Phosphorylation of Cysteine String Protein by Protein Kinase A

IMPLICATIONS FOR THE MODULATION OF EXOCYTOSIS*

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Cyclic AMP-dependent protein kinase (PKA) enhances regulated exocytosis in neurons and most other secretory cells. To explore the molecular basis of this effect, known exocytotic proteins were screened for PKA substrates. Both cysteine string protein (CSP) and soluble NSF attachment protein-α (α-SNAP) were phosphorylated by PKA in vitro, but immunoprecipitation of cellular α-SNAP failed to detect 32P incorporation. In contrast, endogenous CSP was phosphorylated in synaptosomes, PC12 cells, and chromaffin cells. In-gel kinase assays confirmed PKA to be a cellular CSP kinase, with phosphorylation occurring on Ser10. PKA phosphorylation of CSP reduced its binding to syntaxin by 10-fold but had little effect on its interaction with HSC70 or G-protein subunits. Furthermore, an in vivo role for Ser10 phosphorylation at a late stage of exocytosis is suggested by analysis of chromaffin cells transfected with wild type or non-phosphorylatable mutant CSP. We propose that PKA phosphorylation of CSP could modulate the exocytotic machinery, by selectively altering its availability for protein-protein interactions.

Exocytosis is the final stage of the secretory pathway and involves the fusion of secretory vesicles with the plasma membrane in a constitutive or regulated manner (1). In regulated exocytosis, vesicles accumulate in the cytoplasm and only fuse with the plasma membrane upon receipt of an appropriate stimulus (usually, but not always, an increase in intracellular free Ca2+). As regulated exocytosis is the basis of chemical transmission in the brain, much research has been devoted to uncovering its molecular mechanism. This has revealed the involvement of a large number of proteins (2, 3), which can be classified into three groups. The first group, proteins involved in vesicle fusion events in all eukaryotes, includes the SNAP150 receptors, SNAPs, RABs, and the Sec1 family. The second group comprises proteins involved in regulated exocytosis in various cell types and diverse organisms but absent in yeast. This group includes the synaptotagmins and cysteine string proteins (CSP). The third class can be defined as proteins whose role in regulated exocytosis is cell type-specific. An example from this group is the synapsins, which are important modulators of the synaptic vesicle cycle in neurons (4). The complex interactions between the numerous proteins of these classes presumably enables sophisticated fine-tuning of exocytosis to suit the particular physiological needs of each cell type.

In addition to the cell type-specific repertoire of exocytotic proteins expressed, further control over the exocytotic mechanism can be exerted post-translationally (5). Indeed, a large number of studies have implicated protein kinases in the modulation of regulated exocytosis from many cell types by using cell-permeable inhibitors or activators, including Ca2+/calmodulin-dependent protein kinase II (6, 7), mitogen-activated protein kinase (8), cGMP-dependent protein kinase (9), and tyrosine kinases (8). However, one shortcoming of this approach is that the modulation of exocytosis may be indirect, either by effects on membrane receptor or ion channel phosphorylation or via direct steric inhibition of ion channels (e.g. Ref. 10). Thus, application of kinase activators or inhibitors (or indeed purified kinases themselves) to permeabilized cells, where receptors and ion channels are bypassed, is a more rigorous demonstration of a role for protein kinases in the direct regulation of the exocytotic machinery. However, a review of the literature reveals only PKA and PKC or their pharmacological effectors produce an almost universal enhancement of Ca2+-triggered exocytosis in all secretory models studied, for example nerve terminals (11), chromaffin cells (12–14), PC12 cells (15), AtT-20 cells (16), pancreatic acinar cells (17), parotid acinar cells (18), SPOC1 cells (19), neutrophils (20), and mast cells (21). Therefore, identification of PKA or PKC exocytotic substrates will reveal fundamental mechanisms for the direct regulation of exocytosis by phosphorylation.

