the membrane interaction site (Fig. 1). Two major types of VAMPs have been identified; type I/II synaptobrevin is found predominantly in brain and cellubrevin appears to be expressed ubiquitously (McMahon et al., 1993). We have identified a single homologue of the synaptobrevin/VAMP family in sea urchin oocytes that is more similar to synaptobrevin/VAMP than to cellubrevin (Fig. 2A,B; although the same protein was assigned different names by different groups, we refer to this protein as VAMP instead of synaptobrevin to reflect its non-neuronal source). The central part of the protein contains two conserved regions encoding two amphipathic  $\alpha$ -helices, H1 and H2. These regions are thought to be important for interacting with t-SNARE components and H1 has been shown to be required for targeting of the synapto-

brevin protein to synaptic vesicles in PC12 cells (Grote et al., 1995). The cDNA sequence also encodes a hydrophobic transmembrane domain near the C-terminus, and a predicted intravesicular domain of five residues at the C-terminus. This intravesicular domain is more similar to the canonical vertebrate intravesicular domain, i.e., short with a cluster of basic amino acids, than to the intravesicular domain of *Drosophila* synaptobrevin, in which 22 amino acids follow the membrane spanning domain. The region N-terminal to the H1 domain is divergent from synaptobrevin, as are other members of the VAMP family from each other. The partial cDNA clone we have isolated is incomplete by approximately 15 amino acids based on the difference in size predicted from the cDNA translation with the mobility of the native protein in SDS-PAGE (see below).

Antibodies made to a  $\beta$ -galactosidase/VAMP fusion protein showed prominent immunoreactivity to VAMP at the cortex of the egg (Fig. 2Ci). This label is a broad band of approximately 1–2  $\mu$ m and is consistent with its association with cortical granules. Following fertilization (6 min postinsemination (Fig. 2Cii), VAMP remained at the cortex but was restricted to a thin stripe of label that we interpret to be the plasma membrane. This change of pattern is consistent with the incorporation of cortical granule membrane into the plasma membrane and shows a lack of recycling of the VAMP protein following fertilization. All VAMP immunolabel at the cortex was competed away with excess  $\beta$ -galactosidase/VAMP fusion protein and no other organelle of eggs or zygotes was detectably labeled (Fig. 2Ciii).

Immunoblots using VAMP antisera identified a 15-kDa protein, a size predicted for VAMP members, as well as major immunoreactivity at 69 kDa and at >100 kDa (Fig. 2B). The immunolabel in excess of 100 kDa is greatly enriched in the isolated cell surface complex (cortical granules attached to the plasma membrane; isolated according to Kinsey, 1986) relative to the egg, whereas the 15-kDa and the 69-kDa bands are not. We do not know the nature of the high-molecular-weight material but speculate that the immunolabel may result from cross-reactivity to VAMP-like sequences, or it may indicate that VAMP is covalently bound to high molecular weight molecules in the isolated cell-surface complex (CSC). Although the cell surface of the egg and zygote, as well as the contents of the cortical granules have significant protein cross-linking activity (transglutaminases and ovoperoxidases) (Shapiro et al. 1989), we do not see significant crosslinking of other SNARE proteins into high-molecular-weight forms (see below). However, the majority of label is competed away specifically with excess VAMP fusion protein showing that the antibody signal is due to cross-reactivity to VAMP-like sequences.

### Syntaxin

Syntaxin is a transmembrane protein present in the presynaptic plasma membrane and is postulated to interact with both SNAP-25, another t-SNARE, and with the v-SNARE synaptobrevin/VAMP of the synap-

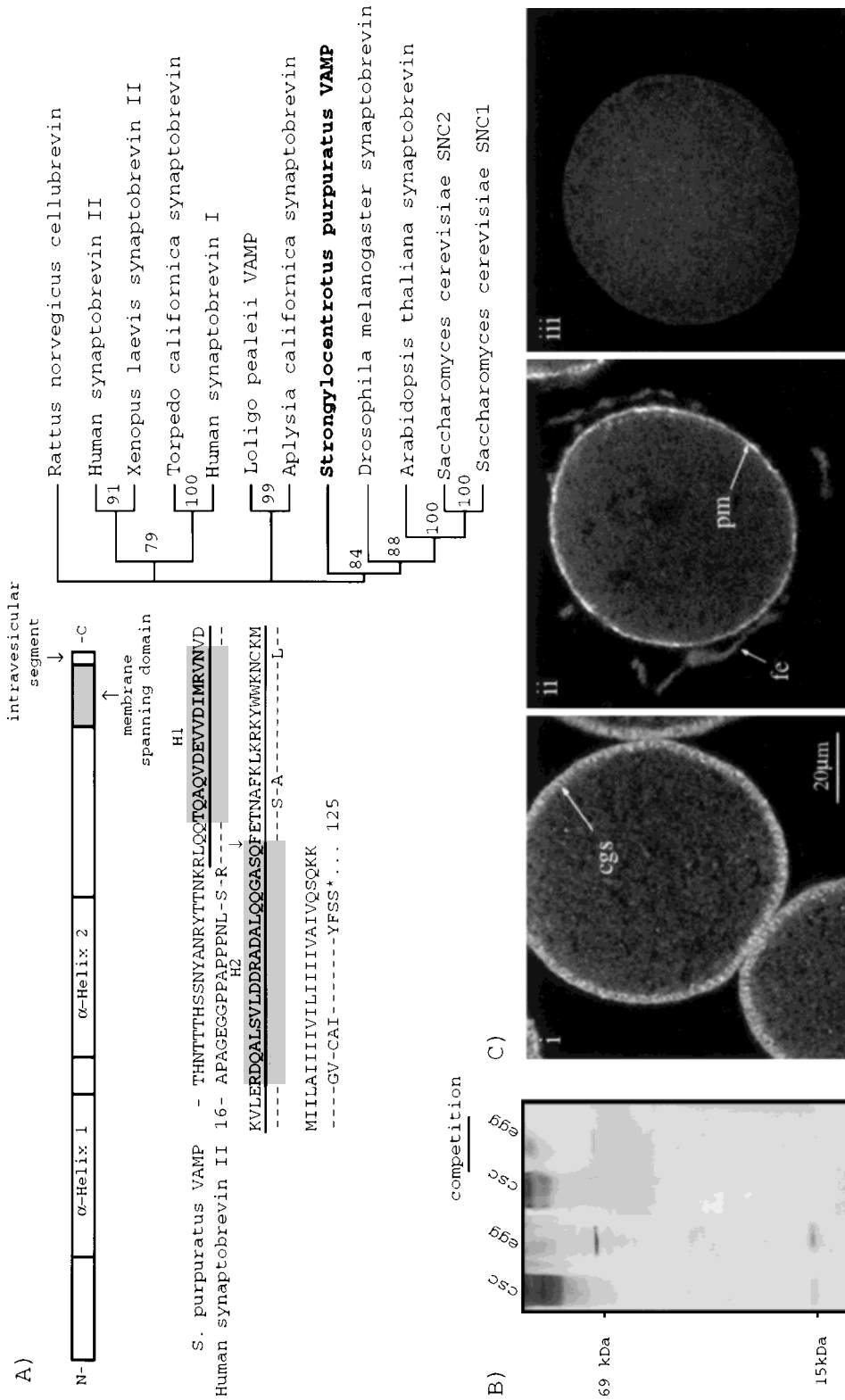
tic vesicle (see Figure 1). This multiprotein interaction is believed to participate in the docking of vesicles to the plasma membrane and to recruit NSF for membrane fusion (Bennett, 1995). The deduced amino acid sequence shows that it contains the expected membrane spanning domain at the C-terminus and strong conservation of the H3  $\alpha$ -helical domain just in front of the membrane spanning domain (Fig. 3A). The H3 helical domain is believed to participate in the multiprotein interactions important for syntaxin function (Kee et al., 1995). The other helical domains, H1 and H2 are strongly conserved, whereas the N-terminus of the encoded protein is distinct from all other syntaxins.

