During exocytosis in the pancreatic acinar cell, zymogen granules fuse directly with the apical plasma membrane and also with granules that have themselves fused with the plasma membrane. Together, these primary and secondary fusion events constitute the process of compound exocytosis. It has been suggested that the sequential nature of primary and secondary fusion is a consequence of the requirement for plasma membrane soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors, such as syntaxin 2, to enter the membrane of the primary fused granule. We have tested this possibility by determining the location of syntaxin 2 in unstimulated and stimulated pancreatic acini. Syntaxin 2 was imaged by confocal immunofluorescence microscopy. Fused granules were detected both through their filling with the aqueous dye lysine-fixable Texas Red-dextran and through the decoration of their cytoplasmic surfaces with filamentous actin. In unstimulated cells, syntaxin 2 was exclusively present on the apical plasma membrane. In contrast, after stimulation, syntaxin 2 had moved into the membranes of fused granules, as judged by its location around dye-filled structures of 1-μm diameter that were coated with filamentous actin. At long times of stimulation (5 min), the majority (85%) of dye-filled granules were also positive for syntaxin 2. In contrast, at shorter times (1 min), more dye-filled granules (20%) were syntaxin 2-negative. We conclude that syntaxin 2 enters the membrane of a fused zymogen granule after the opening of the fusion pore, and we suggest that this movement might permit the onset of secondary fusion.

Regulated exocytosis in the pancreatic acinar cell, in response to a rise in intracellular Ca$^{2+}$ concentration (1), involves the fusion of the membranes of zymogen granules both with the apical plasma membrane and with each other (2–5). This latter process, known as sequential or compound exocytosis, occurs in only a few cell types and, in the case of the acinar cell, is likely to be an adaptation to increase the efficiency of digestive enzyme secretion at the spatially restricted apical pole of the cell.

It is now accepted that membrane fusion events occurring along the secretory pathway are mediated by trans-membrane complexes of soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins (6). The SNARE complex consists of four intertwined amphipathic α-helices (7). The SNAREs of one membrane contribute three helices, each of which contains a strategically located glutamine residue; hence, such SNAREs have been termed Q-SNAREs (e.g. syntaxin and 25-kDa synaptosome-associated protein (SNAP-25)). The fourth helix is provided by the SNARE present on the apposing membrane and contains a similarly placed arginine residue, hence the name R-SNARE (e.g. synaptobrevin). Exocytotic membrane fusion in the acinar cell is known to be SNARE-dependent (8–10). Syntaxin 2, the Q-SNARE present on the apical plasma membrane (8, 9), is required for granule-plasma membrane fusion in vitro (9). In contrast, syntaxin 4, located on the basolateral plasma membrane (8), is likely to be involved in basolaterally directed membrane traffic. Syntaxins 3, 7, and 8 are all present on the granule membrane (8–10), although their roles are still unclear. Surprisingly, SNAP-23, the non-neuronal isoform of SNAP-25, seems to be located on the basolateral plasma membrane (11) and on the granule membrane (10). Both endobrevin (VAMP 8) and synaptobrevin 2 are present on the granule membrane (9, 10). The phenotype of an endobrevin knockout mouse indicates that this protein acts as the major R-SNARE for zymogen granule exocytosis (10). In contrast, synaptobrevin 2 seems to play only a minor role in granule exocytosis (9).

The exocytotic process in the mouse exocrine pancreas has recently been studied in real time in isolated living acini using two-photon excitation imaging (4, 5). In these experiments, a membrane-impermeant fluorescent dye is added to the bathing medium, and the cells are imaged during stimulation with an appropriate secretagogue, such as acetylcholine or carbachol. Shortly after agonist application, Ω-shaped fluorescent spots begin to appear in the subapical region, which have an approximate diameter of 1 μm, consistent with the filling of zymogen granules with dye through the open fusion pore (4, 5). These dye-filled granules are visible at the apical membrane for several minutes, indicating that the fusion pore remains open for extended periods (5). Two types of fusion events can be distinguished: those in which the granule fuses directly with the plasma membrane (primary fusion event) and those in which a granule lying deeper within the cell fuses with a granule that has itself already opened to the exterior (secondary fusion event). Analysis reveals that there are approximately equal numbers of primary and secondary fusion events (4). During the exocytotic response of a pancreatic acinar cell, granules apparently fuse together only when the first granule has made contact with glutamine; SNAP, synaptosome-associated protein; LF, lysine-fixable; F-actin, filamentous actin; GST, glutathione-S-transferase; PBS, phosphate-buffered saline.