To address this issue, our approach was to screen known exocytotic proteins for in vitro kinase substrates. Reasoning that this information would only be relevant if the phosphorylation(s) observed also occurred in the cell, we set out to confirm this and to subsequently determine any functional significance of in vivo phosphorylation. In the present study, we have identified the synaptic vesicle protein CSP as a novel PKA substrate both in vitro and in three different neuronal/neuroendocrine cell preparations, and we mapped the phosphorylation site to Ser10 in the conserved N-terminal domain of CSP. We also show that Ser10 phosphorylation can reduce CSP binding to syntaxin but not to HSC70 or G-protein subunits. Furthermore, mutation of Ser10 to a non-phosphorylatable alanine residue alters the known effects of CSP overexpression on the
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**Materials—** CSP rabbit polyclonal antiserum was as described previously (22). Anti-α-SNAP monoclonal antibody was obtained from Synaptic Systems (Göttingen, Germany). Purified G-proteins and anti-Gα11003 antiserum were obtained from Calbiochem. Anti-Gβ3 monoclonal antibody was obtained from Affiniti (Exeter, UK). Catalytically active PKC was from Alexis Corp. (Nottingham, UK). Synthetic CSP (4–14) peptides, each with an additional N-terminal cysteine residue, were from MWG Biotech (Milton Keynes, UK). The sequences of these peptides were CSP-(4–14), CQRQRLSTSGSE; CSP-(4–14)-S10A, CQRQRLATSSE; and CSP-(4–14)-S10P5, CQRQRLSPSTSGSE (where pS is phosphoserine). [32P]Orthophosphate, [γ-32P]ATP, goat anti-rabbit IgG, glutathione- and protein G-coupled Sepharose FF beads were obtained from Amersham Biosciences. Collagenase was from Lorne Laboratories (Oxford, UK). PKA catalytic subunit, H-89, 8-Br-cAMP, Kemptide, purified HSC70, and all other reagents were obtained from Sigma. Expression and purification of recombinant Hisα105 tagged CSP1, α-SNAP, complexin, and Rabα5A protein were performed as described previously (23). Recombinant GST-syntainax 1A and GST-VAMP2 were expressed and purified as described previously (24, 25). Recombinant purified synaptotagmin and SNAP-25A2 were gifts from Dr. D. Appel (University of Edinburgh, UK) and Dr. M. Wilson (University of New Mexico, Albuquerque, NM), respectively. Recombinant purified neuronal calcium sensor 1 was as described previously (26). pCMV-syntainax (cytosolic domain) was a gift from Dr. M. Bittner (University of Michigan).

**Generation of CSP Mutant Constructs—** Site-directed mutagenesis of pcDNA3.1-nyc-csp (27) was achieved using the Quickchange system (Stratagene). For the S10A mutant, the primers are as follows: sense, 5′–CGCCGCTACCTCCTGCTTGACCTCTGGGGAG–3′ and antisense, 5′–CTCCCATAGGATACGAGCATGGAGCGGC–3′. Nucleotides in parentheses indicate bases that were changed to generate the amino acid substitution. The mutated sequence generated an NdeI restriction site. The plasmid was used to select mutant colonies. Automated sequencing was performed in both directions across the entire coding sequence to ensure introduction of the desired mutation only (University of Durham, Durham, UK).

**Cell Culture and Transfection—** Primary cultures of chromaffin cells were prepared from freshly dissected bovine adrenal glands. Briefly, each gland was flushed three times with 10 ml of Krebs buffer and then transferred to nitrocellulose, followed by exposure of the blots to a Phosphorscreen and then immunoblotting for CSP or α-SNAP. Phos-
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Identification of Exocytotic Proteins That Are PKA or PKC Substrates—The phosphorylation by PKA and PKC of 10 proteins known to have a direct or modulatory role in the late stages of exocytosis was studied (Fig. 1A). Proteins that were not phosphorylated by either kinase were complexin, neuronal calcium sensor-1, syntaxin 1A, and VAMP2. The PKC phosphorylation of nSec1, SNAP-25A, and synaptotagmin confirmed previous observations (37–39). However, CSP, Rab3A, and α-SNAP were novel PKC substrates. Therefore, this initial screen generated an abundance of PKC substrates that are potential candidates for the PKC-mediated regulation of exocytosis, some of which have been studied previously (37–41).

In contrast to PKC, our data for PKA phosphorylation agrees with previous findings (24) that very few exocytotic proteins are PKA substrates. We found that α-SNAP is phosphorylated by PKA, in agreement with Ref. 24, and that CSP is a novel PKA substrate. Because there is a wealth of data supporting a role for PKA in the modulation of exocytosis, the study of any exocytotic PKA substrate is particularly pertinent. We therefore pursued the characterization of CSP and α-SNAP phosphorylation by PKA. Fig. 1B confirms that both CSP and α-SNAP are good in vitro substrates for PKA. Under conditions optimized for maximal phosphorylation, it was found that phosphorylation of CSP by PKA plateaued after 60 min at 30 °C at a stoichiometry of ~1.0 mol of phosphate/mol of protein (Fig. 1B). α-SNAP by comparison was phosphorylated to a lesser extent by PKA with a stoichiometry of only ~0.6 mol of phosphate/mol of protein (Fig. 1B). Because the efficiency of in vitro phosphorylation of a recombinant protein is not a true indication of any in vivo phosphorylation events, we went on to characterize both CSP and α-SNAP phosphorylation in vivo.