Antibodies made to the middle one-third of the protein encoded by the syntaxin cDNA, including the H3 domain, were used to immunolocalize the syntaxin protein (Fig. 3C). In contrast to the immunolabeling pattern for brevin, the syntaxin signal was not consistently detectable by in situ immunolabeling at the cortex. Following fertilization, however, strong syntaxin immunolabeling was apparent and restricted to a thin band at the cell surface (Fig. 3Cii). We interpret this dramatic increased immunolabeling to an increased accessibility of the antibody to syntaxin, perhaps as a result of changes in domains of protein interactions and an unmasking of target epitopes following cortical granule exocytosis. This interpretation is consistent with the proposed role of the H3 helical domain being involved in protein-protein interactions prior to vesicle fusion (Kee et al., 1995).

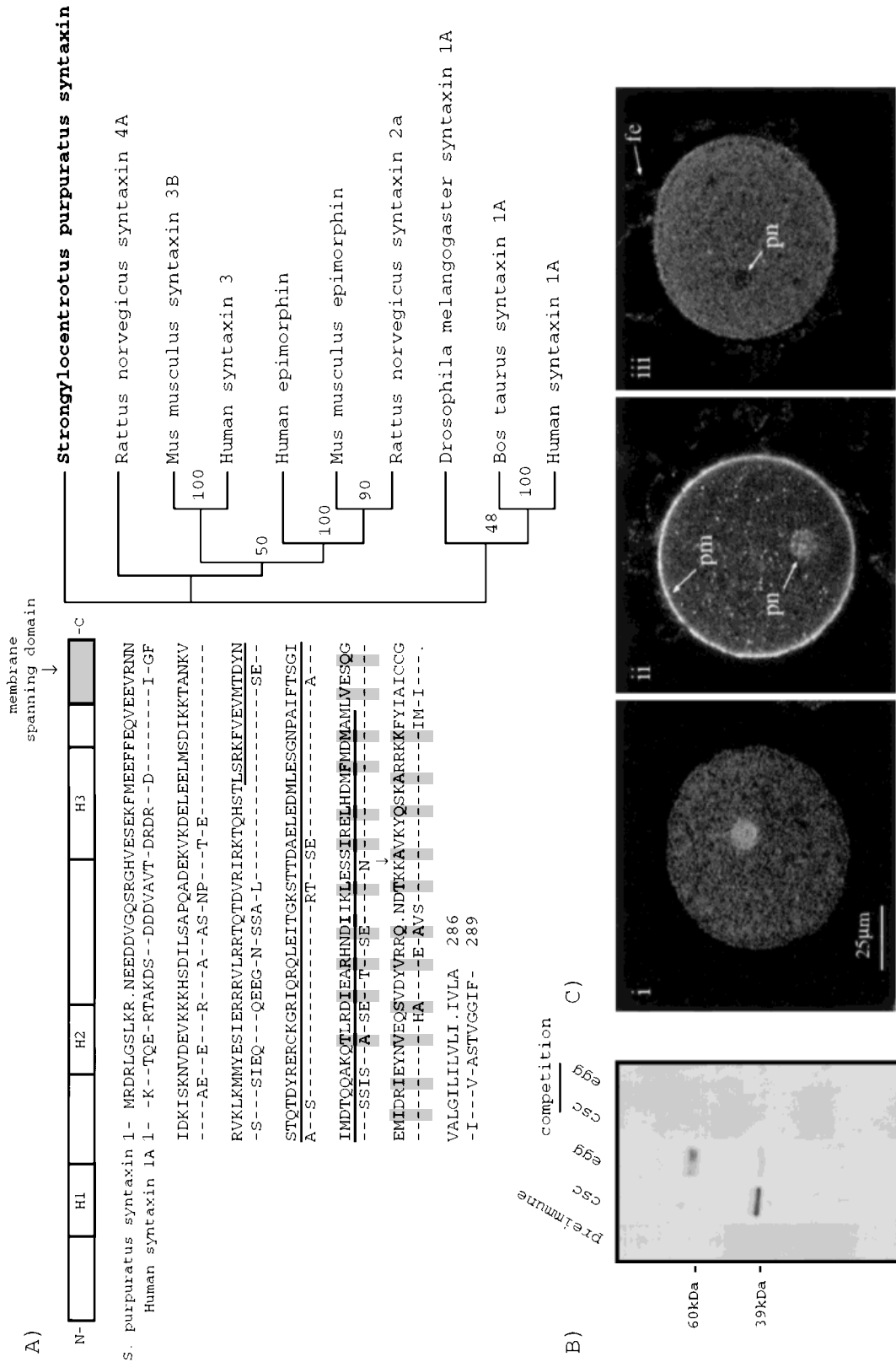
Further support for syntaxin presence at the cell surface comes from immunoblotting in which a 39 kDa band is enriched approximately fivefold in isolated cell surface complex (Fig. 3B). Syntaxin appears to be post-translationally modified, as its apparent 39-kDa mass is greater than the expected 33-kDa form predicted by the cDNA sequence. In addition to the cell surface labeling, we see significant label *within* the pronucleus (Fig. 3C). This signal is apparently associated with a 60-kDa doublet seen by immunoblots of whole eggs, but not of the cortex (Fig. 3B). We do not understand the nature of the pronuclear signal; we believe it is probably a cross-reacting molecule that shares some sequence with syntaxin. Preimmune sera shows no significant label in the pronucleus or in the cell surface either by immunoblots (Fig. 3B) or by immunolocalization (data not shown) and preabsorption of the immune sera with excess syntaxin-fusion protein results in only background levels of cell surface and pronuclear labeling (Fig. 3B,Ciii).

