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A Trimeric Protein Complex Functions as a Synaptic Chaperone Machine

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Summary

We identify a chaperone complex composed of (1) the synaptic vesicle cysteine string protein (CSP), thought to function in neurotransmitter release, (2) the ubiquitous heat-shock protein cognate Hsc70, and (3) the SGT protein containing three tandem tetratricopeptide repeats. These three proteins interact with each other to form a stable trimeric complex that is located on the synaptic vesicle surface, and is disrupted in CSP knockout mice. The CSP/SGT/Hsc70 complex functions as an ATP-dependent chaperone that reactivates a denatured substrate. SGT overexpression in cultured neurons inhibits neurotransmitter release, suggesting that the CSP/SGT/Hsc70 complex is important for maintenance of a normal synapse. Taken together, our results identify a novel trimeric complex that functions as a synapse-specific chaperone machine.

Introduction

The exocytotic release of neurotransmitter from nerve terminals is a fundamental process underlying most intercellular communication in the nervous system. Synaptic vesicles, the central players in this process, undergo a complex cycle of fusion and fission events. They fuse with the presynaptic membrane in response to a rise in the intracellular Ca^{2+} concentration, and release their neurotransmitter cargo into the synaptic cleft. The vesicle membrane is then retrieved by endocytosis (Südhof, 1995, 2000; Scales and Scheller, 1999).

In the last ten years, enormous progress has been made in identifying and characterizing the essential protein machinery involved in organizing and maintaining the synaptic vesicle cycle. One component of this complex protein machinery is the cysteine string protein (CSP), which is thought to have an important function in the exocytotic release of neurotransmitter, hormones, and enzyme precursors (Buchner and Gundersen, 1997). CSP is localized on synaptic vesicles (Mastrogiacomo et al., 1994), chromaffin granules (Chamberlain et al., 1996), and zymogen granules (Braun and Scheller, 1995). It is highly conserved during evolution (Buchner and Gundersen, 1997), reflected by an overall amino acid identity of almost 55% between rat and *Drosophila* CSP. Due to a unique structural feature, a string of 11 cysteines flanked on either side by two additional cysteines, it was named cysteine string protein (Zinsmaier et al., 1990). Most of the cysteine residues are palmitoylated and required for membrane targeting of CSP (Gundersen et al., 1994; Chamberlain and Burgoyne, 1998). Another striking feature of CSP is that it contains a J domain at the N terminus. The J domain comprises a stretch of 70 amino acids evolutionarily conserved from *E. coli* to man (Fink, 1999).

The biological function of CSP has remained elusive. In *Drosophila*, deletion of CSP is lethal for a great majority of flies (Zinsmaier et al., 1994). However, a small percentage of CSP null mutants survived and could be analyzed with electrophysiological methods. CSP "knockout" flies showed an impaired presynaptic neuromuscular transmission accompanied with a dramatic loss of synaptic vesicles. It has been suggested that CSP might function in regulating presynaptic Ca^{2+} channels based on the finding that injection of CSP antisense RNA into *Xenopus* oocytes inhibited the activity of omega-conotoxin sensitive Ca^{2+} channels (Gundersen and Umbach, 1992). Indeed, a direct interaction of CSP with the α1A subunit of P/Q-type Ca^{2+} channels and an indirect interaction via G protein modulation was reported (Leveque et al., 1998; Magga et al., 2000). *Drosophila* CSP null mutants showed a wild-type like release of neurotransmitter when a Ca^{2+} ionophore was used to trigger exocytosis, implying that CSP functions in the coupling of the Ca^{2+} signal to secretion (Ranjan et al., 1998). Consistent with this hypothesis, the temperature-sensitive inhibition of neuromuscular transmission in CSP null mutants is correlated with a rise in the intracellular Ca^{2+} concentration, suggesting that CSP increases the Ca^{2+} sensitivity of the exocytotic machinery (Dawson-Scully et al., 2000). In *Drosophila*, an interaction between CSP and the t-SNARE syntaxin-1A has been reported (Nie et al., 1999; Wu et al., 1999) whereas in vertebrates, CSP was found to bind to synaptobrevin/VAMP but not to syntaxin (Leveque et al., 1998).

Compelling evidence has been presented that CSP interacts with members of the heat-shock family Hsp70 (Braun et al., 1996; Chamberlain and Burgoyne, 1997; Stahl et al., 1999). This interaction was originally found by means of an ATPase assay (Braun et al., 1996). CSP strongly activated the Hsc70 ATPase. Thereafter, a direct binding of Hsc70 to the J domain of CSP could be shown (Stahl et al., 1999). Among other functions,

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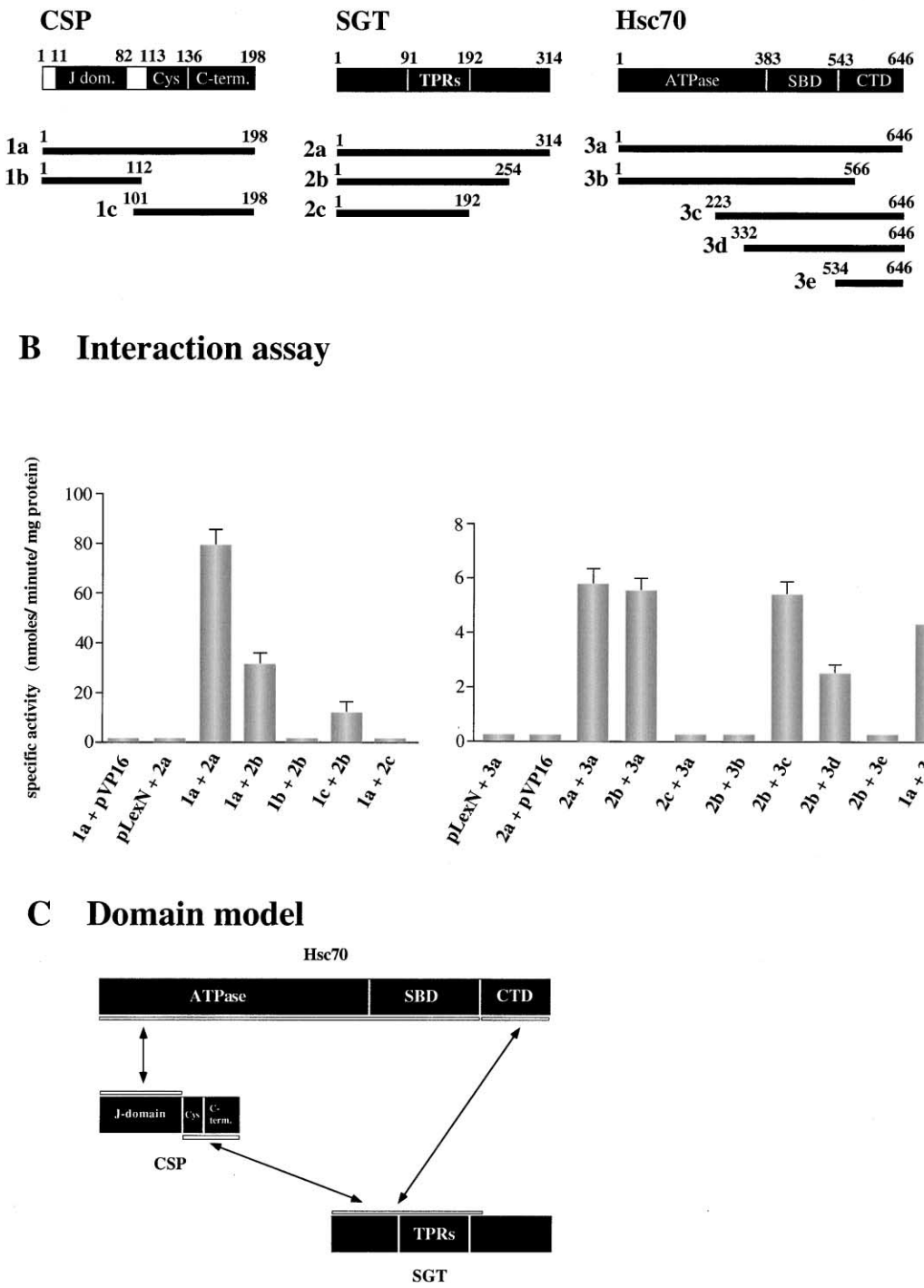

Figure 1. Three Proteins, CSP, SGT, and Hsc70, Directly Interact with Each Other

A yeast-two hybrid screen using full-length rat CSP1 as bait and a rat brain cDNA library as source of preys was performed. As prey, 24 positive clones encoding partial SGT were obtained. The heat-shock protein cognate Hsc70, a known binding partner for CSP, was found in a previous yeast-two hybrid screen using the J domain of CSP as bait. (A) Domain structures of CSP, SGT, and Hsc70. The following abbreviations were used: Cys for a cysteine-rich cluster in CSP, TPRs for tetratricopeptide repeats in SGT, SBD for the substrate binding domain in Hsc70, and CTD for C-terminal domain. Constructs used in a yeast-two hybrid interaction assay are depicted as bars below. The numbers on top of the bars represent the N- and C-terminal amino acids of various constructs. The numbers on the left side of the bars identify individual constructs. (B) The interaction of all binary combinations of CSP, SGT, and Hsc70 was determined by measuring the specific activity of the reporter gene β-galactosidase. β-galactosidase activity was measured in triplicates and is shown as the mean ± SD. CSP

members of the Hsp70 family, like Hsc70, act as molecular chaperones (Hendrick and Hartl, 1995).

