Calcium-dependent synaptic vesicle exocytosis requires three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins: synaptobrevin/vesicle-associated membrane protein in the vesicular membrane and syntaxin and SNAP-25 in the presynaptic membrane. The SNAREs form a thermodynamically stable complex that is believed to drive fusion of vesicular and presynaptic membranes. Complexin, also known as synaphin, is a neuronal cytosolic protein that acts as a positive regulator of synaptic vesicle exocytosis. Complexin binds selectively to the neuronal SNARE complex, but how this promotes exocytosis remains unknown. Here we used purified full-length and truncated SNARE proteins and a gel shift assay to show that the action of complexin on SNARE complex depends strictly on the transmembrane regions of syntaxin and synaptobrevin. By means of a preparative immunoaffinity procedure to achieve total extraction of SNARE complex from brain, we demonstrated that complexin is the only neuronal protein that tightly associates with it. Our data indicated that, in the presence of complexin, the neuronal SNARE proteins assemble directly into a complex in which the transmembrane regions interact. We propose that complexin facilitates neuronal exocytosis by promoting interaction between the complementary syntaxin and synaptobrevin transmembrane regions that reside in opposing membranes prior to fusion.

Membrane fusion requires three proteins, syntaxin, SNAP-25, and synaptobrevin, known as SNAREs. All three SNAREs are molecular targets for clostridial proteolytic toxins that potently block release of neurotransmitters. Syntaxin and SNAP-25 reside in the plasma membrane, whereas synaptobrevin is present on the synaptic vesicle membrane. Whereas SNAP-25 associates with the plasma membrane via its palmitate chains, syntaxin and synaptobrevin each have one transmembrane region (TMR). Formation of SNARE complex, composed of the cytoplasmic parts of all three SNAREs, is followed by the interaction of the complementary TMRs of syntaxin and synaptobrevin, finally mixing the opposing membranes and causing them to fuse. SNARE-mediated synaptic vesicle exocytosis is a highly regulated event that is under the control of a number of cytosolic proteins. Among these, complexin is unique in that it is exclusively present in neuronal cells. Gene knockout studies in mice and peptide injection studies in squid nerve terminals demonstrated that complexin positively regulates a late step in calcium-triggered exocytosis, which, according to current understanding, is the process of the four-helix bundle made of syntaxin, SNAP-25, and synaptobrevin. Interestingly, a number of human neurological diseases have also been linked to deficiency in complexin.

Complexin is a small highly charged cytosolic protein that interacts selectively with the ternary SNARE complex but not with monomeric SNARE proteins. X-ray structures demonstrated that complexin binds to the groove between the synaptobrevin and syntaxin helices. EPR and CD spectroscopy experiments revealed that there are no major structural changes in the cytoplasmic domain of SNARE complex upon complexin binding, whereas an NMR study suggested that complexin stabilizes the C-terminal part of the SNARE complex. Although complexin was proposed to promote assembly and oligomerization of SNARE complex, subsequent studies questioned this hypothesis. Also, complexin was thought to block binding of SNAPs to SNARE complex, but NSF/SNAP-driven disassembly of SNARE complex proceeds whether or not complexin is bound.

Thus, although the physiological importance of complexin has been established in vivo and its binding site has been defined by structural, biophysical and biochemical studies, the molecular link between the binding of complexin and its positive physiological action remains to be established. Here, by using purified full-length and truncated SNARE proteins, we show that the presence of SNARE TMRs dramatically affects the properties of SNARE complex. By taking advantage of this, we provide the first evidence that complexin actively promotes the interaction between the complementary SNARE TMRs implicated in membrane fusion. This novel mechanism readily accounts for the positive role played by complexin in synaptic vesicle exocytosis, as demonstrated in electrophysiological experiments, and is also in good agreement with structural, biophysical, and biochemical studies.