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1 The abbreviations used are: SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; Q-SNARE, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor with glutamine; SNAP, synaptosome-associated protein; LF, lysine-fixable; F-actin, filamentous actin; GST, glutathione-S-transferase; PBS, phosphate-buffered saline.
contact with the plasma membrane, (4, 5), with a mean time
delay between primary and secondary events of 10–20 s (4, 5).
To account for this delay, it has been suggested that secondary
fusion requires the recruitment of components of the fusion
machinery from the plasma membrane (4). This might, for
example, involve the migration of syntaxin 2 into the mem-
brane of the fused granule, although this would raise the issue
of the role of syntaxins 3, 7, and 8, which are already present on
the granule membrane (8–10).
In the present study, we sought to determine whether the
Q-SNARE syntaxin 2, initially present on the apical plasma
membrane, migrates into the membranes of the zymogen gran-
ules as they undergo exocytotic membrane fusion. The answer
to this question will provide important information about the
nature of the fusion pore formed between the granule and
plasma membranes and also about the mechanism underlying
compound exocytosis.

EXPERIMENTAL PROCEDURES

Reagents—A rabbit polyclonal antiserum raised against recombinant rat syntaxin 2 was provided by Dr. R. Jahn (Max Planck Institute for
Biophysical Chemistry, Göttingen, Germany). Alex 488-phalloidin, lys-
sine-fixable (LF) Texas Red-dextran (3,000 Da), fluorescein isothiocya-
nate- and Cy3-conjugated goat anti-rabbit antibodies were from Mole-
cular Probes (Eugene, OR). All other reagents were from Sigma.

Preparation of Recombinant Syntaxins—Full-length syntaxin 2 and
the cytoplasmic domains of syntaxins 7 and 8 were expressed in the
vector pGEX-4T (Amersham Biosciences). Full-length syntaxins 1, 3,
and 4 were expressed in pGEX-2T. Syntaxins tagged with glutathione
S-transferase (GST) were purified from bacterial lysates by binding to
glutathione-Sepharose (Pharmacia). Bead slurries were suspended in
sample buffer and heated to 95 °C to release the GST-syntaxins for
analysis by SDS-PAGE.
Preparation of Rat Pancreatic Zymogen Granules—Zymogen gran-
ules and plasma membranes were isolated from rat pancreas as de-
scribed previously (12). All buffers contained 1 mM EDTA and a prote-
ase inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml
depstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 10 μg/ml soybean tryp-
sin inhibitor, 17 μg/ml benzamidine, and 50 μg/ml bacitracin). Granules
were lysed by incubation for 1 h at 4 °C in 170 mM NaCl and 200 mM
NaHCO3 (pH 7.8; 1:3) to produce granule membranes.

Electrophoresis and Immunoblotting—Proteins were separated by
SDS-PAGE and then electrophotorethetically transferred to nitrocellulose
(Schleicher and Schüll) by semidry blotting. Blots were probed with
primary antibodies at a dilution of 1:1000. Immunoreactive bands were visualized using hors eradish pero xide-conjugated secondary antibod-
yes (1:1000) and enhanced chemiluminescence (Pierce and Warriner).