We have now used the yeast two-hybrid system to find proteins interacting with CSP. A novel CSP binding partner termed SGT was identified. SGT was originally discovered because of its putative interaction with envelope proteins of two viruses, i.e., human immunodeficiency virus type 1 and parvovirus H-1 (Callahan et al., 1998; Czieplucht et al., 1998). A detailed sequence analysis revealed that SGT contains three tandem tetratricopeptide repeat domains (TPRs). The TPR domain is a degenerate 34 amino acid sequence found in a wide variety of proteins, present in tandem arrays of 3 to 16 motifs, which form scaffolds to mediate protein-protein interactions. Currently, more than 50 proteins are known to contain TPR motifs, present in organisms as diverse as bacteria and humans (Blatch and Lässle, 1999).

Our studies define a novel tripartite complex formed by CSP, SGT, and Hsc70. All three proteins can be coimmunoprecipitated from purified synaptic vesicles, and SGT is drastically reduced on synaptic vesicles lacking CSP because of a genetic deletion. These findings strongly support the formation of a trimeric CSP/Hsc70/SGT complex in vivo. The assembly of the trimeric complex depends on ADP, whereas ATP disassembles the complex. The Hsc70 ATPase is strongly activated by a combination of CSP and SGT, thereby providing the free energy for a refolding reaction. The CSP/Hsc70/SGT complex constitutes a synaptic chaperone machine, most likely refolding a misfolded protein in vicinity of the synaptic vesicle surface.

Results

A Yeast Two-Hybrid Screen for CSP Identifies a Novel Binding Partner

We performed a yeast two-hybrid screen with rat CSP1 using a rat brain library as source of preys. Eighty-five million yeast double transformants were screened for interaction with CSP1 (Vojtek et al., 1993). Among the clones isolated in the screen, 29 were found to be positive in a β-galactosidase assay. Sequencing revealed that 24 clones represented partial clones of a single protein with tetratricopeptide repeats (TPRs). This protein was discovered recently because of its putative interaction with two viruses, i.e., human immunodeficiency virus type 1 and parvovirus H-1 (Callahan et al., 1998; Czieplucht et al., 1998). Due to its domain structure, it was termed SGT, an abbreviation for small glutamine-rich TPR protein.

In our yeast two-hybrid screen, we identified partial clones of SGT, the longest of which encoded 81% of the SGT protein (Figure 1A, construct 2b). Screening of a rat brain cDNA library in λZAP II using a partial yeast clone as probe enabled us to clone full-length SGT. A subsequent yeast two-hybrid retransformation assay confirmed that the activation of the reporter gene β-galactosidase depended on both proteins, i.e., CSP1 and SGT (Figure 1B, left diagram). Combinations of CSP1 and SGT, respectively, with prey and bait vectors devoid of inserts, i.e., pVP16 and pLexN, did not result in a β-galactosidase activation (Figure 1B, left diagram). We also investigated whether CSP2, a splice variant of CSP1 differing at the C terminus (Chamberlain et al., 1996), could bind to SGT, and found a similarly strong interaction (data not shown). We then asked which domains mediate the interaction between CSP and SGT. As shown in Figure 1B, the C-terminal domain of CSP is essential for binding to SGT, whereas the N-terminal J domain is not necessary. For the interaction with CSP, the TPRs in SGT are necessary but not sufficient (Figure 1B, left diagram).

SGT Directly Interacts with CSP and Hsc70

Several proteins with TPRs are known to interact specifically with heat-shock proteins, e.g., Hip and Hop bind to Hsc70 and immunophilins and phosphoprotein phosphatase 5 (PP5) interact with Hsp90 (Hohfeld et al., 1995; Young et al., 1998). Since these interactions are mediated by the TPRs, we tested the possibility that SGT binds to Hsc70 via its TPRs. Indeed, such an interaction could be confirmed using a yeast two-hybrid retransformation assay (Figure 1B, right diagram). The TPRs of SGT are necessary but not sufficient for binding of Hsc70 (Figure 1B, right diagram). Interestingly, the C-terminal domain but not the ATPase domain of Hsc70 is essential for the interaction with SGT (Figure 1B, right diagram). The specificity of the SGT/Hsc70 interaction is further underlined by the observation that other heat-shock proteins like Hsp60 and Hsp90 did not interact with SGT (data not shown). The strength of interaction between SGT and Hsc70 is one order of magnitude smaller than that of SGT and CSP (Figure 1B, both diagrams). However, it is on the same order of magnitude as that of CSP and Hsc70 (Figure 1B, right diagram). Please note that the CSP/Hsc70 interaction (Figure 1B, right diagram, col. 10) has already been reported (Stahl et al., 1999).

In sum, our data strongly support the idea that CSP, Hsc70, and SGT can bind to each other. The domains involved in these interactions are depicted in a model (Figure 1C). The J domain of CSP is essential for the interaction with Hsc70 (Braun et al., 1996), whereas the C-terminal domain of CSP mediates the binding of SGT. The cooperation of two distinct domains in Hsc70, i.e., the ATPase domain and the substrate binding domain, is necessary and sufficient for interaction with CSP (Stahl et al., 1999). In contrast, the C-terminal domain but not the ATPase domain of Hsc70 is crucial for binding of SGT. The TPRs of SGT are essential for the binding of both CSP and Hsc70.

CSP, Hsc70, and SGT Form a Trimeric Complex In Vitro

Our yeast two-hybrid data predicted that CSP, Hsc70, and SGT might form dimeric and/or trimeric complexes

directly interacts with SGT (left diagram, col. 3). This interaction is mediated by the C-terminal half of CSP (left diagram, col. 6). The TPRs of SGT are necessary but not sufficient for this type of interaction (left diagram, col. 7). SGT also interacts with Hsc70 (right diagram, col. 3). For this interaction, the C-terminal domain of Hsc70 is essential (right diagram, col. 6). As previously reported, CSP also binds to Hsc70 (right diagram, col. 10). (C) Based on these yeast-two hybrid data, a domain model for the interactions between CSP, SGT, and Hsc70 is presented.