**EXPERIMENTAL PROCEDURES**

Isolation of SNARE Complex from Bovine Brain—All procedures were done at 4 °C. 1 mg of monoclonal anti-SNAP-25 antibody was isolated from 1 ml of ascites fluid (SMI 81) on protein G beads (Amersham Biosciences) using a pH 2.5 elution protocol. Anti-SNAP-25 antibody was covalently coupled to 1 ml of CNBr-activated Sepharose-4B (Amersham Biosciences) according to the manufacturer’s instructions. 2 g of bovine cerebral cortex was homogenized in 50 ml of phosphate-buffered saline (PBS) containing 2 mM EDTA, and membrane material was collected by centrifugation at 12,000 × g. Pelleted membranes were solubilized in 50 ml of PBS in the presence of 2% (v/v) Triton X-100 and Complete protease inhibitor mixture (Roche Molecular Biochemicals).
The lysate was cleared by centrifugation, supplemented with NaCl to
0.5 M, and batch-incubated with the anti-SNAP-25-Sepharose for 2 h.
The gel was then washed in a column with 30 ml of PBS, adjusted to 0.6
M NaCl, 0.2 mM EGTA, 0.1% Triton X-100. Bound proteins were eluted
with 12 ml 0.1 M glycine HCl buffer, pH 2.5, 0.25 M NaCl, 0.2 mM EGTA,
0.1% Triton X-100, and the eluate was neutralized by the addition of
150 µl of 1.5 M Tris-HCl, pH 8.5. Protein concentrations were estimated
using BCA reagent (Pierce) or by Coomassie Blue staining of protein
bands after SDS-PAGE with bovine serum albumin as a standard for
both assays.

Preparation of Monomeric SNARE Proteins—Syntaxin 1A cytoplas-
mic domain (amino acids 1–261), synaptobrevin 2 cytoplasmic domain
(amino acids 1–96), and complexin 2 were expressed in Escherichia coli
strain BL21/DE3 at 37 °C for 3 h (5, 13, 23). After lysis GST fusion
proteins were isolated on glutathione beads. SNARE or complexin re-
combiant proteins were released from GST by incubating beads with
thrombin for 1 h at 37 °C at a thrombin/beads ratio of 80 units/ml. After
addition of a protease inhibitor mixture (Roche Molecular Biochemi-
cals), the released recombinant proteins were used for SNARE complex
formation. His-tagged syntaxin 1A full cytoplasmic domain (amino ac-
ids 1–265) (24) was expressed in E. coli strain BL21/DE3 at 37 °C for 3 h
and purified with a His Trap Kit (Amersham Biosciences). Full-length
SNARE proteins were isolated from bovine brain as described previ-
ously (23).

Re-assembly of SNARE Complexes—All reactions were performed in
150 mM NaCl, 1 mM Na2EDTA, 1 mM dithiothreitol, 0.8% β-octyl glu-
coside, 20 mM Heps, pH 7.2. Monomeric SNARE proteins were mixed
and incubated at room temperature for the indicated times. Assembly
reactions were stopped by addition of sample buffer to give final con-
centrations 2% SDS, 25 mM dithiothreitol, 20 mM Na2EDTA, 5% glyc-
erol, 60 mM Tris-HCl, pH 6.8, and further incubated at room tempera-
ture or 100 °C for 3 min before gel electrophoresis. The latter treatment
disrupts the ternary SNARE complex. SDS-PAGE was carried out
using Bio-Rad 12% Ready gels at 170 V for 45 min at room temperature.
For Western immunoblotting, protein was transferred to Immobilon
membrane (Millipore) at 250 mA for 90 min in freshly prepared transfer
buffer (20 mM Tris, 192 mM glycine, and 20% (v/v) methanol) pre-cooled
to 4 °C. After blocking, the membrane was incubated for 1 h with
primary antibodies (1:5000 dilution), washed 3 times in PBS, 0.1% (v/v)
TWEEN, and incubated with anti-mouse or anti-rabbit secondary anti-
bodies (Amersham Biosciences, 1:5000 dilution) for a further 30 min.
After washing, immunoreactive bands were visualized using a Pierce
Enhanced Chemiluminescence kit (SuperSignal West Dura).

Antibodies—Mouse ascites fluid containing monoclonal antibody to
SNAP-25 (clone SMI 81) was from Sternberger Monoclonals Inc. Mouse
monoclonal antibody to synaptobrevin (clone 69.1) and rabbit polyclonal
antibody to complexin were from Synaptic Systems GmbH (Germany).
Rabbit polyclonal antibodies to syntaxin and SNAP-25 were produced
using recombinant syntaxin 1A (amino acids 1–261) and full-length
SNAP-25A, respectively.