### Rab3

Rab proteins are small, monomeric GTPases that are associated with vesicles in several different membrane trafficking pathways (Pfeffer, 1994). At least 30 different types of rabs have been identified in mammalian cells, each of which appear to be associated either with a specific vesicle pathway, or share pathways but are expressed in different cell types (Zerial and Huber, 1995). The exact function of rabs in vesicle trafficking is



**Fig. 2.** A VAMP homologue is enriched in the cortex of sea urchin eggs. **A:** Cartoon representation of the predicted VAMP protein (above) with the partial amino acid sequence (below) displaying sequence identity with synaptobrevin from the human brain, and a cladogram of the synaptobrevin family (right). The partial cDNA sequence from eggs is missing the N-terminus; it begins with residue 16 of human synaptobrevin, and extends 4 residues longer than the human stop codon (\*) before ending. The underline indicates the region of the protein used for antibody generation, the shaded area indicates the  $\alpha$ -helical domains; arrow, conserved cleavage site for tetanus toxin and Botulinum neurotoxin B. Numbers located at tree branchpoints of the cladogram indicate bootstrap values times 10. **B:** Immunoblot analysis identifies the predicted 15-kDa form of the VAMP protein, in addition to a 69-kDa form and a high-molecular-weight mass. Each of these bands competes for VAMP antibody with the VAMP fusion protein selectively (competition). The high-molecular-weight form of the protein is greatly enriched in the cortex of the egg. Approximately 80  $\mu$ g of protein was loaded in each lane. **C:** (i) VAMP is enriched at the cell cortex of unfertilized eggs associated with cortical granules. Following fertilization, the VAMP signal changes to the plasma membrane (ii). All immunolabeling is lost following competition of the antibody with VAMP recombinant protein (iii). Images are visualized by indirect immunofluorescence using confocal microscopy. cgs, cortical granules; csc, cell surface complex; fe, fertilization envelope; pm, plasma membrane.



**Fig. 3.** Syntaxin is found selectively at the egg cortex and within the pronucleus. **A:** Cartoon representation of the syntaxin peptide (above) with the predicted amino acid sequence (below) compared to human syntaxin. Underline indicates the region of the protein used for antibody generation, the shaded residues refer to amino acids important in the H3  $\alpha$ -helical domain for protein interactions; arrow, conserved cleavage site for Botulinum neurotoxin C. Numbers located at tree branchpoints of cladograms indicate bootstrap values times 10. **B:** Immunoblot analysis for syntaxin indicates an enrichment of a 39-kDa protein in the cell surface complex (csc), and a 60-kDa form in the egg. Both bands compete for syntaxin antibody labeling with the syntaxin fusion protein (competition). Approximately 80 µg of protein is loaded in each lane. **C:** In situ immunolocalizations indicate that syntaxin is enriched in the plasma membrane of the egg (pm) and in the pronucleus (pn) of both unfertilized eggs (i) and in eggs at 6 min post-insemination (ii). Fertilization significantly increases the intensity of syntaxin signal at the plasma membrane, but not in the pronucleus. Competition of antibody with the syntaxin fusion protein reduces signals in the pronucleus and the plasma membrane (cf. images ii and iii). Images are visualized by indirect immunofluorescence using confocal microscopy. fe, fertilization envelope, indicative of the cell having been fertilized.

not known, though their proposed functions include (1) mediating the interactions of SNARE proteins involved in the docking of vesicles; (2) controlling the stability of a SNARE complex; and/or (3) controlling the timing of the fusion event (Rybin et al., 1996). Of all the rab types known, the rab3 family is specifically associated with stimulus-dependent exocytosis and we have isolated a near full-length cDNA sequence from eggs which encodes a rab3 homologue that is most closely related to rab3 from *Drosophila* (Fig. 4A). The predicted rab3 sequence from eggs has complete conservation of the GTP-binding sites and also contains the predicted hypervariable region at the C-terminus that is believed to participate in the targeting of rab proteins to specific vesicle types. The protein sequence ends with the characteristic CXC amino acid sequence necessary for protein prenylation, a modification important for the protein's ability to interact with a membrane.