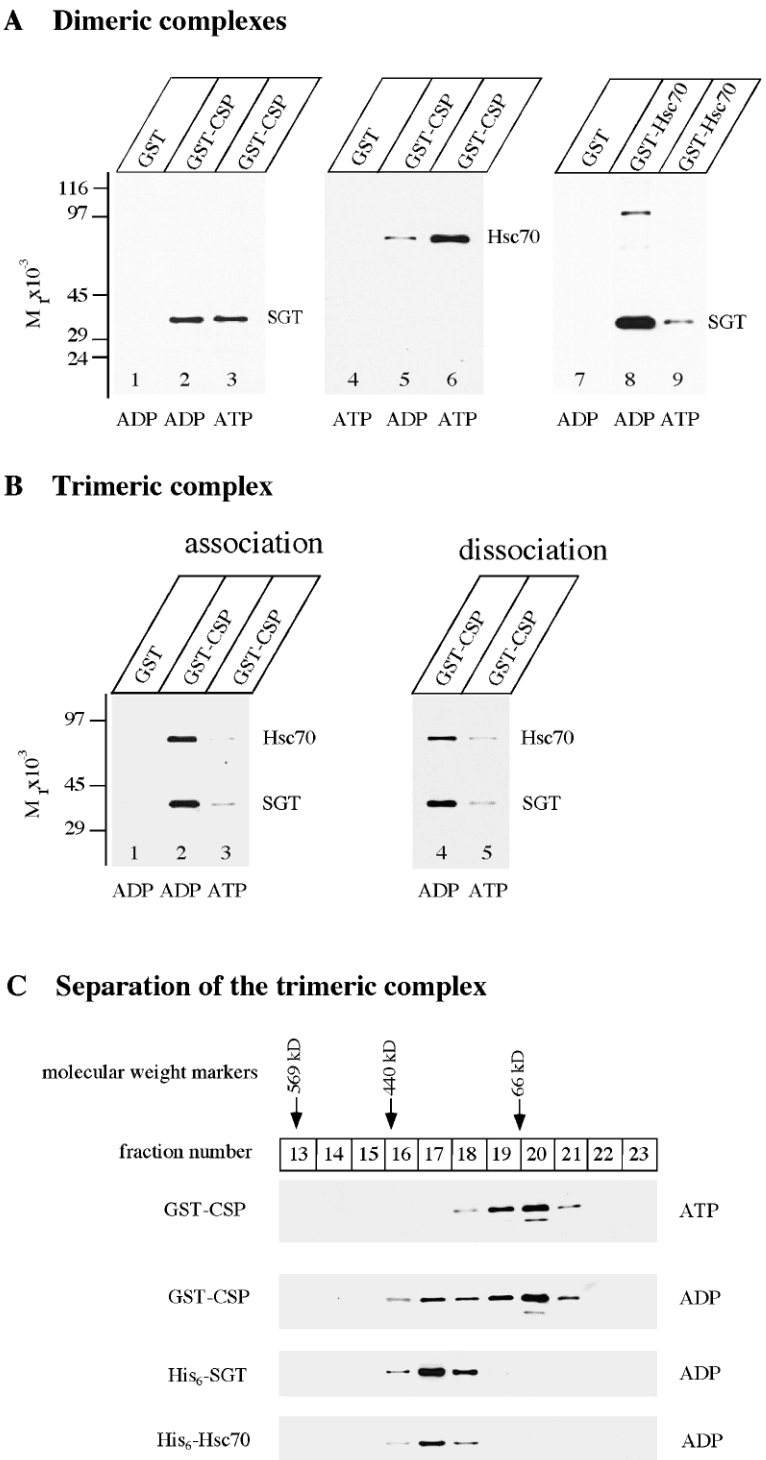

Figure 2. CSP, SGT, and Hsc70 Form a Trimeric Complex in the Presence of ADP

GST fusion proteins of full-length CSP and Hsc70, respectively, were immobilized on glutathione beads. These beads were incubated with His_6-SGT or His_6-Hsc70 in the presence of either ADP or ATP. Proteins bound to these beads were analyzed by SDS-PAGE and immunoblotting for the His_6 epitope. (A) The formation of dimeric complexes between CSP, SGT, and Hsc70 was analyzed. SGT specifically interacted with CSP (lanes 2 and 3), whereas GST alone did not interact (lane 1). Hsc70 bound in an ATP-dependent manner to CSP (lane 6), while SGT interacted with Hsc70 in the presence of ADP (lane 8). (B) A GST fusion protein of full-length CSP was incubated with a combination of His_6-SGT and His_6-Hsc70. In the presence of ADP, Hsc70 and SGT interacted with CSP (lane 2). ATP prevented the formation of a protein complex (lane 3), and induced a dissociation of both components of the complex (lane 5). (C) The complex consisting of GST/CSP, SGT, and Hsc70 was assembled on glutathione beads in the presence of ADP. As a control, the same experiment was performed using ATP instead of ADP. In both cases, the beads were extensively washed. Proteins were then eluted by addition of glutathione and subjected to gel filtration on a Superose 6 column. Fractions were collected and analyzed by immunoblotting. In the presence of ADP, a substantial amount of GST/CSP was shifted to a molecular weight of approximately 300 kDa. GST/CSP, SGT, and Hsc70 were coeluted, strongly suggesting that these proteins formed a trimeric complex under these conditions. However, in the presence of ATP, neither SGT nor Hsc70 was detectable in the column eluate.

under certain conditions (see Figure 1C). We checked this possibility by incubating recombinant SGT or Hsc70 with immobilized CSP or Hsc70. SGT interacted with CSP in a nucleotide-independent manner (Figure 2A, lanes 2 and 3). In contrast, Hsc70 bound to CSP more strongly in the presence of ATP than ADP (Figure 2A, lane 5). Conversely, the third dimeric interaction, the interaction between SGT and Hsc70, depended on ADP (Figure 2A, lane 8).

These results demonstrate that CSP, Hsc70, and SGT can bind to each other. Under which conditions do these proteins assemble to a trimeric complex? We addressed this question by incubating both Hsc70 and SGT with immobilized CSP in the presence of ADP or ATP. Both proteins, Hsc70 and SGT, were found on CSP-beads in the presence of ADP, while ATP abolished the interaction (Figure 2B, lanes 2 and 3). We then studied whether the preformed complex could be dissociated by incuba-

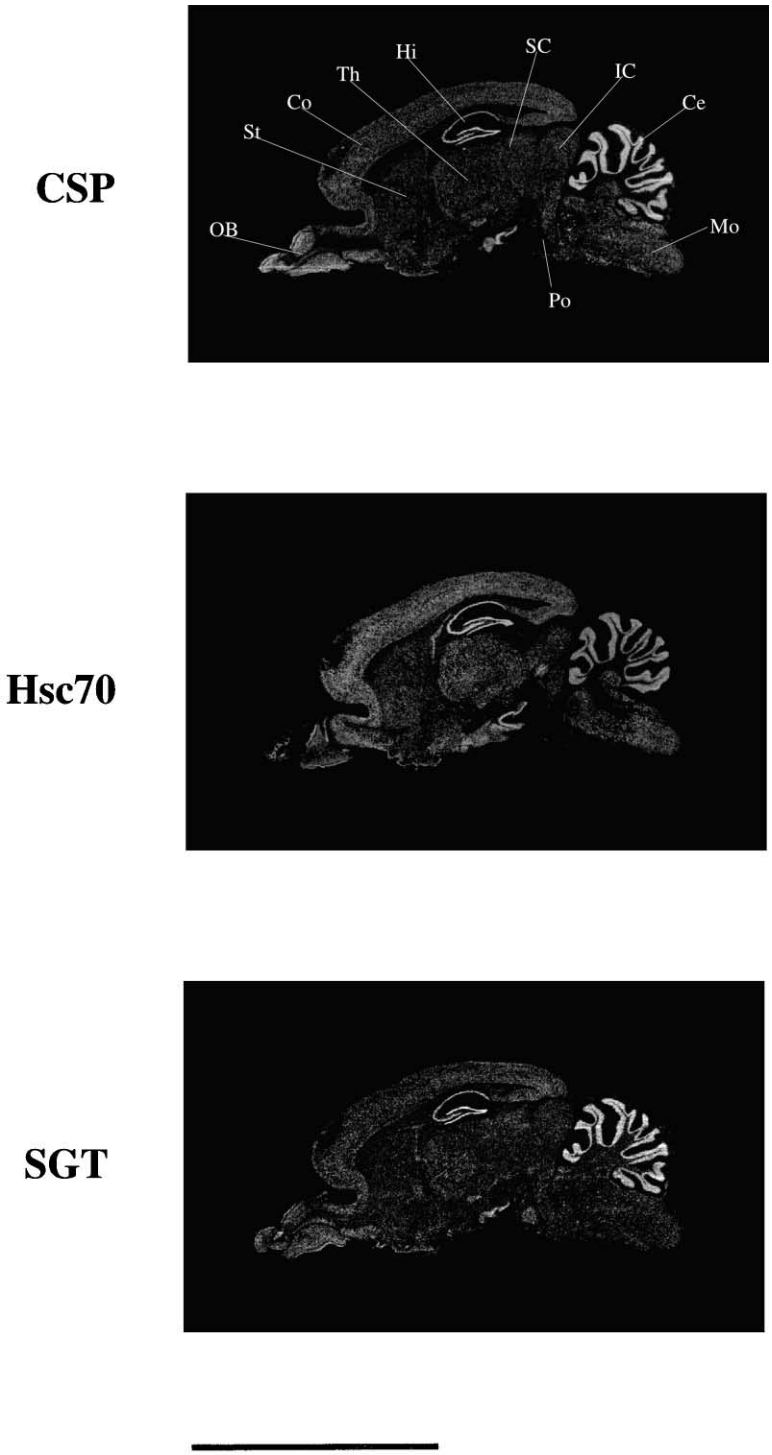

Figure 3. mRNAs of CSP, SGT, and Hsc70 Colocalize in Rat Whole-Brain Sections

The mRNA distribution of CSP, Hsc70, and SGT in adult rat brain was analyzed by applying an in situ hybridization technique. Controls with excess unlabeled oligonucleotides were devoid of signal (results not shown). Negative X-ray film images are shown. Abbreviations: Ce, cerebellum; Co, cortex; Hi, hippocampus; IC, inferior colliculus; Mo, medulla oblangata; OB, olfactory bulb; Po, pons; SC, superior colliculus; St, striatum; Th, thalamus. Scale bar, 1 cm. Note the strong staining of both the hippocampus and the cerebellum for all three mRNA species. A weaker staining is observed in the cortex, thalamus, and striatum.

tion with ATP. In contrast to ADP, ATP was able to dissociate the complex (Figure 2B, lanes 4 and 5).