RESULTS
Because SNARE complex is thermodynamically stable and,
once formed, is not disrupted by SDS, its assembly has been
widely studied by electrophoretic techniques (5, 21, 25, 26).
SNAREs lacking TMRs assembled into a SNARE complex that
migrated as a single band (Fig. 1A). Assembly using full-length
SNARE proteins unexpectedly gave rise to an additional, slow-
migrating form of the complex (Fig. 1A). Because of the impor-
tant role of the TMRs for SNARE function, we decided to
investigate this behavior of the full-length SNAREs in detail.
To analyze which SNARE TMR is responsible for the additional
form of SNARE complex, syntaxin and synaptobrevin with or
without TMRs were mixed with brain-purified SNAP-25, and
complex was allowed to form for 30 min. When SNAP complex
was assembled with either full-length syntaxin or full-length
synaptobrevin, there was a small shift of several kDa in keep-
ing with the addition of a single transmembrane region of ~25
amino acids. Only when both syntaxin and synaptobrevin
TMRs were present did the slow-migrating form of the complex
suddenly appear (Fig. 1B). Of note, the calculated molecular
mass of the two TMRs is only ~5 kDa, but the observed differ-
ence in apparent molecular mass between the two forms is
~100 kDa. Because interaction of the TMRs from syntaxin and
synaptobrevin is SDS-resistant (27), the likeliest explanation
for the dramatic difference in the mobility of the SNARE com-
plexes is that the slow-migrating form represents complex in
which the complementary syntaxin and synaptobrevin TMRs
have interacted.

Next, we used the appearance of slow-migrating SNARE
complex as a simple assay of syntaxin and synaptobrevin TMR-
TMR interaction to investigate the action of complexin. This
was a natural step because complexin has been shown to bind
to syntaxin and synaptobrevin helices close to the transmem-
brane part of the SNARE complex (22). SNARE complex was
prepared by incubating full-length synaptobrevin with
SNAP-25 and either syntaxin-(1–265) lacking TMR or full-
length syntaxin. After 30 min, complexin was added, and the
reaction was allowed to proceed for a further 20 min. Com-
plexin did not cause any changes in SNARE complex when
syntaxin was lacking its TMR (Fig. 2A). But, strikingly, when
the complementary syntaxin and synaptobrevin TMRs were
present, complexin promoted complete transition of preas-
sembled SNARE complex into its slow-migrating form (Fig.
2B).

To determine whether native complexin, even in the pres-
ence of the other neuronal proteins, binds SNARE complex and
promotes the slow-migrating form, we purified SNARE com-
plex from bovine brain cortex. A Triton X-100 brain extract was
loaded on an anti-SNARE immunoadfinity column, made using
an anti-SNAP-25 antibody, clone SMI 81. This monoclonal

FIG. 1. Migration of SNARE complex depends on the comple-
mentary transmembrane regions of syntaxin and synaptobre-
vin. A, two forms of SNARE complex are evident upon reaction of
full-length SNARE proteins but not their truncated versions. SNARE
complex formation was studied using full-length synaptobrevin, recom-
binant synaptobrevin (amino acids 1–96) lacking a transmembrane
region (TMR), full-length SNAP-25, full-length syntaxin, or recombi-
nant syntaxin (amino acids 1–261) without TMR. Synaptobrevin,
SNAP-25, and syntaxin (20–30 pmol each) were incubated for 30 min at
room temperature. 30-µl reactions were stopped by the addition of
SDS-containing sample buffer, and samples were analyzed by SDS-
PAGE in a 12% Bio-Rad Ready gel. Protein was visualized by Coomas-
sie staining. B, both syntaxin and synaptobrevin transmembrane re-
regions are required for assembly of the slow-migrating form of SNARE
complex. Full-length or truncated SNARE proteins were incubated for
30 min at room temperature, and SNARE complex formation was
analyzed by SDS-PAGE. Protein was visualized by Coomassie staining.

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an anti-SNAP-25 antibody, clone SMI 81. This monoclonal
antibody is highly specific for SNAP-25, can bind SNAP-25 containing complexes, and does not cross-react with complexin (data not shown). A typical chromatography round yielded ∼1 mg of SNARE proteins per 2 g of brain cortex, co-purifying with stoichiometric amounts of complexin (Fig. 3A). Because the interaction of complexin with SNAREs is sensitive to SDS, the co-purifying complexin migrated in its monomeric position in both boiled and non-boiled samples. Eluted SNAP-25 complex migrated as a major band (Fig. 3A) corresponding to the slow-migrating form identified in our SNARE re-assembly reactions (Fig. 1). When boiled in sample buffer, the slow-migrating complex is disrupted into monomeric syntaxin, SNAP-25, and synaptobrevin (Fig. 3A), as expected. The anti-SNAP-25 immunoaffinity column also pulled down monomeric SNAP-25, as expected (Fig. 3A).

To ensure that we had extracted all available forms of SNARE complex and thus excluded the possibility of selective isolation of the slow-migrating complex, we tested the efficiency of the isolation procedure. We compared equal aliquots of the flow-through and the original loading material, both samples being boiled before electrophoresis to ensure that SNAP-25 complex was disrupted and SNAREs were being compared in their monomeric state. Fig. 3B shows that the affinity column completely removed SNAP-25 (and thus all SNAP-25-containing ternary SNARE complexes) from the loaded brain detergent extract. Interestingly, complexin concentration in the flow-through material did not change significantly, showing that complexin amounts exceed maximal SNARE complex quantities in brain.