To generate antibodies to rab3, the hypervariable region of the predicted protein sequence was used as an immunogen, as it is the only region that is distinct between members of the rab GTPase subfamily. The resulting antibodies identified a band in immunoblots of 24 kDa and sometimes a 21-kDa band; both are enriched in the cortex of eggs approximately 100-fold (Fig. 4B). The predicted size of rab3 is 24 kDa, which suggests that the 21-kDa band is a proteolytic product. Similar results have been found in immunoblot analysis of a sea urchin rab10 GTPase (Leaf and Blum, 1996).

The cortical location of the rab3 protein is verified by immunolocalizations where it appears to be associated with the cortical granules (Fig. 4Ci). This rab immunolabeling is around vesicles approximately 1  $\mu\text{m}$  in diameter and completely lines the cortex, consistent with a cortical granule association. Following fertilization of the egg, the rab3 immunolabel shifts to vesicles different from cortical granules. Although the identity of these vesicles is unknown, they are clearly not cortical granules, since cortical granules are no longer present in the fertilized egg and are smaller in diameter than the post-fertilization rab3-positive vesicles (1  $\mu\text{m}$  vs 1.5–2.0  $\mu\text{m}$ ) in diameter (Fig. 4Cii). No immunolabel is detected when using preimmune sera (data not shown) or when the immune sera is preabsorbed with the peptide used as immunogen (Fig. 4Ciii).

### Tagmin

Stimulus-dependent exocytosis is a calcium-dependent phenomenon, and the requirement of calcium ions in cortical granule exocytosis at fertilization is well established (see Shen, 1995, for review). The best candidate currently for a sensor of free calcium in regulating exocytosis is synaptotagmin (Südhof, 1995). This protein contains two calcium binding domains that are similar to the calcium binding motif of protein kinase C, and it undergoes a calcium-dependent conformational change (Chapman et al., 1996; Kee and Scheller, 1996). Since other members of the SNARE model appeared to be present in association with the cortical granules at fertilization, we tested for the

presence of tagmin homologues in eggs. Our cloning approaches were successful only in identifying the second C2 domain of tagmin (Fig. 5A). Without additional sequence to confirm the identity of the protein our assignment is tentative, but the encoded amino acid sequence is more similar to the PKC-like domain of synaptotagmin (greater than 80% identity) than to the PKC domains of other proteins, including rabphilins and PKCs (identity less than 50%, data not shown).

Antibodies generated to a synthetic peptide specific to the C2 domain of tagmin show that tagmin is localized at the plasma membrane of the egg. Tagmin, does not appear to be associated with the entirety of the cortical granule membrane, as seen for VAMP, since a distinct lack of fluorescence is noted in the cortical granule-rich region underlying the plasma membrane (Fig. 5B i,ii). We can not, however, exclude an association of tagmin with cortical granules where they dock to the plasma membrane. Following fertilization, the tagmin signal at the cell surface was far more intense than in unfertilized eggs, as in the case of the syntaxin (see above). We interpret this result to mean that the tagmin epitopes are more accessible to the antibody following fertilization which is consistent with experiments that suggest that the C2 domain (the region used for antibody generation) is important for protein interactions (Bommert et al., 1993). The nature of the punctate labeling throughout the cytoplasm is unknown and, although the antisera show excellent titers to the immunogen (in excess of 10,000-fold), we have been unable to identify a tagmin protein by immunoblotting.

### DISCUSSION

Cortical granule exocytosis at fertilization is necessary to block polyspermy and to create or to modify the extracellular environment of the embryo. This report shows that molecules believed to participate in the secretory function of synaptic vesicles are also present in eggs, in a place consistent with their involvement in cortical granule exocytosis. Although the biology of synaptic vesicles and cortical granules has significant differences, synaptic vesicles are recycled, have vastly different contents, are smaller than cortical granules, and are a heterogeneous population of docked and predocked vesicles, these two vesicle types share several similarities as well. The similarities include a stimulus dependent exocytosis, dependence on a transient calcium exposure for exocytosis, a unique population of contents destined for secretion, and we now add the similarity that they share several molecules implicated in regulating exocytosis.

A functional contribution of SNARE components in eggs of the sea urchin was previously implicated by the use of neurotoxins. Steinhardt et al. (1994) and Bi et al. (1995) injected *Botulinum* neurotoxins A, and B (BoNT/A, BoNT/B) and tetanus toxin (TeNT) into eggs and early embryos and found that resealing, or healing of membranes damaged by mechanical or laser wounds, was inhibited. Cortical granule exocytosis was also

inhibited when first treated with tetanus toxin. The evidence we present here demonstrates that neurotoxin target sequences are present in the SNARE proteins of these eggs. The VAMP sequence contains a perfect match for the predicted cleavage site of TeNT and BoNT/B, ASQ ↑ FET found in rat VAMP-2. Especially relevant in this sequence is the threonine in the last position, instead of a serine as found in VAMP-1, which renders the protein *insensitive* to both TeNT and BoNT/B. Thus we predict the egg VAMP to be TeNT and BoNT/B *sensitive* and one of the target proteins that participates in membrane resealing seen by Steinhardt et al. (1994). Steinhardt's group also found a *Botulinum* neurotoxin C (BoNT/C) -sensitive target protein that was important for membrane resealing (Bi et al., 1995). The predicted BoNT/C cleavage site, DTKK ↑ AVKY, for syntaxin homologues, e.g., syntaxin 1A and 1B of rat (Schiavo et al., 1995) is also present precisely in sea urchin syntaxin. Taken together, these results show that syntaxin and VAMP sequences are localized to cortical granules and the plasma membrane in sea urchin eggs, and that they are required for cortical granule exocytosis.