CSP Is Phosphorylated in Vivo—To assess whether endogenous CSP and α-SNAP are phosphorylated in vivo, we employed two alternative neuronal model systems commonly used for studying regulated exocytosis, bovine adrenal chromaffin cells, and rat brain synaptosomes. The cells or synaptosomes were labeled with [32P]orthophosphate and subjected to stimulation with a secretagogue (nicotine or KCl, respectively) or the cell-permeable PKA agonist, 8-Br-cAMP, and then lysed. CSP and α-SNAP were immunoprecipitated from the lysates with specific antisera, subjected to two-dimensional gel electrophoresis, and transferred to nitrocellulose membrane. Incorporated 32P was detected by Phosphorcreen (Fig. 2), whereas location of the protein on the two-dimensional membrane was confirmed by immunoblotting with the immunoprecipitating antibody. Densitometric analysis of immunoprecipitated CSP demonstrated that it was phosphorylated in synaptosomes under resting conditions. Interestingly, in chromaffin cells, phosphorylation of CSP was induced by nicotine or 8-Br-cAMP treatment (Fig. 2A). α-SNAP was not detectably phosphorylated under any condition in either chromaffin cells or synaptosomes (Fig. 2B).

The direct phosphorylation of CSP by PKA in vitro together with the stimulation of CSP phosphorylation by cAMP treatment in vivo suggests that PKA is a cellular CSP kinase. We employed an in-gel kinase assay to confirm that PKA from cell and tissue lysates could phosphorylate CSP. Triton-soluble lysates from rat brain tissue, synaptosomes, PC12 cells, and chromaffin cells and purified PKA catalytic subunits were resolved on an SDS-PAGE gel with or without CSP contained in the gel matrix. Following denaturation and renaturation of the lysate proteins, the gels were incubated in a kinase reaction buffer containing [32P]ATP, washed, and exposed to a Phosphorcreen. No significant kinase autophosphorylation was observed in the control gel (Fig. 3B). In the CSP-containing gel the catalytic subunit of PKA (Fig. 3C, lane 1) produced an intense band of ~40 kDa (the predicted mass of this protein) corresponding to the band on the Coomassie stain of the same gel (Fig. 3A, lane 1). A band of ~40 kDa was observed in all of the lysate lanes of the same molecular weight as the catalytically active enzyme.
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subunit of PKA (Fig. 3C, lanes 2–5). Addition of the PKA inhibitor H-89 (42) to the kinase reaction buffer for a CSP-containing gel almost abolished phosphorylation of the 40-kDa band in all lanes (Fig. 3D), confirming that the 40-kDa band observed in Fig. 3C, lanes 2–5, was PKA.

Identification of the in Vivo PKA Phosphorylation Site of CSP—By having established that CSP is a probable in vivo substrate for PKA, we sought to identify the phosphorylation site(s) by using preparative quantities of recombinant His₆-CSP phosphorylated in vitro by PKA with [γ-³²P]ATP. PKA-and mock (identical conditions in the absence of kinase)-phosphorylated His₆-CSP was prepared, and the proteins were digested with trypsin and the resulting peptides separated by RP-HPLC (Fig. 4, A and B). It was found that the HPLC A₂₄₅ trace for PKA-phosphorylated CSP contained an additional peak (the peak denoted by * in Fig. 4B) when compared with the trace for the mock-phosphorylated protein (Fig. 4A). Fractions collected manually containing peaks of peptide content were subjected to Cerenkov counting, and only the additional peak found in the PKA-phosphorylated sample contained ³²P. All of the peptide fractions from the trypptic digestion were sequenced by Edman degradation, and it was found that the phosphorylated peptide had the same sequence as an adjacent peak (the peak denoted by # in Fig. 4, A and B) found in both the mock and phosphorylated samples and corresponding to CSP-(8–24) with the sequence SLSTSGESLYHVLGLDK (Fig. 4C). Because this peptide contains 4 serines and 1 threonine the specific residue(s) phosphorylated by PKA could not be instantly identified. Thus, to determine the location of ³²P-labeled residues in CSP-(8–24), the peptide was covalently attached to a Sequelon membrane and subjected to Edman degradation. The sequentially released amino acid derivatives were counted for radioactivity, and virtually all of the radioactivity contained in CSP-(8–24) was in Ser¹⁰ (Fig. 4D).

To demonstrate further that PKA only phosphorylates Ser¹⁰ and not either of the 2 serines or 1 threonine residue immediately surrounding Ser¹⁰, we performed in vitro PKA phosphorylation of a synthetic peptide corresponding to CSP-(4–14) and two peptides with either an alanine or phosphoserine residue substituted at the 10-position (Table I). Kemptide, an ideal PKA peptide substrate (43), was assayed in parallel for comparison. Under conditions optimized for kinetic measurements, the CSP-(4–14) peptide was phosphorylated with Kₘ and Vₘₐₓ values comparable with that of Kemptide (Table I). However, the alanine- and phosphoserine-substituted peptides, CSP-(4–14)-S10A and CSP-(4–14)-S10pS, respectively, were not detectably phosphorylated at concentrations of up to 30 μM. The phosphorylation of CSP by PKA in vitro is therefore specific to Ser¹⁰.