This observation can be explained by two hypotheses—either two dimeric complexes of CSP/Hsc70 and CSP/SGT formed in equal amounts or a trimeric complex consisting of CSP, Hsc70, and SGT assembled. The fact that incubation with ATP resulted in a dissociation of both proteins, Hsc70 and SGT, and not only of Hsc70, strongly supports the second hypothesis. To test this hypothesis, we studied the behavior of the complex on a gel filtration column (Superose 6). The CSP/SGT/Hsc70 complex was eluted from the beads by incubation with glutathione, and subjected to gel filtration in the presence of ADP. Two separate column runs were performed. As a negative control, beads depleted of Hsc70 and SGT were used. Upon gel filtration, GST/CSP from the control beads eluted at a size of ~50 kDa (Figure 2C, first panel). In a second column run, proteins from

the assembled complex were analyzed. To avoid a disassembly of the complex, ADP was added to the running buffer. This time, a substantial part of GST/CSP shifted to a molecular weight of ~300 kDa (Figure 2C, second panel). Consistent with our hypothesis of a trimeric complex, GST/CSP, SGT, and Hsc70 eluted at the same size of ~300 kDa. This molecular weight is twice the size as expected, suggesting that the stoichiometry of the trimeric complex is 2:2:2 rather than 1:1:1.

CSP, Hsc70, and SGT Form a Trimeric Complex on the Synaptic Vesicle Surface

As a first step to characterizing the localization of CSP, Hsc70, and SGT, we performed in situ hybridizations on brain sections from adult rats (Figure 3). Two different oligonucleotides for each mRNA sequence were used; they exhibited essentially the same labeling pattern for the respective mRNAs. This labeling pattern was abolished when an excess of unlabeled oligonucleotide was added to the hybridization mix (results not shown).

Detailed analyses of expression patterns of CSP, Hsc70, and SGT were performed with one oligonucleotide for each mRNA. Autoradiographs of hybridized rat brain sections revealed a high degree of overlap in the expression of the CSP, Hsc70, and SGT mRNAs (Figure 3). These mRNAs are expressed throughout the brain, with highest levels in the cerebellum and the hippocampus. Strong labeling is observed in the CA regions and the dentate gyrus. Lower levels of mRNA expression are found in the cortex, olfactory bulb, thalamus, and striatum. In sum, the mRNA expression patterns for CSP, Hsc70, and SGT are consistent with a colocalization of the corresponding proteins in various brain regions.

To assemble to a trimeric complex, it is a prerequisite that all three proteins are located in the same cellular compartment. In brain, CSP is primarily located on synaptic vesicles (Mastrogiacomo et al., 1994). Hsc70, a cytosolic protein, is enriched in the nerve terminal (Ungewickell et al., 1995). To determine the localization of SGT, we raised a polyclonal antibody against recombinant SGT. As shown in Figure 4A, the antibody was specific for SGT, a protein with a molecular weight of 35 kDa. This molecular weight agrees well with the expected value based on the amino acid composition of SGT.

Since the antibody against SGT did not give a specific signal in immunocytochemistry, we performed subcellular fractionations. Antibodies directed against CSP, SGT, Hsc70, GDI, synaptophysin, and rab3a were used to characterize these fractions. As depicted in Figure 4B, CSP was mainly found in the synaptic vesicle fraction (LP2). It was not detectable in cytosolic fractions (S3, LS2), implying that the entire pool of CSP is subject to palmitoylation and membrane bound. SGT was predominantly found in cytosolic fractions (S3, LS2), consistent with a hydrophilic structure predicted from its amino acid sequence. However, significant amounts of SGT and Hsc70 were found in the synaptic vesicle fraction (LP2), strongly suggesting that SGT, Hsc70, and CSP colocalize on synaptic vesicles.

Synaptophysin, a synaptic vesicle marker (Jahn et al., 1985), was highly enriched in LP2 and not detectable in cytosolic fractions. Rab3a, a small G protein of the ras superfamily, was located on synaptic vesicles (LP2) and, as expected, found in soluble fractions (S3, LS2). GDI (Sasaki et al., 1990) is a cytosolic protein that was not found in membrane fractions (LP1, LP2). Therefore, we could exclude the possibility that LP2 was contaminated with LS2, supporting the idea that SGT and Hsc70 are recruited to vesicles by a resident synaptic vesicle protein.

We then asked whether CSP, Hsc70, and SGT form a trimeric complex on synaptic vesicles. Therefore, we immunoprecipitated SGT from purified synaptic vesicles using a polyclonal mouse antibody raised against SGT (Figure 4C). In the resulting immunoprecipitate, Hsc70 and CSP but not rab3a, rabphilin, syntaxin, and SNAP25 could be detected. These results demonstrate that SGT, Hsc70, and CSP specifically coimmunoprecipitate from purified synaptic vesicles without contamination of other synaptic vesicle proteins.

To test if the interaction of CSP with SGT is functionally important in vivo, we utilized a CSP knockout mouse that we generated in an independent study, and that will be described separately (R.F.-C. and T.C.S., unpublished observations). CSP knockout mice are viable until 4–5 weeks after birth, making it possible to study them biochemically. In total brain homogenates, no significant changes in SGT and Hsc70 levels were detected compared to wild-type controls (Figure 4D). However, when we examined synaptic vesicles purified from CSP knockout mice, we observed a selective decrease of SGT on synaptic vesicles. Quantitations revealed that SGT was decreased more than 70% on the vesicles, while the levels of other proteins were unaffected (Figure 4E). These data suggest that CSP functionally interacts with SGT in vivo and is required for the recruitment of SGT to synaptic vesicles.

SGT and CSP Activate the ATPase of Hsc70

As already reported (Braun et al., 1996; Chamberlain and Burgoyne, 1997), CSP strongly activated the Hsc70 ATPase with stimulation factor of ~12. We were therefore interested in whether SGT could affect the ATPase activity of Hsc70 in a similar manner.

[α-^{32}P] ATP was incubated with various combinations of bacterially expressed rat CSP, SGT, and Hsc70 in stoichiometric ratios of 1:1:1. At the indicated times, hydrolysis of [α-^{32}P] ATP was determined by thin layer chromatography and autoradiography. SGT alone did not induce ATP hydrolysis (Figure 5A). Hsc70 exhibited a weak intrinsic ATPase activity. This ATPase activity was significantly increased by SGT, demonstrating a functional interaction between SGT and Hsc70 (Figure 5A). Compared to the ATPase activation by CSP (~12-fold), the stimulation by SGT was weaker (~3-fold). A combination of CSP and SGT resulted in a dramatic ATPase activation (~19-fold) (Figure 5A). Since all three components are present in equimolar amounts, the effects of CSP and SGT on the Hsc70 ATPase activity are superadditive. Interestingly, the ATPase activation by CSP could not be increased by higher concentrations of CSP (data not shown), implying that CSP and SGT bind to distinct domains in Hsc70. This conclusion is indeed confirmed by mapping the respective binding domains (see Figure 1C).