Because the eluate contained SNAP-25 in its monomeric state (Fig. 3A), we tested whether complexin was purified strictly with the ternary SNARE complex rather than with SNAP-25. We immobilized GST–complexin on glutathione beads and tested SNAP-25 and the SNARE complex for binding. Fig. 3C shows that the immobilized complexin did not bind monomeric SNAP-25 but stoichiometrically interacted with complex composed of syntaxin, SNAP-25, and synaptobrevin. SNAREs are likely to assemble in vivo in the presence of cytoplasmic complexin. Therefore, we analyzed the behavior of SNARE complex when made to assemble in the presence of complexin. Notably, it was the slow-migrating complex that appeared (Fig. 4), indicating that the three SNARE proteins were assembling directly into the form in which complementary TMRs interact. Thus complexin not only binds rapidly (21) but also exerts its function promptly on the assembling SNARE complex. In other words, it is SNARE complex assembly, rather than complexin function, that is the rate-limiting step in this reaction.

DISCUSSION

The TMRs of SNARE proteins play a critical role in membrane fusion, which requires directed movement of two membranes by proteins under conditions of high membrane tension and curvature (28). It is thought that assembly of the cytoplasmic part of the SNARE complex exerts force on the transmembrane anchors, thereby generating inward and lateral movement in both membranes (2, 9). Moreover, it has been shown that syntaxin and synaptobrevin transmembrane regions actually interact via coiled-coil interactions (27, 29). Zippering of the four-helix SNARE bundles is thought to be followed by interaction of the transmembrane parts of syntaxin and synaptobrevin leading to membrane fusion (29). Indeed, in a liposome fusion system, insertion of a linker between the transmembrane domain and the cytoplasmic part of syntaxin decreased fusion efficiency (30). Furthermore, replacing the membrane anchors of syntaxin and synaptobrevin with short phospholipids that do not span the bilayer prevented lipid mixing (31). In the present study, we were able to utilize highly purified full-length SNAREs and a gel shift assay to follow interactions between complementary SNARE TMRs and thus to address the function of complexin.

A recent structural analysis of complexin bound to the truncated SNARE complex showed that complexin seals the C-terminal interface between synaptobrevin and syntaxin (22). By using NMR spectroscopy, Rizo and colleagues (22) further demonstrated that this action of complexin extends beyond the interacting region toward the C terminus where the membrane anchors would normally be located. By taking advantage of full-length SNARE proteins, we now provide the first evidence that complexin promotes interaction of the transmembrane regions of syntaxin and synaptobrevin. This function of complexin takes place after SNARE complex assembly (Fig. 2B), consistent with binding studies (20, 21) and in vivo results that showed that complexin acts at the last step of exocytosis (16). In the presence of complexin, the full-length SNARE proteins actually assemble directly into the slow-migrating complex (Fig. 4), which is the major form observed in bovine brain (Fig. 3A). Importantly, our experiments suggest that interaction between complementary SNARE TMRs can take place in the absence of complexin, but complexin potently stabilizes SNARE complex in the form in which TMRs interact (Figs. 1 and 2). Indeed, whereas complexin is not obligatory for synaptic vesicle exocytosis, it does increase evoked neurotransmitter release by 60–70% as demonstrated by complexin gene knockout in mice (16).

Our experimental results are consistent with the findings of an independent study (21) that questioned the recent proposal that complexin causes two contiguous SNAP-25 helices to oli-
Samples were boiled to disrupt SNARE complex prior to analysis by SDS-PAGE and Coomassie staining. The incubated with monomeric SNAP-25, SNARE complex, or control buffer for 30 min. After washing, protein was eluted by addition of sample buffer.