Analysis of the CSP Phosphorylation Site(s) in Vivo—To ascertain whether endogenous CSP is phosphorylated on Ser¹⁰ in vivo, we needed to immunoprecipitate a large quantity of CSP from ³²P-labeled cells for analysis by trypptic digestion and HPLC separation. For maximal CSP immunoprecipitation, we employed a PC12 cell line that overexpresses CSP¹ (29). As shown in Fig. 5A, CSP is highly overexpressed in PC12 clone 1 cells (29) compared with wild type cells, as shown previously (29). We first confirmed that overexpressed CSP in PC12 cells was subject to phosphorylation as shown previously for chromaffin cells and synaptosomes. ³²P-Labeled clone 1 cells were treated with Krebs (control), a secretogogue (300 μM ATP), or 500 μM 8-Br-cAMP and lysed, and CSP was immunoprecipitated. Fig. 5B shows CSP phosphorylation is easily detectable under control conditions and not significantly altered by secretogogue or kinase agonist stimulation, similar to that seen in synaptosomes. Because the constitutive CSP phosphorylation in untreated control cells was high and the in-gel kinase assay suggested PKA is the only PC12 CSP kinase (Fig. 3A, lane 3), we decided to use ³²P-labeled control 1 PC12 cells for the in vivo site determination of CSP. 10 × 10⁶ PC12 cells were labeled for 4 h with 1.5 μCi of ³²P, and lysed. All of the CSP immunoprecipitated from the PC12 lysate and 2 μg of ³²P-labeled His₆-CSP that had been phosphorylated in vitro by PKA were sep-
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Recombinant CSP is phosphorylated on Ser^{10} by PKA in vitro. 2 μg of His_{6}-CSP was incubated with 2 μCi of [γ-^{32}P]ATP in the absence (A, mock) or presence (B, + PKA) of 0.4 μg of the catalytic subunit of PKA. The proteins were digested with trypsin and separated by RP-HPLC. A and B show the A_{214} peptide traces for the mock- and PKA-phosphorylated CSP. The major peptide peaks were sequenced by Edman degradation and subjected to liquid scintillation counting (C). Only one peptide peak, that corresponding to the extra peak in (B), was found to contain ^{32}P (*). This peak had the same sequence as a non-radioactive peak immediately next to it, found also in the mock sample (#). D, in order to discover the ^{32}P-labeled residue(s) in the radioactive phosphopeptide, the peptide was covalently attached by its C terminus to a Sequelon membrane and subjected to Edman degradation. The released amino acid fractions were assayed for radioactivity by Cerenkov counting.

Bovine serum phosphatase was added to the mock sample (Fig. 5C) thus, in PC12 cells CSP is phosphorylated in the same region (CSP-(8–24)) as that observed in His_{6}-CSP. Our data demonstrating that PKA is a major CSP kinase in the cell and the absolute specificity of PKA for Ser^{10} phosphorylation is convincing evidence that Ser^{10} is the likely CSP phosphorylation site in vivo.

Phosphorylation of CSP Inhibits Its Binding to Syntaxin in Vitro—One of the major functional effects of protein phosphorylation is to change the affinity of interaction of a protein with its binding partners. CSP has been shown to interact directly in vivo with syntaxin, HSC70, and the α- and β-subunits of heterotrimeric G-proteins (44–48). To ascertain what effect phosphorylation of CSP by PKA might have upon its biochemical function, we studied its interactions with these proteins. We performed a GST pull-down assay with PKA- or mock-phosphorylated recombinant His_{6}-CSP and GST-syntaxin 1A. Equal amounts of syntaxin were eluted from the beads under all conditions (data not shown). Eluted CSP protein was quantified by Western blotting, which included the use of a ^{125}I-labeled secondary antibody that allowed linear quantitation of CSP protein across the range of concentrations used (Fig. 6A). A small amount of CSP bound to GST alone (Fig. 6A, 7th lane), and this was subtracted from the binding to GST-syntaxin to give the absolute amount of CSP bound to syntaxin (Fig. 6A). CSP bound GST-syntaxin in a dose-dependent manner with a maximal ~5% of total input CSP being recovered with syntaxin, similar to previous observations (35, 44, 45). Phosphorylation of CSP by PKA resulted in a profound decrease in the affinity of CSP for syntaxin (Fig. 6A). These data suggest a potential functional effect of CSP phosphorylation upon regulated exocytosis through modulation of syntaxin.