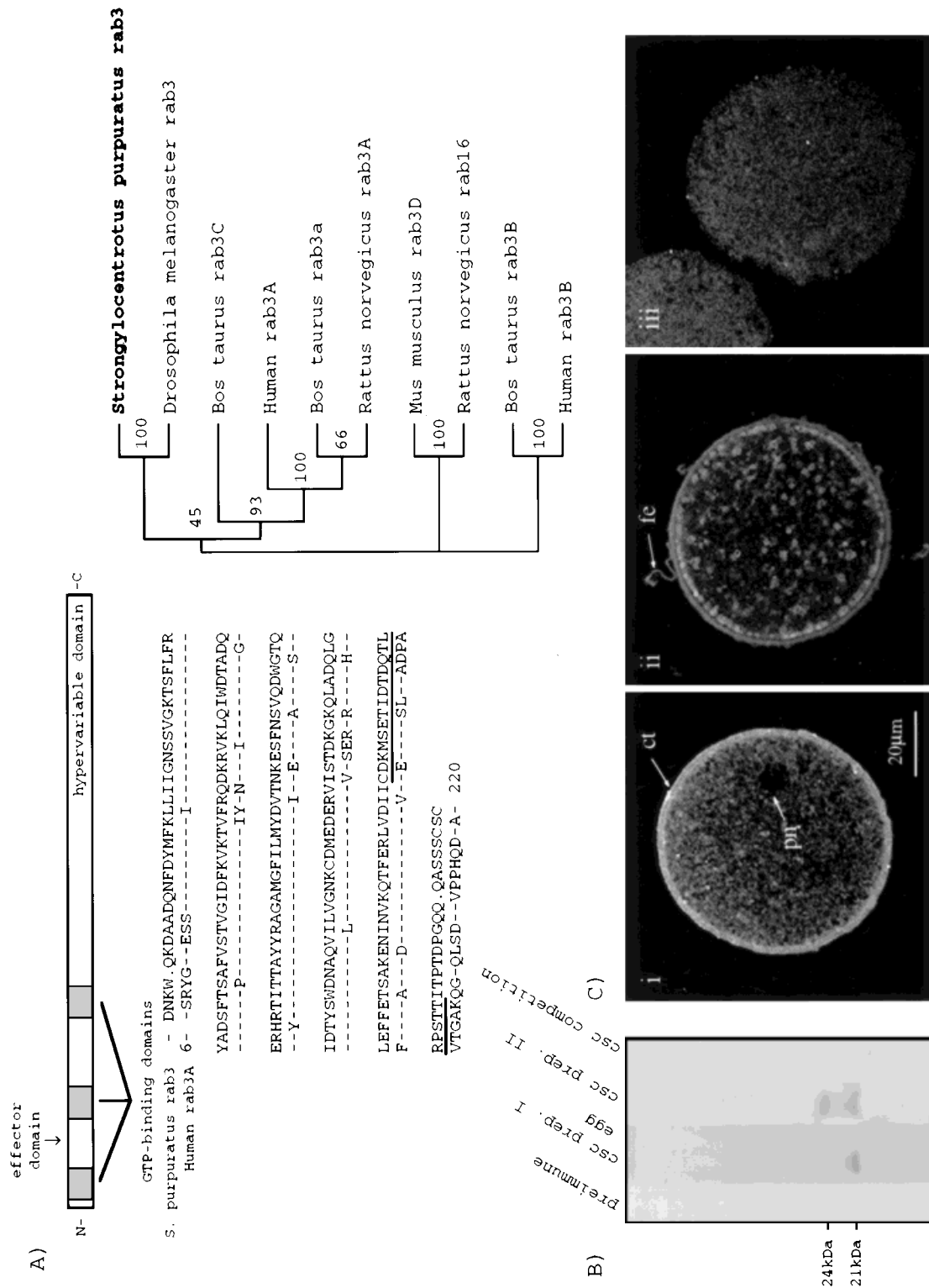
How do the secretory compartments retain their distinct identities? Membrane retrieval must be important for recycling throughout the secretory pathway, since the membrane area of each step remains relatively constant; how this works is unclear. In neurons, synaptic vesicles form from recycled, presynaptic plasma membrane, which would contain a complete complement of SNAREs, but somehow the cell sorts v- and t-SNAREs to retain their targeting functions. Syntaxin 1 retrieval was shown to occur in neurons following the exocytosis of synaptic vesicles (Walch-Solimes et al., 1995). This t-SNARE was recycled in what appeared to be an inactive form, but whether synaptobrevin and synaptotagmin follow similar recycling is unknown. Fertilization presents a different problem. During fertilization in sea urchins, 15,000 cortical granules with a membrane surface area of 47,000  $\mu\text{m}^2$  is added to the existing cell surface of 31,000  $\mu\text{m}^2$ , resulting in a new surface area 250% of the original egg. Recent evidence from Whalley et al. (1995) demonstrated that retrieval of this enlarged plasma membrane begins immediately after fertilization, and continues for 15 minutes post-insemination. At no time during this retrieval step, however, do we detect retrieval of syntaxin, VAMP, or tagmin, as each of the proteins remains on the surface during this retrieval period. Since cortical granules are only used at one time in development, an immediate recycling of SNARE components may not be required. However, this implies that the fertilized egg selectively retains the SNAREs at the plasma membrane and does not use these components for exocytosis of other secretory vesicles that occurs shortly after fertilization (Alliegro et al., 1992). Whether the maternally derived syntaxin, VAMP, and tagmin remain at the plasma membrane through later development is unknown.