A quantitative analysis of the hydrolysis kinetics (Figure 5B) provided initial rate constants as follows (given in nmol $\times$ l^{-1} $\times$ h^{-1}): v_0(Hsc70) = 0.42; v_0(Hsc70 and

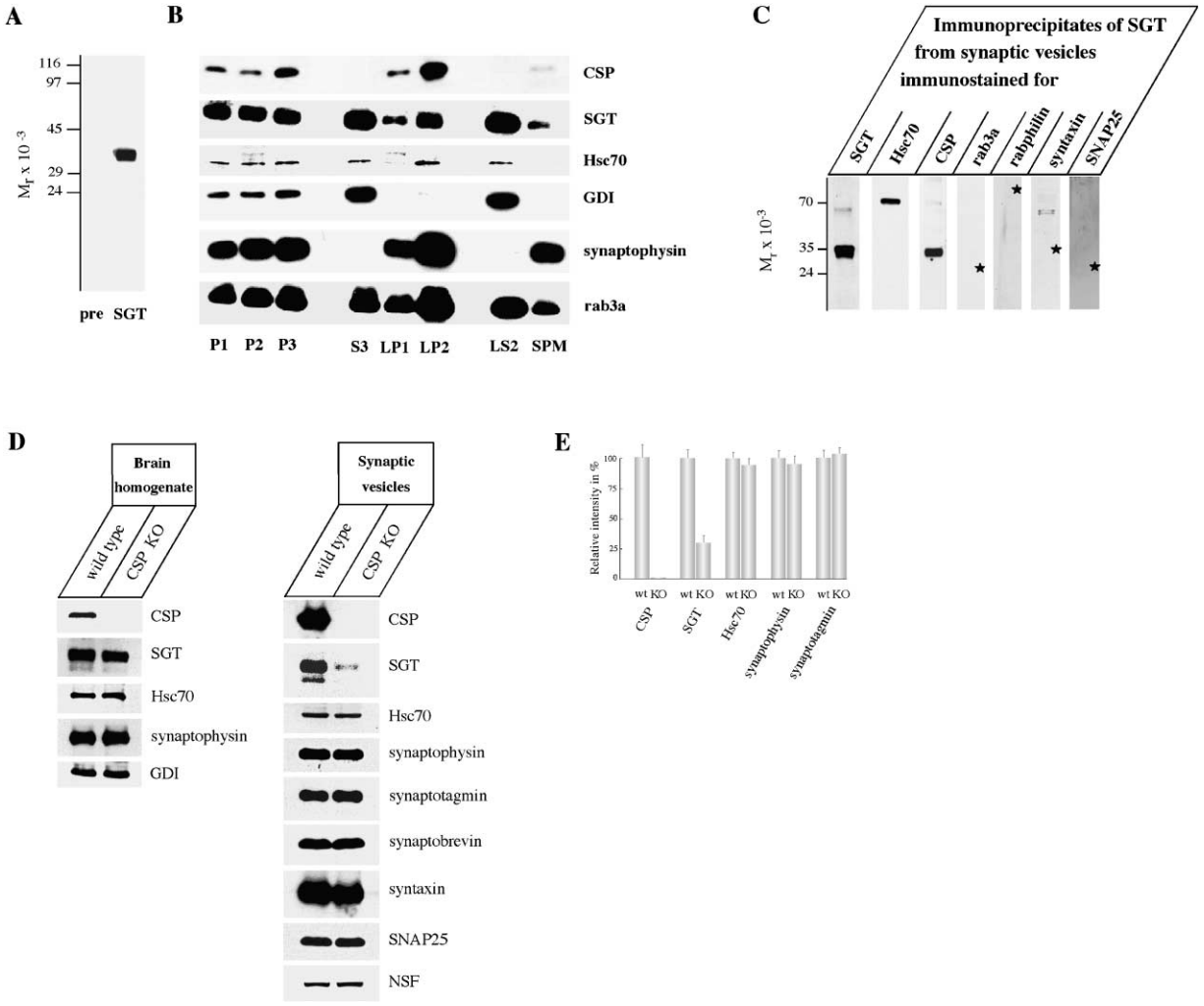

Figure 4. CSP, SGT, and Hsc70 Form a Trimeric Complex on Synaptic Vesicles

(A) A polyclonal rabbit antibody raised against recombinant SGT specifically recognized a protein with a molecular weight of 35 kDa. (B) Equal amounts of subcellular fractions from rat brain (20 μg/lane) were analyzed with specific antibodies. Subcellular fractions are designated as follows: P1, crude pellet with nuclei; P2, crude synaptosomal pellet; P3, light membrane pellet; S3, cytosolic fraction; LP1, lysed synaptosomal membranes; LP2, crude synaptic vesicle fraction; LS2, cytosolic synaptosomal fraction; SPM, synaptic plasma membranes. CSP is absent in cytosolic fractions (LS2 and S3) and highly enriched in LP2. SGT is predominantly found in cytosolic fractions (LS2 and S3). However, a substantial amount of SGT as well as Hsc70 is found in the synaptic vesicle fraction LP2. (C) SGT was immunoprecipitated from isolated synaptic vesicles using a polyclonal mouse antibody. These immunoprecipitates were analyzed with rabbit antibodies directed against diverse synaptic vesicle proteins. SGT, Hsc70, and CSP coimmunoprecipitated, demonstrating an interaction of these proteins on synaptic vesicles. Other synaptic vesicle proteins, such as rab3A, rabphilin, syntaxin, and SNAP25, lack in these immunoprecipitates and, therefore, are marked by asterisks. (D) Synaptic vesicles were prepared from CSP knockout and wild-type mice. In a synaptic vesicle fraction from CSP knockout mice, SGT was drastically reduced compared with wild-type mice. Other synaptic proteins were unchanged. (E) A quantitative analysis of synaptic vesicle proteins revealed a reduction of SGT by 72% in CSP knockout mice.

SGT) = 1.22; v_0(Hsc70 and CSP) = 4.95; v_0(Hsc70, SGT, and CSP) = 7.91. Please note that equimolar amounts of all three proteins were used to determine these rate constants. An exponential fit of the ATP hydrolysis data revealed a first-order kinetics during the first hour of incubation. Thereafter, a significant deviation from first-order kinetics was observed, suggesting that a partial inactivation of Hsc70 occurred during prolonged incubation times.

The Trimeric Complex Consisting of CSP, SGT, and Hsc70 Has Chaperone Activity

Three reasons prompted us to study a potential chaperone activity of the trimeric complex composed of CSP, SGT, and Hsc70. (1) As typical for chaperones, the CSP/Hsc70/SGT complex undergoes an association-dissociation cycle as a function of ADP and ATP, respectively. (2) CSP and SGT combined strongly activate the Hsc70 ATPase, most likely providing the free energy for refolding of a misfolded protein. (3) Hsc70 in combination with certain co-chaperones is known to act as chaperone (Hohfeld et al., 1995). To address the possibility that our trimeric complex behaves as a chaperone, we used denatured firefly luciferase as an artificial substrate. This assay is well established to study chaperone activities of proteins and protein complexes because the enzymatic activity of luciferase can easily be determined by measurement of bioluminescence (Szabo et al., 1994).