Another protein reported to bind CSP in vivo is HSC/HSP-70 (48). The activation of HSC70 ATPase activity by CSP (36, 49) provides a sensitive assay for measuring any alterations in the binding of CSP to HSC70. PKA- or mock-phosphorylated His_{6}-CSP was incubated at a range of concentrations (0–1 μM) in an ATP containing buffer with and without 1 μM HSC70. Free phosphate generated by HSC70 activation was determined by a spectrophotometric assay (36). An approximate 5–10-fold stimulation of ATPase activity was observed at the maximal concentration of CSP (1 μM), confirming previous observations (36, 49). The phosphorylation of CSP by PKA had no significant effect upon its ability to activate HSC70 (Fig. 6B), demonstrating there is specificity in the phosphorylation-dependent binding of CSP to syntaxin. G-protein α- and β-subunits have recently been added to the list of CSP-binding proteins (47), and so the phosphorylation dependence of these interactions was also determined. A pull-down approach assaying binding of purified G-protein subunits to immobilized PKA- or mock-phosphorylated His_{6}-CSP was employed for these studies. Over a series of experiments, it was found that similar levels of both α- and β-subunits bound to CSP regardless of phosphorylation (data not shown), thus further reinforcing the phospho-specificity of the CSP-syntaxin interaction.

Effect of Ser^{10} Mutation on Exocytosis—To address the role of CSP phosphorylation in vivo, we substituted the Ser^{10} codon in pcDNA3.1-myc-csp for alanine or glutamate codons. Our rationale was that the S10A mutation would render CSP non-phosphorylatable and therefore act as a permanently dephosphorylated CSP, whereas the negative charge of the S10E mutation might potentially mimic permanently phosphorylated CSP. To test this experimentally, we studied the effect of the mutations on syntaxin binding, which we have established as a phosphorylation-dependent interaction (Fig. 6A), by co-transfecting wild type or mutant CSPs with the cytoplasmic domain of syntaxin 1A in HeLa cells. In theory, CSP(S10A)
should bind equal or higher levels of syntaxin than wild type CSP, whereas a phosphomimetic CSP(S10E) should exhibit a marked reduction in syntaxin binding. Indeed, readily detectable amounts of syntaxin co-immunoprecipitated with wild type CSP and CSP(S10A) (Fig. 7A). This demonstrates an in vivo interaction between the two mammalian proteins and confirms that the Ser10 mutation does not cause gross conformational defects in the mutant protein. The increased syntaxin binding by CSP(S10A) relative to wild type may reflect constitutive phosphorylation of wild type CSP in the HeLa cells. Unfortunately, similarly increased levels of syntaxin binding were also observed with CSP(S10E), indicating that this mutation failed to create the desired phosphomimetic protein (data not shown). We therefore used the non-phosphorylatable CSP(S10A) construct to investigate the role of Ser10 phosphorylation in exocytosis.

Overexpression of CSP in chromaffin cells has two distinct effects on exocytosis as assessed by carbon-fiber amperometry (28) as follows: first, a gross inhibition of exocytosis evident as a reduction in amperometric spike number; and second, a more subtle effect on the kinetics of the residual release events. To determine whether Ser10 phosphorylation modulates these effects of CSP, chromaffin cells were transfected with wild type or CSP(S10A) plasmids. Both constructs were co-transfected with green fluorescent protein (to detect transfected cells), and exocytosis from permeabilized cells was elicited by application of 20 μM digitonin and 10 μM Ca2+. Catecholamine release was detected by amperometric recording (28). The example traces in Fig. 7B demonstrate that, as previously described, overexpression of CSP reduced the number of evoked spikes. We observed a similar reduction in spike number for the overexpression of CSP(S10A) However, analysis of the individual spike kinetics (as defined in Fig. 7C) revealed significant differences between overexpression of the wild type CSP and the Ser10 mutant (Fig. 7D). Wild type CSP altered the time course of amperometric spikes, manifested as an approximate 44% increase in the rise time and 62% increase in half-width (28). In contrast, spikes from cells expressing CSP(S10A) exhibited rise time and half-width values similar to control spikes (Fig. 7D). Taken together, these data suggest that the gross inhibition of exocytosis due to CSP overexpression is independent of Ser10 phosphorylation but that this residue is critical for the modulation of exocytosis kinetics by CSP.

**DISCUSSION**

**PKA Targets in Exocytosis**—The mechanism by which protein kinases modulate the late stages of regulated exocytosis is largely unknown. Because the enhancement of exocytosis in most secretory models by PKA or its activator cAMP is a universal but poorly understood phenomenon, we have focused upon identifying proteins involved in exocytosis that are targets of PKA action. In this study, we have discovered that CSP is a novel PKA substrate. Previously identified exocytotic PKA substrates include raphilin 3A and α-SNAP. α-SNAP is a good