Rab3 GTPase, in contrast, can dissociate and reassociate with membranes through a GTP hydrolysis and

GDP exchange process, and does not necessarily require membrane recycling for its reutilization (Pfeffer, 1994). In the fertilized egg, rab3 quickly reassociates with vesicles both near the cell surface and within the cytoplasm. Although we do not know the identity of these putative vesicles associated with the rab3, the size (1.5–2.0  $\mu\text{m}$ ), the abundance, and the distribution are each similar to the endocytic vesicles described by Whalley et al. (1995). Association with an endocytic vesicle, though, would be unique for a rab3 homologue. Alternatively, the rab3 protein may reassociate with any one of several other classes of vesicles that are activated for secretion at fertilization. These include vesicles containing extracellular matrix molecules (Wessel et al., 1984), fibropellins (Bisgrove and Raff, 1993), and others defined by antibodies (reviewed in Alliegro et al., 1992). Since fertilization activates many transport pathways that are stalled in a mature, quiescent egg, rab3 GTPase association with these maternally stored vesicles may be important in the regulation of timing of other exocytotic events in early development.

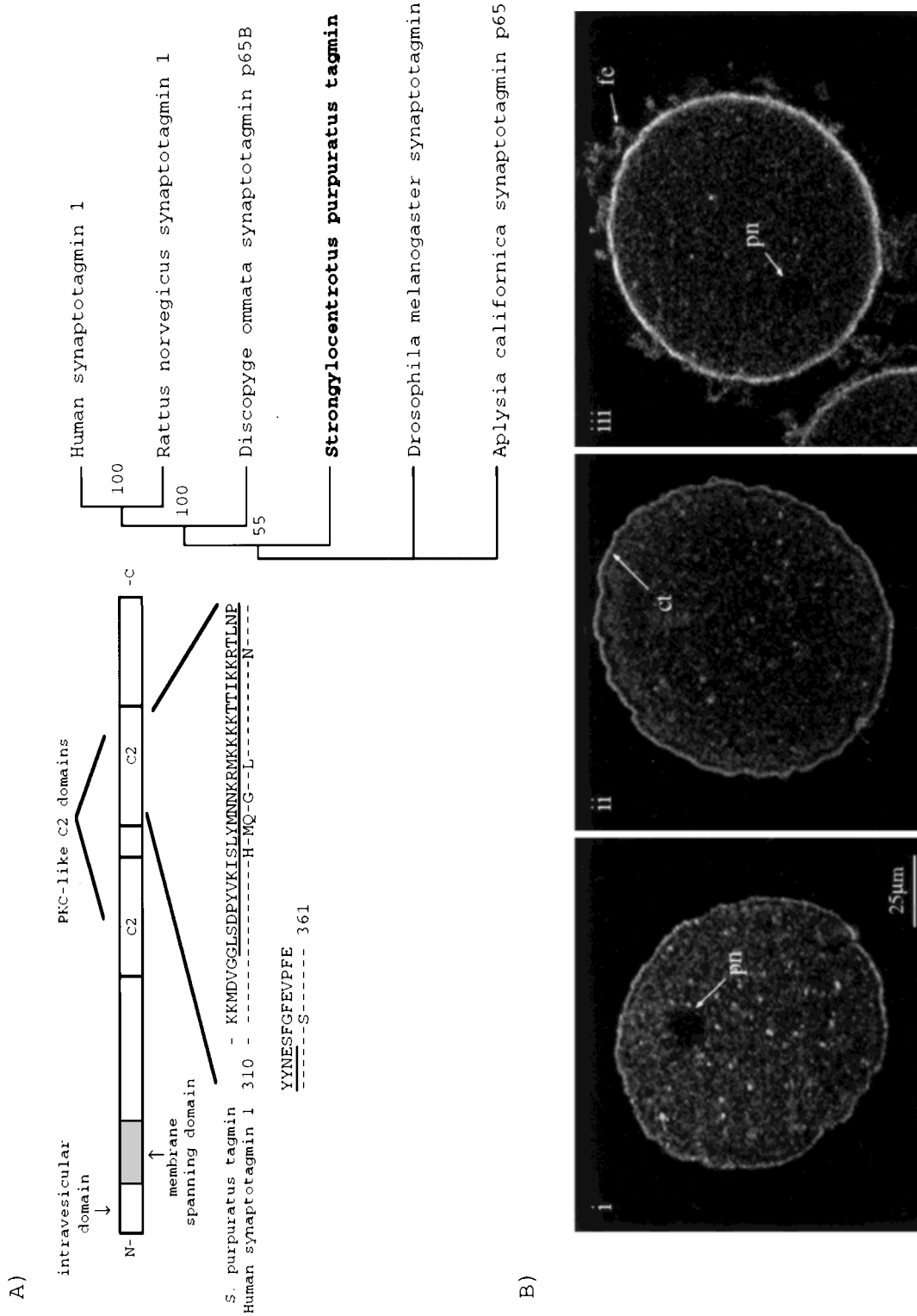
A second consequence of fertilization on the SNAREs identified here is the increased intensity of signal for syntaxin and tagmin by immunofluorescence. We interpret this increased signal as an increased accessibility of the antibodies to the proteins following fertilization, and not to additional protein synthesis, as we see no significant change in the abundance of syntaxin protein by immunoblots (although tagmin has not been detected consistently by immunoblotting). Prior to exocytosis, the SNARE components are believed to be within a tightly aggregated, SDS-insoluble complex (Söllner et al., 1993). Following calcium stimulated exocytosis, however, the SNARE complex dissociates, which may make the individual proteins accessible to immunolabeling. It is significant in this context that the regions of syntaxin and tagmin used to generate antibodies includes those evolutionarily conserved regions that participate in specific protein interactions of the SNARE complex. The notion of inaccessibility of the antibodies to these regions of the protein prior to fertilization is supported by experiments using neurotoxins to inactivate syntaxin prior to exocytosis. *Botulinum* neurotoxin C (BoNT/C) was not effective in cleaving the syntaxin target unless cortical granules were first dislodged from the plasma membrane (Bi et al., 1995).