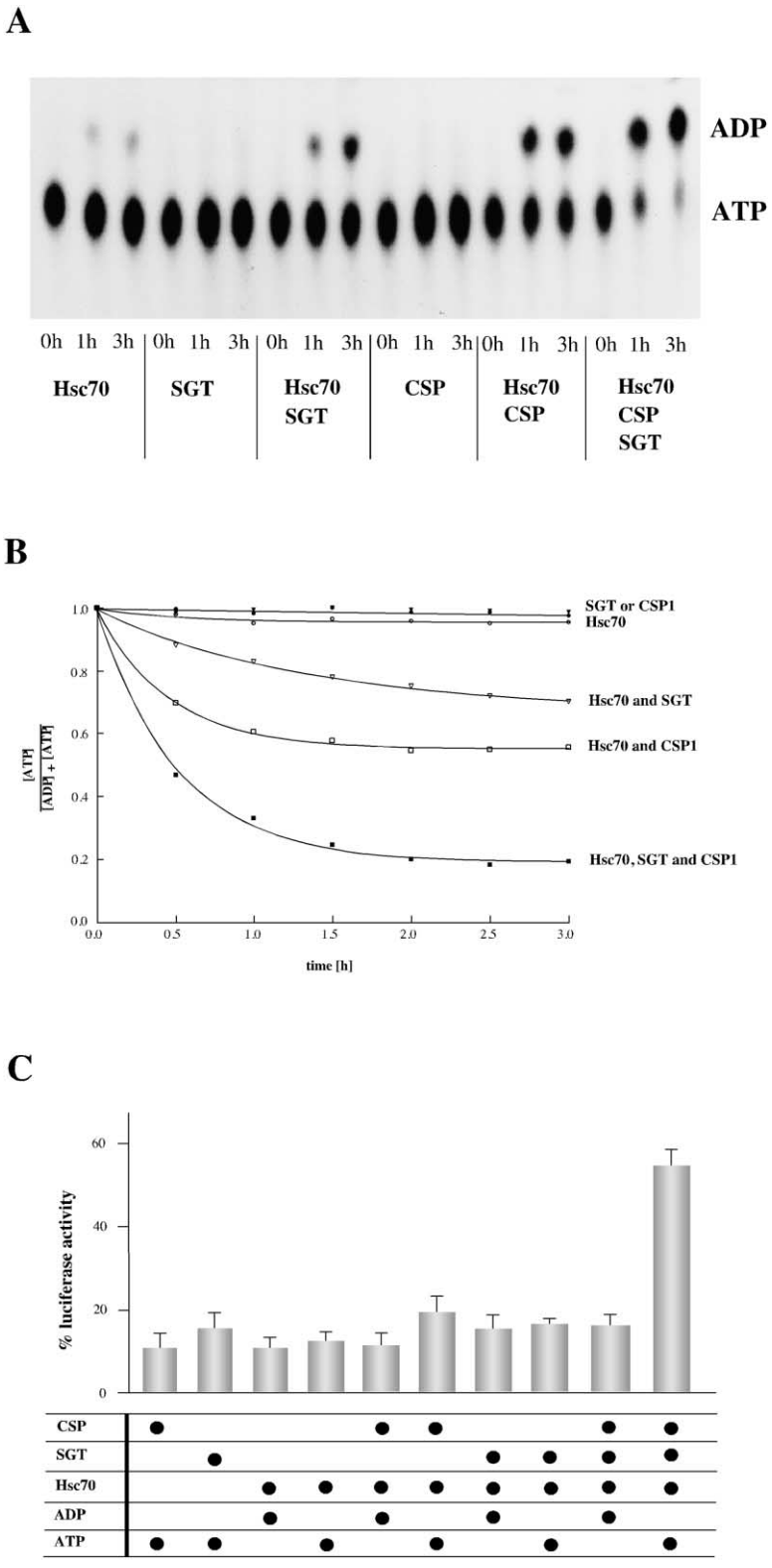

Figure 5. As Components of a Trimeric Chaperone Complex, SGT and CSP Strongly Activate the Hsc70 ATPase

(A) Various combinations of rat CSP, SGT, and Hsc70 were analyzed for ATPase activity. At the indicated times, hydrolysis of [α-^{32}P] ATP was determined by thin layer chromatography and autoradiography. Hsc70 revealed a weak intrinsic ATPase activity, whereas SGT and CSP did not show any detectable activity. Each Hsc70 binding protein by itself, i.e., CSP and SGT, activated Hsc70. A combination of both proteins, CSP and SGT, caused a superadditive effect on the ATPase activity of Hsc70. (B) A quantification of these data with the aid of a Phosphoimager revealed a first-order kinetics for short incubation times (within 1 hr). A combination of all three proteins induced a strong activation of the Hsc70 ATPase (19-fold). (C) Denatured firefly luciferase was diluted 100-fold into a buffer containing various combinations of CSP, SGT, and Hsc70 in the presence of either ATP or ADP. Luciferase activities were determined after 1 hr incubation at 30°C. Activities are expressed as percentage of native luciferase activity. SGT revealed a weak and nucleotide-independent chaperone effect (col. 2). The binary combination of CSP and Hsc70 showed a significant and ATP-dependent refolding activity toward denatured luciferase (col. 5 and 6). A combination of all three proteins strongly reactivated denatured luciferase in the presence of ATP (col. 10), demonstrating that the trimeric complex functions as a chaperone.

SGT seems to have a weak and ATP-independent chaperone activity (Figure 5C, col. 2). Hsc70 alone did not mediate the refolding of luciferase (col. 3 and 4). A combination of CSP and Hsc70 achieved a weak chaperone activity as a function of ATP (col. 5 and 6). However, when unfolded luciferase was incubated with a mixture of CSP, SGT, and Hsc70, renaturation of ~60% of the enzyme was observed (col. 10). Efficient renaturation required all three proteins, and was dependent on the presence of ATP (compare col. 9 with 10). We conclude

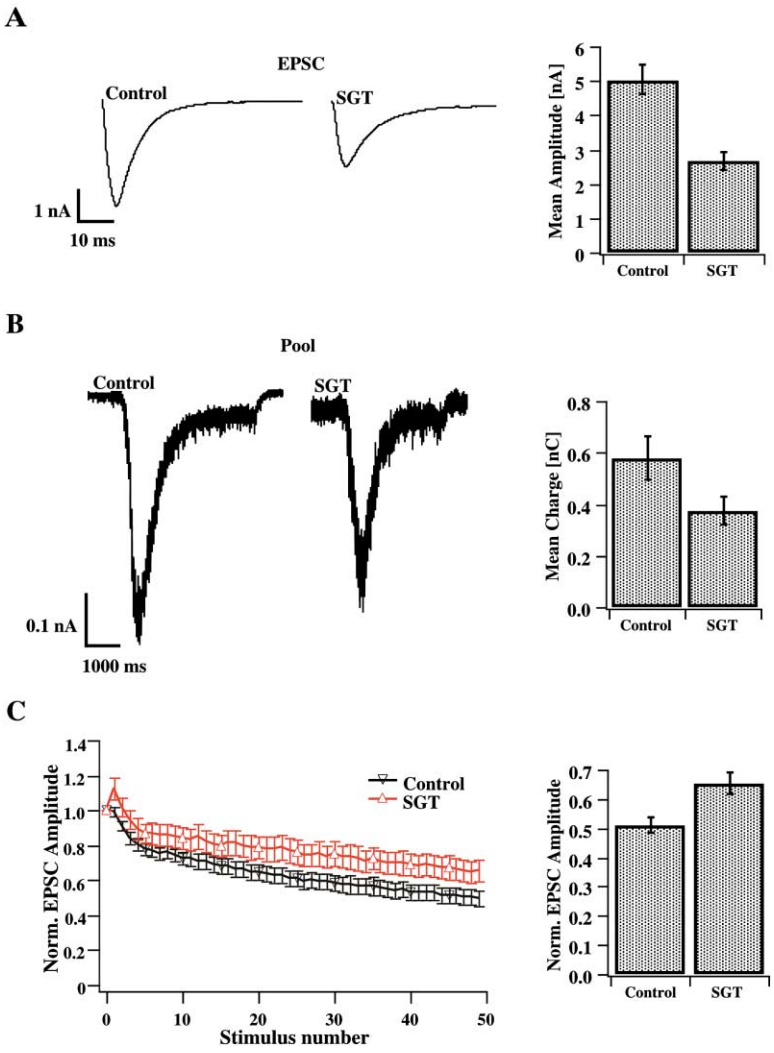

Figure 6. Overexpression of SGT in Hippocampal Neurons Leads to a Reduction in Synaptic Transmission

SGT/GFP was transiently overexpressed in hippocampal neurons. (A) Neurons were stimulated at a frequency of 0.2 Hz and the resulting excitatory postsynaptic currents (EPSC) were recorded. A reduction in EPSC amplitude to about 50% of the amplitude of control cells was observed. (B) A reduction in the readily releasable vesicle pool size to about 30% of the value of control cells was determined by application of hypertonic sucrose solution. (C) Neurons transfected with SGT/GFP were stimulated with 50 stimuli at 10 Hz. Compared with control cells, SGT-transfected cells showed a less pronounced synaptic depression rate.

from these results that SGT increases the efficiency of the CSP/Hsc70 system in this refolding reaction through both its ability to stabilize the CSP/Hsc70 complex and to activate the ATPase of Hsc70.

SGT Overexpression in Intact Synapses Leads to a Reduction of Synaptic Transmission

We next investigated the physiological consequences of the CSP/Hsc70/SGT interaction on synaptic transmission at intact synapses. For this purpose, we transiently overexpressed a fusion protein consisting of SGT and green fluorescent protein (GFP) in hippocampal neurons grown in microisland culture (Bekkers and Stevens, 1991). Six to eighteen hours after infection, isolated neurons were stimulated at a frequency of 0.2 Hz and the resulting autaptic postsynaptic currents were measured. As shown in Figure 6A, overexpression of SGT resulted in a marked and statistically highly significant reduction in EPSC amplitude to about 53 % of the amplitude of control cells (2.66 $\pm$ 0.25 nA, n = 58 for SGT-overexpressing cells versus 5.00 $\pm$ 0.44 nA, n = 66 for control cells; $p < 0.0001$). Since we have shown previously that neurons infected with green fluorescent protein alone display no reduction in mean amplitude (Lao et al., 2000), we can conclude that the reduction is a specific effect of the overexpression of SGT.