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**TABLE I**

| Peptide            | Sequence               | $K_m$ | $V_{max}$ |
|--------------------|------------------------|-------|-----------|
| Kemptide           | LRRASLG                | 10.8  | 1.3       |
| CSP-(4-14)         | (C)QRQSLSTSGE         | 17.4  | 0.5       |
| CSP-(4-14)S10A     | (C)QRQSLATSGE         | Not detectably phosphorylated at 30 μM |
| CSP-(4-14)S10pS    | (C)QRQSLpSTSGE        | Not detectably phosphorylated at 30 μM |

**Fig. 5. The tryptic peptide of CSP that contains Ser10 is phosphorylated in vivo.** A, CSP immunoblot of equal amounts (10 μg of protein) of PC12 cell lysates from either wild type (WT) or CSP-overexpressing stable cell lines (clone 1 (29)). Arrows indicate the monomer (lower) and dimer (upper) forms of CSP. B, CSP-overexpressing PC12 cells were labeled with 1.5 mM [32P]ATP, 4 h, with Krebs (control), 300 μM ATP, or 500 μM 8-Br-cAMP for 15 min and lysed on ice. CSP was immunoprecipitated from the lysates and processed for 32P incorporation (top panel) or CSP immunoblotting (bottom panel). Phosphorylation expressed as a percentage of control cells was calculated from the PhosphorImager and normalized to the protein content in the immunoblot. C and D, a whole CSP immunoprecipitate from 10 × 10⁶ [32P]-labeled CSP clone 1 PC12 cells was resolved in a single lane by SDS-PAGE alongside 2 μg of His₆-tagged CSP that had been phosphorylated by PKA in vitro. The CSP-containing bands were excised, subjected to in-gel trypsin digestion, and the peptides separated by RP-HPLC (C, PKA in vitro phosphorylation; D, PC12 immunoprecipitation). The graphs show the 32P incorporated (in cpm) into each HPLC fraction; notice that the only radioactive peaks in each sample are found in the same peptide fraction.
in vitro substrate for PKA (this study and Ref. 24); however, we have found that α-SNAP is not detectably phosphorylated in either chromaffin cells or rat brain synaptosomes despite immunoprecipitation of readily detectable amounts of protein from both sources. Rabphilin 3A, an effector of the GTPase Rab3, is phosphorylated in vitro by PKA (50) and in vivo in response to forskolin or long term potentiation in the CA3 region of the hippocampus (51). However, no functional effects of rabphilin phosphorylation (for example modulation of its established interaction with Rab3) have yet been reported, and in addition, the rabphilin 3A knockout mouse displays no defects in neurotransmission or long term potentiation (52). In contrast, Drosophila CSP mutants exhibit severe defects in neurotransmission (53, 54), and not only is CSP phosphorylated in vivo, but we have also demonstrated functional implications of its phosphorylation. During preparation of this manuscript, data were published (55) revealing the synaptic vesicle protein Snapin to be a novel PKA substrate acting in exocytosis. As we have reported here for CSP, Snapin is phosphorylated in vivo, and phosphorylation alters its function in both biochemical (increased in vitro binding to GST-SNAP-25) and cellular assays (overexpression of a Ser-Ala mutant in chromaffin cells alters exocytosis kinetics). The presence of two PKA substrates (CSP and Snapin) on the synaptic vesicle may contribute to the sophisticated regulation of neurotransmitter release by phosphorylation. However, as Snapin is neuronal specific (56), CSP appears a more likely candidate effector of PKA in exocytosis in other cell types.

The Specificity of CSP Phosphorylation—Analysis of the mammalian CSP amino acid sequence by the Net Phos and Phosphobase data bases (57, 58) reveals potential phosphorylation sites for a variety of kinases, including Ca²⁺/calmodulin-dependent protein kinase II (Ser⁸), casein kinase I (Ser¹², Thr¹³, Thr¹⁷, Thr¹⁸¹, and Ser¹⁹¹), casein kinase II (Thr¹⁴, Thr²⁷, Ser¹⁰, and Ser¹⁷²), p70 S6 kinase (Ser¹⁰), PKA (Ser⁸), and PKC (Ser³⁰ and Thr⁸⁶). However, our data are consistent with PKA being a principal CSP kinase and Ser¹⁰ as its site of action. For instance, in vitro, CSP is phosphorylated by PKA only at Ser¹⁰, whereas an alanine-substituted peptide, CSP-(4–14)-S10A, was not phosphorylated. In vivo, CAMP can stimulate CSP phosphorylation in chromaffin cells, and phosphorylation of CSP within intact PC12 cells occurs in a single tryptic peptide containing Ser¹⁰. Furthermore, an in-gel kinase assay found PKA to be the only reconstituted kinase activity from cell lysates that could phosphorylate CSP. Interestingly, computer prediction programs based on primary sequence data do not identify Ser¹⁰ as a PKA site. This suggests that the tertiary structure of the protein has a profound influence on kinase specificity and hence emphasizes the need to empirically determine protein phosphorylation sites.