In conclusion, we demonstrate that SNARE components are in the right place and at the right time to function in the exocytosis of the cortical granules, allowing us to focus on the mechanisms of stimulus dependent exocytosis of these vesicles at fertilization. Although cortical granules have been studied intensively over many years, and many content proteins identified, the results with VAMP and rab3 here provide the first markers for a cortical granule membrane protein. Finally, because oocytes and eggs from sea urchins provide a synchronous system to examine the stepwise progression from granule biogenesis, to docking, and finally secretion, and because each of these steps is temporally and experimentally distinct, this



**Fig. 4.** A rab3 homologue is enriched at the cortex of the egg. **A:** Cartoon representation of the predicted rab3 protein (above) with the deduced partial amino acid sequence and comparison to human rab3 (below), and a cladogram of the rab3 family (right). The partial cDNA sequence begins at residue 6 of human rab3A and ends with the predicted amino acid sequence CXC, which is characteristic of rabs. The underlined amino acid sequence in the hypervariable region represents the peptide used for antibody generation. Numbers located at three branchpoints of cladograms indicate bootstrap values times 10. **B:** A polyclonal antibody to rab3 identifies two proteins of 24 kDa and 21 kDa, that are enriched in the cell surface complex (csc). Both bands are completely competed by preabsorption of the rab3 antibody to the rab3 peptide used as an immunogen. Each lane contains 25 µg of protein. **C:** In situ immunolocalization of rab3 shows an enrichment at the cortex of the egg (i) in association with the cortical granules. At 6 min following fertilization (ii), rab3 signal is redistributed to vesicles larger than cortical granules dispersed throughout the cytoplasm. Competition of the antibody with the peptide used as an immunogen competes completely with signal in egg (iii). Visualization by indirect immunofluorescence using confocal microscopy. ct, cortex; pn, pronucleus; fe, fertilization envelope.

**Fig. 4.** A rab3 homologue is enriched at the cortex of the egg. **A:** Cartoon representation of the predicted rab3 protein (above) with the deduced partial amino acid sequence and comparison to human rab3 (below), and a cladogram of the rab3 family (right). The partial cDNA sequence begins at residue 6 of human rab3A and ends with the predicted amino acid sequence CXC, which is characteristic of rabs. The underlined amino acid sequence in the hypervariable region represents the peptide used for antibody generation. Numbers located at three branchpoints of cladograms indicate bootstrap values times 10. **B:** A polyclonal antibody to rab3 identifies two proteins of 24 kDa and 21 kDa, that are enriched in the cell surface complex (csc). Both bands are completely competed by preabsorption of the rab3 antibody to the rab3 peptide used as an immunogen. Each lane contains 25 µg of protein. **C:** In situ immunolocalization of rab3 shows an enrichment at the cortex of the egg (i) in association with the cortical granules. At 6 min following fertilization (ii), rab3 signal is redistributed to vesicles larger than cortical granules dispersed throughout the cytoplasm. Competition of the antibody with the peptide used as an immunogen competes completely with signal in egg (iii). Visualization by indirect immunofluorescence using confocal microscopy. ct, cortex; pn, pronucleus; fe, fertilization envelope.



**Fig. 5.** Tagmin is present in the cortex of the sea urchin egg. **A:** Cartoon representation of synaptotagmin I from human showing the region of partial cDNA sequence obtained from sea urchin eggs as compared to the human sequence. A cladogram of this sequence with the synaptotagmin family is shown, numbers located at tree branchpoints indicate bootstrap values times 10. **B:** Antibodies generated to the peptide sequence underlined in A were used to localize the tagmin protein by indirect immunofluorescence. Signal is present at the cortex of unfertilized eggs that appears to be associated with the plasma membrane (i, ii), and not surrounding the cortical granules (cf. Fig. 2Ci, VAMP). Following fertilization (iii), tagmin signal in the plasma membrane increases significantly. pn, pronucleus; ct, cortex; fe, fertilization envelope.

system should be useful for contributing to dissection of the steps of SNARE protein function.