 SNAP-25 and thus all ternary complex. Complexin immunoreactivity in the flow-through sample does not dramatically change despite stoichiometric binding to SNARE complex. FT

 anti-SNAP-25 immunoaffinity chromatography. Equal aliquots of loading material (Load), flow-through (FT), and pH 2.5 eluate after neutralization were analyzed by SDS-PAGE, and protein was visualized by Coomassie staining. Neuronal SNARE complex, co-purifying with stoichiometric amounts of complexin, is represented by its slow-migrating form. Demonstration of total extraction of SNARE complex from brain on anti-SNAP-25 column. Western immunoblot of equal aliquots of the flow-through (FT) and the loading material (Load), using anti-synaptobrevin, anti-synaptobrevin, and anti-complexin antibodies. All samples were boiled prior to SDS-PAGE and thus represent total amounts of SNARE proteins. SNAP-25 immunoreactivity is absent in the flow-through material indicating that the anti-SNAP-25 column extracts all SNAP-25 and thus all ternary complex. Complexin immunoreactivity in the flow-through sample does not dramatically change despite stoichiometric binding to SNARE complex. GST-complexin immobilized on glutathione beads was incubated with monomeric SNAP-25, SNARE complex, or control buffer for 30 min. After washing, protein was eluted by addition of sample buffer.

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 In our study SNARE complex, assembled using syntaxin and synaptobrevin lacking TMRs, migrates as a single band. However, the original studies of the SDS-resistant properties of SNARE complex by Hayashi et al. (5) reported that SNARE complexes assembled using truncated syntaxin and synaptobrevin, and bacterially expressed full-length SNAP-25 migrates as a number of bands. The difference is probably because of the fact that the recombinant full-length SNAP-25, used previously, contains exposed cysteine residues that can be cross-linked via disulfide bonds, causing artifactual aggregation of SNARE complexes. Full disruption of disulfide bonds in a protein sample requires boiling of the sample with beta-mercaptoethanol; this boiling procedure cannot be used in studies of SNARE complex as it also disrupts the complex (5). In contrast, we used SNAP-25 purified from brain where cysteine residues are likely to be post-translationally palmitoylated (33, 34). Revealingly, when mixed with syntaxin and synaptobrevin lacking their TMRs, bacterially expressed SNAP-25 in which the four cysteines within the hinge region were replaced by alanines gives only a single band of SNARE complex (17). Thus, it is likely that the behavior of SNARE complex reported in this study has not been observed previously in re-assembly experiments because we used, for the first time, brain-purified SNAP-25, as well as full-length syntaxin and synaptobrevin.

 Despite the important role of complexin in synaptic neurotransmission, molecular understanding of its action apart from its binding to the SNARE complex has been elusive. The present study yields new insights into the function of complexin on SNARE complex. Our data indicate that the binding of complexin to syntaxin and synaptobrevin helices, demonstrated in structural studies (22), in fact promotes interaction of their complementary TMRs. Because the TMRs of syntaxin and synaptobrevin are in the two opposing membranes prior to membrane fusion, complexin-driven TMR-TMR interaction would inevitably promote lipid bilayer mixing and membrane fusion. This model readily accounts for the physiological activity of complexin and is in good agreement with the current understanding of synaptic vesicle exocytosis, namely that SNARE proteins assemble directly into the slow-migrating form of the SNARE complex in the presence of complexin. Full-length SNARE proteins were mixed in the presence or absence of complexin, and at indicated times the reaction was stopped by the addition of sample buffer. SNARE complex formation was detected by Western immunoblotting using monoclonal anti-synaptobrevin antibody, clone 69.1.

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 Fig. 3. Complexin is the major binding partner of SNARE complex in brain. A, SNARE complex extraction from bovine brain by anti-SNAP-25 immunoaffinity chromatography. Equal aliquots of loading material (Load), flow-through (FT), and pH 2.5 eluate after neutralization were analyzed by SDS-PAGE, and protein was visualized by Coomassie staining. Neuronal SNARE complex, co-purifying with stoichiometric amounts of complexin, is represented by its slow-migrating form. B, demonstration of total extraction of SNARE complex from brain on anti-SNAP-25 column. Western immunoblot of equal aliquots of the flow-through (FT) and the loading material (Load), using anti-syntaxin, anti-SNAP-25, anti-synaptobrevin, and anti-complexin antibodies. All samples were boiled prior to SDS-PAGE and thus represent total amounts of SNARE proteins. SNAP-25 immunoreactivity is absent in the flow-through material indicating that the anti-SNAP-25 column extracts all SNAP-25 and thus all ternary complex. Complexin immunoreactivity in the flow-through sample does not dramatically change despite stoichiometric binding to SNARE complex. C, selective binding of complexin to SNARE complex. GST-complexin immobilized on glutathione beads was incubated with monomeric SNAP-25, SNARE complex, or control buffer for 30 min. After washing, protein was eluted by addition of sample buffer. Samples were boiled to disrupt SNARE complex prior to analysis by SDS-PAGE and Coomassie staining. The asterisk indicates a breakdown product of the GST fusion protein.

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complex must zipper beyond its cytoplasmic part to force fusion of two membranes (3, 9, 31).

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