To determine if the observed reduction of EPSCs in SGT-overexpressing neurons is due to an underlying reduction in the size of the readily releasable vesicle pool, we assayed the size of this pool by application of hypertonic sucrose solution. We found that excitatory postsynaptic responses induced by application of hypertonic sucrose solution were markedly reduced in neurons overexpressing SGT (Figure 6B, left). However, the degree of this reduction was less than that observed for synaptically evoked responses (total charge transfer 0.58 $\pm$ 0.086 nC, n = 33, in control cells; 0.38 $\pm$ 0.052 nC, n = 32, in SGT-overexpressing cells, $p < 0.05$; Figure 6B, right). Consequently, the ratio between total charge transfer during an EPSC and that during sucrose stimulation was also altered when SGT-overexpressing cells were compared to wild-type control cells (0.088 $\pm$ 0.006, n = 33, in control cells; 0.060 $\pm$ 0.006, n = 32, in SGT-overexpressing cells, $p < 0.002$). These data suggest that SGT overexpression leads, in addition to a reduction in pool size, also to a reduction in vesicular release probability. To further confirm this possibility, we stimulated

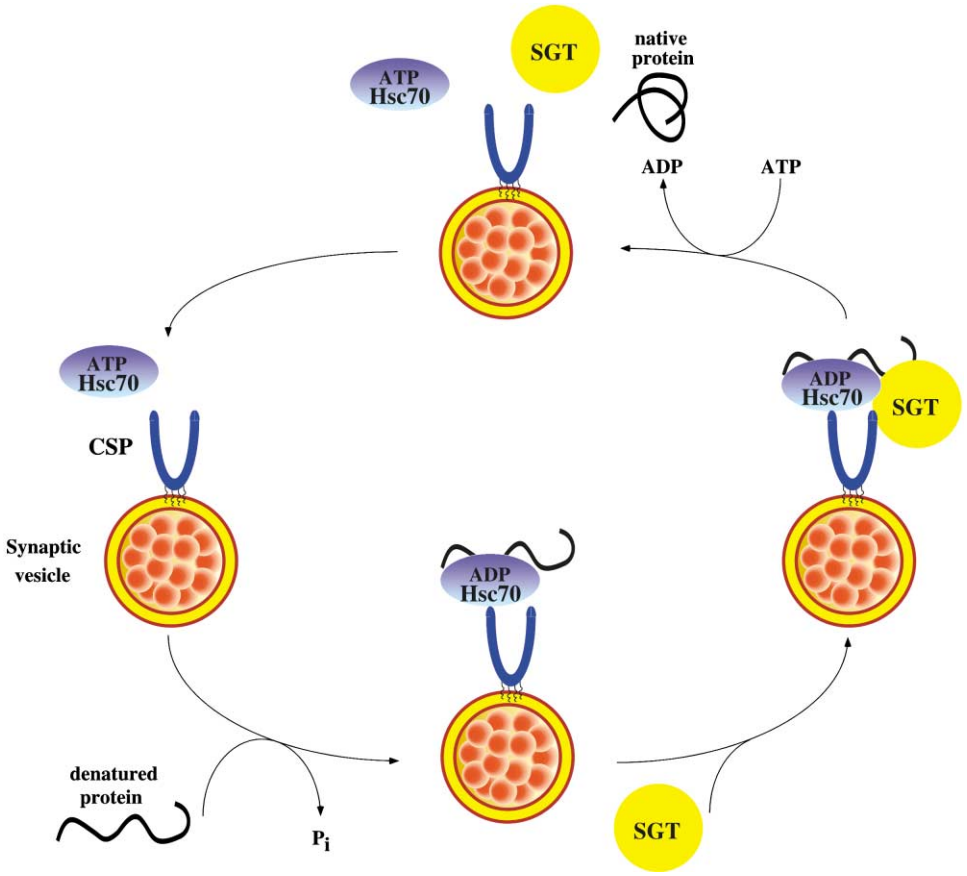

Figure 7. Model Showing the Association-Dissociation Cycle of the CSP/Hsc70/SGT Complex on the Synaptic Vesicle Surface

CSP is a synaptic vesicle protein inserted into the vesicle membrane by its hydrophobic palmitoyl side chains attached to cysteine residues. Based on the exclusive localization of CSP on synaptic vesicles, the analysis of CSP knockout mice, and coimmunoprecipitation results, we postulate that CSP recruits Hsc70 as well as SGT to the synaptic vesicle surface, thereby forming a trimeric protein complex. In the presence of ATP and available substrate, this protein complex dissociates. As a consequence of ATP hydrolysis and chaperone catalysis, unfolded protein substrates in the vicinity of the synaptic vesicle surface are refolded and reactivated. The physiological substrate for this chaperone machine is unknown. However, preliminary evidence suggests that presynaptic Ca^{2+} channels or SNARE proteins might be its physiological substrates.

cells with 50 stimuli at 10 Hz. Since autaptic responses in cultured hippocampal neurons are characterized by a marked activity-dependent depression (Mennerick and Zorumski, 1995; Rosenmund and Stevens, 1996), a reduction in release probability should be reflected in a less pronounced depression. Indeed, SGT-overexpressing cells displayed an average steady-state depression level of 65.3 $\pm$ 6.1 % ($n = 22$), which is significantly higher ($p < 0.05$) than that from control cells, which had an average steady-state depression level of 50.0 $\pm$ 4.8% (Figure 6C). Therefore, we conclude that the observed decrease in synaptic transmission upon overexpression of SGT is caused by both a reduction in the size of the readily releasable pool and a reduction in vesicular release probability.

Discussion

CSP is a small, abundant, and evolutionarily conserved synaptic vesicle protein which contains an N-terminal J domain and is bound to the synaptic vesicle membrane by a string of palmitoylated cysteine residues (Buchner and Gundersen, 1997). In addition to synaptic vesicles, CSP is found on other secretory vesicles, suggesting a general function in regulated secretion (Braun and Scheller, 1995; Chamberlain et al., 1996). In *Drosophila*, null mutants in CSP exhibit a fascinating phenotype: most mutant flies die, but a small percent of the flies survive, indicating that CSP is important but not essential for synaptic transmission. The survivors exhibit changes in synaptic transmission, including alterations of short-term synaptic plasticity and a temperature-induced block of neurotransmitter release (Zinsmaier et al., 1994). Interestingly, the Ca^{2+} sensitivity in the mutant nerve terminals is reduced (Dawson-Scully et al., 2000), and the block in temperature-dependent inhibition of neurotransmitter release can be rescued with Ca^{2+} ionophores (Ranjan et al., 1998). These results demonstrated that CSP has a specific action in coupling the Ca^{2+} signal to secretion in synaptic nerve terminals.

The temperature-induced phenotype of the fly CSP mutants agrees well with the notion that CSP may be a component of a synaptic chaperone machine, as was suggested by the J domain in CSP and by the interaction of the J domain with the heat-shock protein cognate Hsc70 (Braun et al., 1996; Chamberlain and Burgoyne,

1997; Stahl et al., 1999). However, the nature of this chaperone machine, its composition, and function were unknown. We have now characterized a tripartite complex composed of CSP, Hsc70, and a TPR-domain protein called SGT, and demonstrate that this complex constitutes a synaptic chaperone machine. Using a yeast two-hybrid screen for CSP-interacting proteins, we first identified a novel binding partner for CSP, a small glutamine-rich tetratricopeptide repeat (TPR)-containing protein called SGT. We then showed that SGT binds not only to CSP, but also to Hsc70. Hsc70, in turn, directly interacts with CSP (Braun et al., 1996; Chamberlain and Burgoyne, 1997; Stahl et al., 1999). Based on coimmunoprecipitation experiments from purified synaptic vesicles and the strong reduction of SGT on vesicles lacking CSP because of a genetic deletion, we concluded that CSP, Hsc70, and SGT form a tripartite protein complex on synaptic vesicles. Five features highlight the significance of this trimeric complex. (1) It undergoes an association-dissociation cycle driven by ATP hydrolysis. (2) Simultaneous binding of CSP and SGT to Hsc70 maximally stimulates the ATPase activity of Hsc70 to a level 19-fold higher than that of Hsc70 alone. (3) The trimeric complex functions as a chaperone and exhibits a strong refolding activity as measured with a denatured model substrate, luciferase, whereas the respective monomers or dimers were inactive. (4) On synaptic vesicles CSP, SGT and Hsc70 form a stable protein complex. (5) Overexpression of SGT in hippocampal neurons (Figure 6) and CSP in chromaffin cells (Graham and Burgoyne, 2000) as well as hypomorphic mutations in *Drosophila* Hsc70 (Bronk et al., 2001) lead to a similar reduction in synaptic transmission. We conclude from these data that the CSP/Hsc70/SGT complex is the functional form of CSP and acts as a chaperone machine on the synaptic vesicle surface.