Ser¹⁰ Phosphorylation Defines a Novel Functional Domain of CSP—An interaction between CSP and syntaxin both in vitro and in vivo is reported in the literature (35, 44, 45). In Drosophila, CSP and syntaxin can be co-immunoprecipitated, and the phenotype of mutant flies overexpressing syntaxin can be rescued by the simultaneous overexpression of CSP (44). We have found that mammalian recombinant His₆-CSP binds GST-syntaxin 1A in vitro with similar efficiency to that shown in previous studies (44, 45) using the corresponding Drosophila proteins. We have also co-immunoprecipitated CSP and the cytoplasmic domain of syntaxin from a heterologous system, demonstrating a cellular interaction of the two mammalian proteins. Whereas it is known that the J domain of CSP is responsible for binding HSC70 (36, 46, 49), the CSP domain(s) that interacts with syntaxin is unknown. Because Ser¹⁰ lies outside the CSP J domain that is known to interact with HSC70 (Fig. 7E), it is perhaps not surprising that we have found phosphorylation does not affect its stimulation of HSC70 ATPase activity. In addition, we saw no marked effect of phosphorylation on the recently reported binding of CSP to Go or Goβ subunits (47). Inhibition of binding to syntaxin by phosphorylation of CSP on Ser¹⁰ suggests the extreme N terminus of CSP has a role in syntaxin binding. This is consistent with the observation that both mammalian and Drosophila CSP bind syntaxin because the C-terminal domains of each protein share little homology (59), whereas the N termini, particularly surrounding the phosphorylation site (residues 1–15), have high homology (Fig. 7E). Thus, the total conservation of a CSP Ser¹⁰ phosphorylation site across species from Drosophila to man.

**Fig. 6. Phosphorylation of CSP by PKA inhibits its interaction with syntaxin but not HSC70 or G-proteins.** A. CSP (specified concentrations) was incubated with GST-syntaxin (1 μM) and glutathione-agarose beads. Bound proteins were visualized by immunoblotting, using a 125I-anti rabbit IgG secondary antibody to ensure a linear signal. The relative amounts of PKA-phosphorylated (filled circles) and mock-phosphorylated (open circle) CSP bound to syntaxin 1A were calculated by densitometry of the CSP immunoblot and then by subtraction from each condition of the small amount of CSP bound to GST alone. B, the effect of CSP phosphorylation upon binding to HSC70 was assessed by measuring the CSP-dependent activation of HSC70 ATPase activity. The indicated concentrations of PKA-phosphorylated (filled circles) and mock-phosphorylated (open circle) CSP were incubated with 0.2 μM HSC70, for 2 h and the inorganic phosphate liberated was assayed using a spectrophotometric assay with KH₂PO₄ as a standard. Data are from a representative experiment expressed as mean ± S.E., n = 8.
FIG. 7. Mutation of Ser10 alters the effect of CSP on exocytosis kinetics. A, immunoblots of input lysate and CSP immunoprecipitates from HeLa cells cotransfected with pCMV-syntaxin (cytosolic domain) and pcDNA3-CSP or -CSP(S10A). B, examples of amperometric traces recorded from control (nontransfected) chromaffin cells or cells co-transfected with pEGFP and pcDNA3-CSP or -CSP(S10A). C, example of an amperometric spike plot with an expanded time scale to indicate the characteristics that were analyzed. D, the mean spike number, spike height, spike half-width, and spike rise time from CSP- or CSP(S10A)-transfected cells are plotted as the percentage difference from the corresponding values of control spikes from nontransfected cells in the same dishes (*, p < 0.005; **, p < 0.001). Data are expressed as mean ± S.E. and are derived from 17 to 30 cells and 64 to 539 spikes for each condition. E, sequence alignment surrounding the Ser10 phosphorylation site of CSP and comparison of the N-terminal domain of CSP from various species.

(Fig. 7E) may represent an evolutionarily conserved regulatory mechanism.

The Role of Ser10 in Late Fusion Events—We now have evidence that the previously reported effects of overexpressing wild type CSP in chromaffin cells on amperometric spike characteristics (28) involve Ser10. Overexpression of wild type CSP results in an increase in the half-width and rise time values of residual amperometric spikes, thus slowing the kinetics of vesicular release. However, substitution of Ser10 to alanine, thus making CSP non-phosphorylatable, results in spikes with control values for half-width and rise time. This effect is not due to low expression levels of the mutant protein because this construct produced a gross reduction in spike number similar to wild type CSP (Fig. 7), and because both CSP proteins were expressed to similar extents upon transfection in HeLa cells (data not shown). Thus, the effects of wild type CSP on spike kinetics are likely to involve Ser10 phosphorylation because the only observed difference between the mutant and wild type CSP is that the mutant cannot be phosphorylated at the 10-position. As the rise time parameter is thought to represent the rate of expansion from fusion pore to full membrane fusion (60), this suggests a role for CSP phosphorylation at a late stage of exocytosis.