Preparation of Mouse Pancreatic Acini—Mouse pancreatic acini were prepared essentially as described previously (13). A 5–8-week-old mouse was killed by
exposure to a rising concentration of CO2, followed by cervical disloca-
tion. The pancreas was excised and immediately transferred to ice-cold
extracellular medium (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM
glucose, 2 mM CaCl2, and 10 mM HEPES, pH 7.7). Collagenase (1000
units, type A, in 5 ml of extracellular medium; Sigma) was injected
into the pancreas and the tissue was incubated at 37 °C for 7 min. The
supernatant was discarded and the pancreas was washed twice in
extracellular medium, followed by trituration with a large-bore plastic
pipette. Once the cell suspension flowed freely, trituration was carried out
twice more with pipettes with progressively narrower bores. Cells were
pelleted from the suspension by centrifugation at 1000 × g for 1 min.
The pellet was resuspended in 10 ml of extracellular medium and the acini were gently washed twice in the same medium. Cells were
kept at room temperature for 10 min before 100-μl samples were plated
onto poly-L-lysine (0.1%) pre-treated coverslips.

Confocal Immunofluorescence Microscopy—All steps were carried out at
room temperature, except where otherwise indicated. Acini attached to
coverslips were used either unstimulated or after stimulation with 2 μM
dipalmitoylphosphatidylcholine for 5 min. When appropriate, the fluid phase
countermarker LF Texas Red-dextran (Molecular Probes; 1 mg/ml) was added
5 min before acetylecholine stimulation. After incubation with acetylecho-
line, the cells were washed twice with 2 ml of phosphate-buffered saline
(PBS; 10 mM sodium phosphate, pH 7.4, and 150 mM NaCl) for 10 min
each time and then fixed in 4% (w/v) paraformaldehyde in sucrose/
phosphate buffer (210 mM sucrose and 30 mM sodium phosphate, pH
6.8), for 30 min. For studies using LIF Texas Red-dextran, the initial
washes were omitted, and the cells were washed three times in 4%
parafomaldehyde over 30 min. After fixation, the cells were washed
twice for 5 min in PBS, twice for 5 min in high-salt PBS (20 mM sodium
phosphate, pH 7.4, and 500 mM NaCl), and then incubated for 15 min in
permeabilizing buffer (PBS containing 0.1% (w/v) Triton X-100). Cells
were washed three times for 5 min in permeabilizing buffer containing
5% (w/v) goat serum (blocking buffer) and then incubated with primary
antibody overnight at 4 °C on Parafilm. The cells were washed twice
with high-salt PBS for 5 min and once for 5 min in blocker, before
incubation with secondary antibody for 90 min. Cells were washed twice
with high-salt PBS, once with PBS, and finally once with low-salt PBS (10
mM sodium phosphate, pH 7.4, and 5 mM NaCl). The
coverslips were mounted on glass slides in Vectashield mounting me-
dium (Vector Laboratories, Burlingame, CA), sealed with nail varnish,
and stored in the dark at 4 °C.

Specimens were imaged using a Zeiss 100 M Axioscope confocal laser
scanning microscope, with a 63× objective lens of numeric aperture 1.3,
capturing an optical slice of ~1 μm. Images were collected with the
appropriate filters: fluorescein isothiocyanate and Alexa Fluor 488-
phalloidin were excited using the 488-nm line of a krypton/argon laser
and imaged with a 505–550 band-pass filter. Cy3 and LF Texas Red-
dextran were excited with a 543 nm line of a Helium/Neon laser
and collected with a long-pass 560 nm filter. All images were captured using
Zeiss LSM 510 software. Images were thresholded to background signals.
Using this mode, cross-talk was estimated to be less than 2%.
Analysis was performed using LSM 510 and ImageJ software.
The statistical significance of differences between means was assessed using
Student’s t test for unpaired data.

RESULTS
Because the pancreatic acinar cell is known to contain syn-
taxins isoforms 2, 3, 4, 7, and 8 (8–10), it was important to
establish that the antibody we intended to use to detect syn-
taxin 2 was specific for that isoform. To this end, we prepared
these isoforms (along with syntaxin 1, which is not expressed in
the acinar cell) as GST fusion proteins, and tested the ability of
the anti-syntaxin 2 antibody to discriminate between them.
Syntaxins 1–4 were full-length proteins, whereas syntaxins 7
and 8 were cytoplasmic domains. The Coomassie blue-stained
gels shown in Fig. 1A demonstrate the approximately equal
loading of the various syntaxin isoforms. The major bands seen
on the gels migrate at positions appropriate to the combined
molecular masses of syntaxin (28–35 kDa) and GST (25 kDa).
The anti-syntaxin 2 antibody was a rabbit polyclonal raised
against the entire cytoplasmic domain and has been used pre-
viously (9). As shown in Fig. 1A, the antibody gave a strong
signal when blotted against GST-syntaxin 2 but did not detect
syntaxins 1, 3, 4, 7, or 8. Fig. 1B shows a Coomassie blue-
stained gel of plasma membrane and zymogen granule mem-
brane fractions isolated from rat pancreas. As expected from
previous results (9), the anti-syntaxin 2 antibody detected a
35-kDa band in the plasma membrane fraction but not in
the zymogen granule membrane fraction.