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### REFERENCES

- Alliegro MC, Black SD, McClay DR (1992): Deployment of extracellular matrix proteins in sea urchin embryogenesis. *Microsc Res Technique* 22:2-10.
- Bennett MK (1995): SNAREs and the specificity of transport vesicle targeting. *Curr Opin Cell Biol* 7:581-586.
- Bennett MK, Calakos N, Scheller RH (1992): Syntaxin: A synaptic protein implicated in the docking of synaptic vesicles at presynaptic active zones. *Science* 257:255-259.
- Bi G-Q, Alderton JM, Steinhart RA (1995): Calcium-regulated exocytosis is required for cell membrane resealing. *J Cell Biol* 131:1747-1758.
- Bisgrove BW, Raff RA (1993): The SpEGF III gene encodes a member of the fibropellins: EGF-repeat containing proteins that form the apical lamina of the sea urchin embryo. *Dev Biol* 157:526-538.
- Bommert K, Charlton MP, DeBello WM, Chin GJ, Betz H, Augustine GJ (1993): Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* 363:163-165.
- Chapman ER, An S, Edwardson JM, Jahn R (1996): A novel function for the second C2 domain of synaptotagmin. *J Biol Chem* 271:5884-5889.
- Devereux H, Haerberli S, Smithies O (1984): A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 12:387-395.
- Ducibella T, Duffy P, Buetow J (1994): Quantification and localization of cortical granules during oogenesis in the mouse. *Biol Reprod* 50:467-473.
- Feinberg A, Vogelstein B (1983): A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6-13.
- Foltz KR (1995): Sperm-binding proteins. *Int Rev Cytol* 163:249-303.
- Geppert M, Archer BT, Südhof TC (1991): Synaptotagmin II. A novel differentially distributed form of synaptotagmin. *J Biol Chem* 266:13548-13552.
- Grote E, Hao JC, Bennett MK, Kelly RB (1995): A targeting signal in VAMP regulating transport to synaptic vesicles. *Cell* 81:581-589.
- Harlow E, Lane D (1988): "Antibodies: A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Higgins DG, Bleasby AJ, Fuchs R (1991): CLUSTAL V: Improved software for multiple sequence alignment. *CABIOS* 8:189-191.
- Hoodbhoy T, Talbot P (1994): Mammalian cortical granules: Contents, fate, and function. *Mol Rep Dev* 39:439-448.
- Ikonen E, Tagaya M, Ullrich O, Montecucco O, Simons K (1995): Different requirements for NSF, SNAP, and Rab proteins in apical and basolateral transport in MDCK cells. *Cell* 81:571-580.
- Kee Y, Scheller RH (1996): Localization of synaptotagmin-binding domains on syntaxin. *J Neurosci* 15:1975-1981.
- Kee Y, Lin RC, Hsu SC, Scheller RH (1995): Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron* 14:991-998.
- Kinsey WH (1986): Purification and properties of the egg plasma membrane. In Schroeder, T.E. (ed.): "Methods in Cell Biology." San Diego: Academic Press, pp. 139-152.
- Lacy P, Thompson N, Tian M, Solari R, Hide I, Newman TM, Gomperts BD (1995): A survey of GTP-binding proteins and other potential key regulators of exocytotic secretion in eosinophils. Apparent absence of rab3 and vesicle fusion protein homologues. *J Cell Sci* 108:3547-3556.
- Laidlaw M, Wessel GM (1994): Cortical granule biogenesis is active throughout oogenesis in sea urchins. *Development* 120:1325-1333.
- Leaf D, Blum G (1997): Cloning and localization of a sea urchin rab10 GTPase homologue. (in press.)
- Lindsay L, Hedrick JL (1995): Isolation and characterization of oochymase, a chymotrypsin-like protease released during *Xenopus laevis* egg activation. *Dev Biol* 167:513-516.
- McClay DR (1986): Embryo dissociation, cell isolation, and cell reassociation. In Schroeder, T.E. (ed.): "Methods in Cell Biology." San Diego: Academic Press, pp 304-323.
- McMahon HT, Ushkaryov YA, Edelman L, Link E, Binz T, Niemann H, Jahn R, Südhof TC (1993): Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* 364:346-349.
- Moller CC, Wassarman PM (1989): Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. *Dev Biol* 132:103-112.
- Nonet ML, Grundahl K, Meyer BJ, Rand JB (1993): Synaptic function is impaired but not eliminated in *C. elegans* mutants lacking synaptotagmin. *Cell* 73:1291-1305.
- Perin MS, Fried VA, Mignery GA, Jahn R, Südhof TC (1990): Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* 345:260-263.
- Perin MS, Johnston PA, Ozcelik T, Jahn R, Südhof TC (1991): Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. *J Biol Chem* 266:615-622.
- Pfeffer SR (1994): Rab GTPases: Master regulators of membrane trafficking. *Curr Opin Cell Biol* 6:522-526.
- Rothman JE, Warren G (1994): Implication of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr Biol* 4:220-233.
- Rybin V, Ullrich O, Rubino M, Alexandrov K, Simon I, Seabra C, Goody R, Zerial M (1996): GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. *Nature* 383:266-269.
- Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463-5467.
- Schuel H (1985): Secretory functions of egg cortical granules. In Metz, C.B., and Monroy, A. (eds.): "Biology of Fertilization." Vol. 3. San Diego: Academic Press, pp 1-44.
- Schulze KL, Broadie K, Perin MS, Bellen HJ (1995): Genetic and electrophysiological studies of *Drosophila* syntaxin-1A demonstrate its role in nonneuronal secretion and neurotransmission. *Cell* 80:311-320.
- Schiavo G, Shone CC, Bennett MK, Scheller RH, Montecucco C (1995): Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J Biol Chem* 270:10566-10570.
- Shapiro BM, Somers C, Weidman PJ (1989): Extracellular remodeling during fertilization. In Schatten, H., and Schatten, G. (eds.): "Cell Biology of Fertilization." San Diego: Academic Press, pp 251-276.
- Shen SS (1995): Mechanisms of calcium regulation in sea urchin eggs and their activities during fertilization. In Pederson, R.A., and Schatten, G.P. (eds.): "Current Topics in Developmental Biology." Orlando, FL: Academic Press, Vol. 30, pp 63-101.
- Short JM, Fernandez JM, Sorge JA, Huse WD (1988): Lambda ZAP: A bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res* 16:7583-7600.
- Söllner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman J (1993): A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75:409-418.
- Steinhart RA, Bi G, Alderton JM (1994): Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science* 263:390-393.
- Südhof TC (1995): The synaptic vesicle cycle: A cascade of protein-protein interactions. *Nature* 375:645-653.
- Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350-4354.

- Trimble WS, Cowan DM, Scheller RH (1988): VAMP-1: A synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci USA* 85:4538-4542.
- Walch-Solimena C, Blasi J, Edelmann L, Chapman ER, Fischer von Mollard G, Jahn R (1995): The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J Cell Biol* 128:637-645.
- Wessel GM (1997): Biogenesis and function of cortical granules in the sea urchin egg. *Prog Dev Biol* (in press).
- Wessel GM, Marchase RB, McClay DR (1984): Ontogeny of the basal lamina in the sea urchin embryo. *Dev Biol* 103:235-245.
- Whalley T, Terasaki M, Cho M-S, Vogel SS (1995): Direct membrane retrieval into large vesicles after exocytosis in sea urchin eggs. *J Cell Biol* 131:1183-1192.
- Whitaker M, Swann K (1993): Lighting the fuse at fertilization. *Development* 117:1-12.
- Yu H, Leaf DS, Moore H-PH (1993): Gene cloning and characterization of a GTP-binding Rab protein from mouse pituitary AtT-20 cells. *Gene* 132:273-278.
- Zerial M, Huber LA (1995): "Guidebook to Small GTPases." Oxford: Oxford University Press.