In the CSP/Hsc70/SGT complex, CSP is the most restricted component in terms of evolutionary conservation, localization, and expression. Hsc70, in turn, is the most general component, and SGT is somewhat intermediate. SGT and Hsc70 are widely expressed in many tissues, and are evolutionarily conserved in all eukaryotes down to yeast (SGT) or even bacteria (Hsc70). In contrast, CSP is primarily expressed in brain and in tissues specialized for exocytosis (Mastrogiacomo et al., 1994; Braun and Scheller, 1995; Chamberlain et al., 1996), and is not present in lower eukaryotes such as yeast. These differences in expression patterns in tissues and in evolution imply that SGT and Hsc70 perform functions independent of CSP, possibly by collaborating with other J domain proteins. According to this view, CSP would provide localization and substrate specificity to a more indiscriminate chaperone activity executed by Hsc70 and SGT. Indeed, Hsc70 has been shown to interact with a number of different J domain and TPR domain proteins (Ungewickell et al., 1995; Liu et al., 1999), and at least one trimeric chaperone machine similar to the one described here has been previously characterized (Hohfeld et al., 1995).

The physiological substrate for the CSP/Hsc70/SGT complex remains unknown. Since CSP is attached to the synaptic vesicle membrane, it is likely that the substrate of the complex is found in vicinity of the synaptic vesicle surface. Based on our data, we propose a speculative working model for the function of the tripartite complex on the synaptic vesicle surface (Figure 7). According to this model, the J domain of CSP recruits Hsc70 to the synaptic vesicle surface. The C-terminal domain of CSP binds to SGT, thereby forming a trimeric protein complex. This complex is stabilized by interactions between Hsc70 and SGT. Our data suggest that the tripartite complex has a chaperone activity for a misfolded protein or for a protein complex that does not spontaneously dissociate. In vertebrates, direct and indirect interactions of CSP with voltage-gated Ca^{2+}-channels have been demonstrated (Leveque et al., 1998; Magga et al., 2000). CSP has also been proposed to bind to the SNAREs syntaxin (Nie et al., 1999) or synaptobrevin/VAMP (Leveque et al., 1998), raising the possibility that the CSP/Hsc70/SGT complex may restore the biological activity of voltage-gated Ca^{2+} channels either by transferring them into a SNARE complex, or rescuing them from such a complex.

Experimental Procedures

cDNA Cloning, Construction of Expression Vectors, Sequencing, Expression of Recombinant Proteins

Full-length cDNAs for rat CSP1 and Hsc70 were generated by polymerase chain reaction (PCR) using primers specific for the respective 5′- and 3′-ends of their coding regions and rat brain cDNA (Clontech) as template. For amplification by PCR, *Pfu* polymerase (Stratagene) was used. The PCR products were subcloned into pGEX-KG (Guan and Dixon, 1991) using restriction enzyme sites placed into the oligonucleotide sequences. A partial sequence of SGT obtained by a yeast two-hybrid screen was used as probe to screen a rat brain cDNA library in λZAP II at high stringency. Yeast bait vector constructs were generated by subcloning of CSP1 and SGT cDNA into pLexN (Vojtek et al., 1993). Prey vector constructs for Hsc70 and SGT were obtained by subcloning of PCR fragments into pVP16 (Vojtek et al., 1993). His_6-tagged Hsc70 and SGT were created by cloning the respective BamHI/NotI fragments into pET-28a (Novagen). A Semliki Forest Virus construct encoding full-length SGT in-frame with EGFP was constructed by cloning of a blunt-end SGT-EGFP cDNA into the SmaI site of pSFV1 (GIBCO-BRL). The identity of most DNA constructs was confirmed by sequencing. GST-tagged fusion proteins were expressed in *Escherichia coli* (strain XL1-Blue) and purified according to a standard procedure (Smith and Johnson, 1988). His_6-tagged fusion proteins were expressed in the *E. coli* strain BL21(DE3) and purified as described (Noji et al., 1997).

Yeast Two-Hybrid Screen and Interaction Assay

Yeast strain L40 (Vojtek et al., 1993) was sequentially transfected with the bait vector (full-length rat CSP cloned into pLexN) and a rat brain cDNA library (Vojtek et al., 1993) using lithium acetate (Schiestl and Gietz, 1989). All the following steps were performed as described (Stahl et al., 1999).

Gel Filtration

Proteins bound to immobilized GST/CSP on glutathione beads were eluted with 25 mM glutathione in 0.2 M Tris (pH 8.0). Following centrifugation at 100,000 × g for 10 min, the eluate was subjected to gel filtration on a FPLC Superose 6 column (Pharmacia) according to the instructions of the manufacturer. When indicated, the column run was performed in the presence of 1 mM ADP and ATP, respectively. Thirty fractions with a fraction size of 1.8 ml were obtained. To increase the protein concentration for a subsequent SDS gel electrophoresis, fractions were concentrated 25-fold using a standard procedure.

In Situ Hybridization

Male Wistar rats (8 weeks old) were anaesthetized and decapitated. All the following steps were performed as described (Augustin et

al., 1999). Antisense oligonucleotides representing the following sequences were chosen as probes: bp 37–81/466–510 of CSP (GenBank accession number S81917); bp 1040–1084/1335–1379 of Hsc70 (GenBank accession number X70065); bp 226–270/612–656 of SGT (GenBank accession number AJ222724).

Determination of Hsc70 ATPase Activity

Hsc70 ATPase activity was assayed with minor modifications as described (Braun et al., 1996). In brief, the rate of ATP hydrolysis was analyzed after incubation of 0.5 μM GST/Hsc70 with 0.5 μM GST/CSP and 0.5 μM GST/SGT in 100 mM Tris (pH 8.4), 100 mM KCl, 0.5 mM DTT, 2 mM $MgCl_2$, 5 nM [α-^{32}P] ATP (20 mCi/mmol) for the indicated times at 37°C. Afterwards, radioactive nucleotides were separated by thin layer chromatography on PEI cellulose and analyzed with a Phosphoimager (Fuji).

Luciferase Refolding Assay

Firefly luciferase (Boehringer Mannheim) was denatured at a concentration of 3 μM in 6 M guanidinium-HCl, 30 mM Tris (pH 7.4), 3 mM DTT at 25°C for 1 hr. The unfolded protein was diluted 100-fold into 10 mM MOPS (pH 7.2), 50 mM KCl, 3 mM $MgCl_2$, 5 mM DTT containing approximately 0.5 μM His_6-SGT, 0.5 μM GST/CSP, 0.5 μM His_6-Hsc70 and 1 mM ATP when indicated. Luciferase activities were measured after 1 hr incubation at 30°C.

Generation and Maintenance of CSP Knockout Mice

A knockout vector was constructed from a mouse genomic CSP clone. Exon 1 and flanking introns were replaced by a neomycin resistance gene cassette. Embryonic stem cells were electroporated with the vector and selected with G418 (Life Technologies, Inc.) and FIAU essentially as described (Rosahl et al., 1993). Resistant embryonic stem cell clones were analyzed by polymerase chain reaction for homologous recombination. Positive clones were injected into blastocysts, resulting in the generation of a single mouse line that was bred to homozygosity. These knockout mice are available for distribution.

Hippocampal Neuron Culture and Electrophysiology

Microisland culture preparation of hippocampal neurons was performed according to a modified version of published procedures (Bekkers and Stevens, 1991). After 10–14 days in culture, cells were infected with 50 μl of an activated Semliki Forest virus containing the cDNA of full-length SGT coupled to green fluorescent protein (GFP) at its C-terminal end. All measurements were performed 12 to 18 hours after infection. Only dots containing a single neuron forming excitatory synapses (autapses) were used. Data are expressed as mean ± standard error. Statistical significance was tested by Mann-Whitney U test.

Antibodies

Polyclonal rabbit and mouse antibodies directed against rat CSP and SGT were generated with GST fusion proteins containing full-length constructs of both proteins. A goat antibody against Hsc70 was purchased from Santa Cruz Biotechnology, and a mouse antibody specific for the His_6 epitope was from Qiagen. Antibodies to GDI (GDP dissociation inhibitor, cl. 81.2), synaptophysin (cl. 7.2), synaptotagmin (cl. 604.4), synaptobrevin (cl. 69.1), syntaxin (cl. 78.2 and R31), SNAP25 (cl. 71.1 and R28), NSF (cl. 83.1), rabphilin (l734), and rab3A (cl. 42.2 and R9) were kindly provided by Dr. R. Jahn (Max Planck-Institute for Biophysical Chemistry, Göttingen, Germany).

Miscellaneous Procedures and Materials

All chemicals were of the highest available purity and were purchased from standard sources. SDS-polyacrylamide gel electrophoresis and immunoblotting were performed with minor modifications as described (Laemmli, 1970; Towbin et al., 1979). Immunoprecipitation of synaptic proteins was performed as described (Stahl et al., 1996). Immunoreactive bands were visualized with enhanced chemiluminescence (Amersham Corp.). Subcellular fractionation of brain tissue was done according to a standard procedure (Walch-Solimena et al., 1995).

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