We used confocal immunofluorescence microscopy with the
anti-syntaxin 2 antibody to locate syntaxin 2 within mouse
pancreatic acini. As shown in Fig. 2A, syntaxin 2 was exclu-
sively localized to the apical membranes of the acinar cells (red
staining). The figure also shows the location of filamentous
actin (F-actin), as detected using Alexa 488-phalloidin (green
staining). F-actin is known to be concentrated at the apical pole
of the acinar cell, just beneath the apical membrane (14–16). In
the set of magnified images shown in Fig. 2B syntaxin 2 is
clearly concentrated inside the “tram-line” arrangement of F-
actin, consistent with its location on the plasma membrane.

To follow exocytosis in the acini, we used LF Texas Red-
dextran (3,000 Da), an extracellular dye. As explained above,
the dye will enter a fusing granule through the fusion pore,
enabling the detection of single exocytotic fusion events. As
shown in Fig. 3A, unstimulated acini showed little staining for LF Texas Red-dextran, although some dye entered the spaces between the cells (compare the red signal with the typical tramline appearance of F-actin beneath the apical membrane). In contrast, after a 5-min stimulation of the acini with acetylcholine (2 μM), the LF Texas Red-dextran could be seen in clusters of granular structures close to the apical membranes (arrowheads, Fig. 3B). The diameter of these features (~1 μm) is consistent with the filling of fused zymogen granules with the extracellular dye. Stimulation of the acini also caused a dramatic remodeling of the actin-based cytoskeleton, so that the fused granules became surrounded by actin cytoskeleton (arrows). Two distinct types of fusion event can be observed (C): granule-plasma membrane fusion (arrowhead) and granule-granule fusion (arrow). Scale bars, A and B, 10 μm; C, 5 μm.

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**Fig. 1. Immunoblot analysis of syntaxin 2 in the pancreatic acinar cell.** A, specificity of the anti-syntaxin antibody. Recombinant GST-tagged full-length syntaxins 1–4, and GST-tagged cytoplasmic domains of syntaxins 7 and 8 (~0.5 μg of protein), were analyzed by SDS-PAGE. Coomassie blue-stained gels (top) show the protein loading. Proteins were electrophoretically transferred to nitrocellulose and probed with an antibody against syntaxin 2, followed by a horseradish peroxidase-conjugated secondary antibody (bottom). Immunoreactive bands were visualized by enhanced chemiluminescence. The positions of molecular mass markers (kilodaltons) are indicated on the right. B, presence of syntaxin 2 in the plasma membrane but not the zymogen granule membrane. Purified plasma membrane and zymogen granule membrane proteins (20 μg) were analyzed by SDS-PAGE. A Coomassie blue-stained gel (top) shows the protein patterns of the two membranes. The positions of molecular mass markers (kilodaltons) are indicated on the right. Proteins were transferred to nitrocellulose and probed with anti-syntaxin 2 antibody (bottom).

**Fig. 2. Localization of syntaxin 2 and F-actin in unstimulated acini.** Unstimulated acini were fixed, permeabilized, and incubated with anti-syntaxin 2 antibody (1:30 dilution). Cells were then incubated with Cy-3 conjugated anti-rabbit IgG (1:500) and Alexa 488-phalloidin (1:100). Note the “tram-line” appearance of the syntaxin 2 staining (arrowheads in B, middle). Scale bars, A, 10 μm; B, 5 μm.

**Fig. 3. Visualization of exocytosis in pancreatic acini.** Acini were attached to glass coverslips and either left unstimulated (A) or stimulated with 2 μM ACh for 5 min (B and C). For both conditions, LF Texas Red-dextran (1 mg/ml) was added to the extracellular medium before incubation. Acini were fixed with 4% paraformaldehyde, permeabilized, and incubated with Alexa 488-phalloidin (1:100). In unstimulated cells (A), F-actin was localized beneath the apical plasma membrane, and there was little LF Texas Red-dextran labeling. In stimulated cells (B), LF Texas Red-dextran labeled granules that had fused with the apical plasma membrane (arrowheads). These fused granules were also surrounded by actin cytoskeleton (arrow). Two distinct types of fusion event can be observed (C): granule-plasma membrane fusion (arrowhead) and granule-granule fusion (arrow). Scale bars, A and B, 10 μm; C, 5 μm.
...granules that were filled with LF Texas Red-dextran (1 mg/ml) for 5 min before fixation. Cells were then permeabilized and incubated with anti-syntaxin 2 antibody (1:30), followed by fluorescein isothiocyanate-conjugated secondary antibody (1:30). Two representative images are shown. Note the “tram-line” appearance of the syntaxin 2 staining (bottom left). Scale bar, 10 μm.

We next examined the location of syntaxin 2 before and after stimulation of exocytosis. As shown in Fig. 4, syntaxin 2 lined the apical membrane of unstimulated acinar cells. (Note the “tram-line” appearance of the staining, indicated by the arrowhead). The LF Texas Red-dextran had moved into the narrow spaces between the cells and gave a very similar signal to that of syntaxin 2. After stimulation (Fig. 5), the pattern of staining was dramatically different. Granules stained with LF Texas Red-dextran were seen as before, with the majority of granules now also stained for syntaxin 2 (Fig. 5A, top, arrowhead). Quantitation of LF Texas Red-dextran and syntaxin 2 staining in 11 images showed that after a 5-min stimulation there were 9.6 ± 0.7 (n = 38 cells) LF Texas Red-dextran positive exocytotic events per acinar cell, of which 8.3 ± 0.6 (i.e. 85 ± 2%) also stained for syntaxin 2. In many instances, the syntaxin 2 staining patterns were clearly circular, and surrounded the central red staining of the LF Texas Red-dextran (Fig. 5A, bottom). In a control experiment (data not shown), we found that the migration of syntaxin 2 into the granule membranes did not require the presence of LF Texas Red-dextran. Further, a pre-immune serum, taken from the same rabbit used to generate the anti-syntaxin 2 antibodies, gave no specific staining (data not shown).

When acini were stimulated with acetylcholine for 5 min, 15% of the granules that were filled with LF Texas Red-dextran were not stained for syntaxin 2 (e.g., Fig. 5A, arrow), and these granules were always terminal events. Because a finite time will be required for syntaxin 2 to diffuse around the open fusion pore into the granule membrane, these features may represent cases where fixation has “frozen” a granule after fusion pore opening but before significant syntaxin 2 migration. After 5 min of stimulation, the peak of the exocytotic burst is virtually complete (17). To study an earlier phase of the exocytotic response, we imaged cells that had been fixed after a 1-min stimulation, when exocytosis is occurring robustly (17). Analysis of 10 images taken at this time showed that there were 5.1 ± 0.5 (n = 33 cells) LF Texas Red/dextran-positive exocytotic events per acinar cell, of which 3.7 ± 0.5 (i.e. 71 ± 3%) also stained for syntaxin 2. Hence, the number of granules that had fused with the plasma membrane was significantly smaller after 1 min of stimulation than at 5 min (p < 0.001); of these fused granules, a significantly smaller proportion had taken up syntaxin 2 (p < 0.001). Fig. 5B shows an image taken after a 1-min stimulation. It can be seen that, compared with Fig. 5A, the number of granules filled with LF Texas Red-dextran is small; the fused granules line the apical membrane, and there are no detectable secondary fusion events. Further, although some of the fused granules stain positively for syntaxin 2 (arrowhead), others do not (arrows). This result supports the suggestion made above that the movement of syntaxin 2 into the membrane of fused granules lags behind the opening of the fusion pore; this entry of syntaxin 2 into that granule membrane may permit the secondary fusion seen so clearly at later times during the response (Fig. 5A).

In a final experiment, we compared the staining pattern of syntaxin 2 in stimulated acini with that of F-actin, visualized using Alexa 488-phalloidin. As shown in Fig. 6A, circular features were visible near the apical plasma membrane in both the red channel (syntaxin 2) and the green channel (F-actin). At higher magnification, it is clear that the red labeling is inside the green labeling (Fig. 6B, arrowheads). This relationship between the two staining patterns is consistent with the presence of syntaxin 2 on the cytoplasmic surface of the granules, and the coating of the surfaces of fused granules with F-actin.

DISCUSSION

Herein, we present evidence that in mouse pancreatic acinar cells the plasma membrane Q-SNARE syntaxin 2 moves into the granule membrane on cell stimulation. At rest, syntaxin 2 is present in the apical domain of the acinar cells in a band peripheral to F-actin, consistent with its localization on the apical plasma membrane. After cell stimulation, syntaxin 2 is observed in regions around zymogen granules that have undergone exocytosis, as marked with the aqueous dye LF Texas Red-dextran. We suggest that this movement of syntaxin 2 may play a role in the control of secondary exocytotic events during compound exocytosis.

Previous work in pancreatic acinar cells provides abundant...
movement of the Q-SNARE SNAP-23 (22) into the secretory granule apparently precedes exocytosis. SNAP-23 is therefore not simply diffusing from the plasma membrane into the granule membrane but rather is actively transported.

The nature of the fusion pore in pancreatic acinar cells is unknown. Because granule contents consist of digestive enzymes (i.e., proteins), the fusion pore must have a diameter of several nanometers, and there is evidence from AFM imaging of live acinar cells that the pores are in fact as large as 100–180 nm (23). In contrast, the fusion pores in neurons and neuroendocrine cells have diameters of less than 1 nm (24). Recently, it has been reported that the fusion pore in PC-12 cells is lined by the transmembrane segments of five to eight syntaxin molecules (25). However, it seems unlikely that the much larger fusion pores in the acinar cell are similarly lined by a ring of proteins, which would act as a physical barrier to mixing between the granule and plasma membranes and also prevent the collapse of the granule membrane into the target membrane after fusion. If movement of lipids and/or proteins between the two membranes is in some way controlled, this might instead depend on features of the membranes themselves that render them immiscible or on the operation of a scaffold on the cytoplasmic surface of the granule (possibly involving F-actin) that acts as a molecular “picket fence” (26).

Perhaps the most intriguing possibility is that the differences between our results and those of Thorn et al. (5) arise from the use different types of stimulation. In this study, we intentionally used maximal agonist concentrations, bath-applied for long periods, to elicit a large response. In contrast, Thorn et al. (5) examined single-granule exocytosis, which was either spontaneous or induced by the focal uncaging of caged carbachol, a stimulus that is small and transient. Under these conditions, compound exocytosis was observed only rarely. It is therefore possible that single vesicle exocytotic events preserve granule integrity, whereas loss of granule integrity and movement of SNAREs is a requirement to trigger compound exocytosis. To test this possibility, we would need to extend the present findings and follow the movement of SNAREs in living cells, where we can track the progress of single granules and determine whether or not compound exocytosis occurs. To do this, however, we would have to generate a syntaxin 2 construct labeled (e.g., with green fluorescent protein) so as to minimize effects on the properties of the protein and to express this construct efficiently in acinar cells, which are notoriously difficult to transduce, even using adenoviral expression systems (27). The “ideal” experiment, therefore, presents enormous technical obstacles.

In conclusion, we have demonstrated the movement of syntaxin 2 into the membrane of the fusing zymogen granule during exocytosis. Although we have not shown that this movement is necessary for the triggering of secondary granule fusion, the apparent lag between the opening of the fusion pore (allowing the entry of LF Texas Red-dextran) and the redistribution of syntaxin 2 is certainly consistent with this notion.

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