A recent amperometric study in chromaffin cells has demonstrated that application of forskolin or other agents that increase cellular cAMP levels have the same effects upon initial spike kinetics as overexpression of CSP, namely increased half-width and rise time values (61). Furthermore, these effects were abolished by pretreatment with the PKA-selective inhibitor H-89, suggesting a role for PKA in the slowing of spike kinetics (61). Because PKA activation or overexpression of PKA-phosphorylatable (wild type) CSP slow the late stages of exocytosis and PKA inhibition or overexpression of non-phosphorylatable CSP abolish these effects, the modulation by PKA of the late stages of exocytosis observed by Machado et al. (61) could be explained by its phosphorylation of CSP on Ser10.

The Functional Significance of CSP Phosphorylation—The amperometric data suggest there may be two distinct effects of CSP on regulated exocytosis as follows: (i) a phosphorylation-independent reduction in the overall number of exocytotic events, and (ii) a phosphorylation-dependent slowing of release kinetics in the remaining fusions. In (i), the gross reduction in spike number is likely to be due to phosphorylation-dependent protein-protein interactions of CSP. HSC70 is an obvious candidate here, as its interaction with CSP is unaffected by Ser10 phosphorylation. Furthermore, HSC70 is itself critical for synaptic vesicle exocytosis in vivo, and interaction with CSP is required for this function (48). Binding of G-protein α- and β-subunits by CSP is similarly phosphorylation-independent. Although the interaction of CSP with Gα and Gβ has been interpreted in the context of Ca2+ channel regulation (47), direct effects of heterotrimeric G-proteins on the exocytotic machinery have been well documented in various secretory cells, including neurons (62). However, it cannot be ruled out that the gross reduction in exocytosis is mediated by excess non-phosphorylated CSP binding to syntaxin. There is a precedent for this in Drosophila where overexpression of CSP can titrate out the effects of syntaxin overexpression upon neurotransmission (44). Although the molecular basis of the general inhibition of exocytosis by CSP is unclear, it is not restricted to chromaffin cells, as transient CSP overexpression in insulin-secreting cells also inhibits overall exocytosis (27, 63).

We propose that the second effect of CSP overexpression on exocytosis, the slowing of vesicular release, is dependent upon phosphorylation of CSP because it is not observed in cells expressing non-phosphorylatable CSP. In addition, the same effects upon spike kinetics are observed in chromaffin cells following stimulation of PKA activity (61). Our biochemical data imply that the effect of CSP overexpression is unlikely to be due to titration of syntaxin by CSP, because phosphorylated CSP has an extremely low affinity for syntaxin. Furthermore, amperometric analysis from cells where syntaxin function has been ablated by botulinum neurotoxin C1 expression reveals gross inhibitory effects upon spike number (as we have ob-
served with wild type and mutant CSP constructs) but no changes to release kinetics (64). How then could phosphorylated CSP induce the observed effects on spike kinetics? One possibility is that phosphorylation of CSP frees up syntaxin to engage with other binding partners that then act to slow the late stages of exocytosis. This theory fits with the proposed physiological role of CSP as a syntaxin chaperone (35, 44, 45, 59). Although a variety of proteins could potentially bind syntaxin following CSP dissociation, nSec1/munc18 is the most likely candidate, by virtue of the extremely high affinity of this interaction (65). Intriguingly, overexpression of an nSec1/munc18 mutant with reduced affinity for syntaxin accelerates the late stages of exocytosis, manifesting as a decrease in amperometric rise time and half-width parameters (60), the exact opposite of CSP overexpression. In view of the inferred ability of endogenous wild type nSec1/munc18 to slow the kinetics of membrane fusion, it is tempting to speculate that the effect of CSP phosphorylation on exocytosis kinetics is due to increased formation of nSec1/munc18-syntaxin complexes. An alternative explanation of the data is that an unknown protein(s) involved in the late stages of exocytosis binds phosphorylated CSP preferentially.

Previous studies have implicated CSP in the regulation of Ca2+ channels and/or modulation of a direct Ca2+-dependent fusion step of exocytosis (28, 44, 45, 46–70). In this study, we propose a refinement of the physiological functions of CSP through its phosphorylation by PKA. This could modulate exocytosis by facilitating the donation of syntaxin into protein complexes involved in vesicle docking and fusion or by interactions with unknown phospho-CSP-binding proteins. In addition, phosphorylation of CSP could potentially also affect Ca2+ signaling via reduced binding to syntaxin, which is well known as a modulator of presynaptic ion channel function (71, 72). Therefore, regulating CSP phosphorylation could influence multiple stages in synaptic vesicle exocytosis, thus enabling sophisticated control of neurotransmitter release. Furthermore, as CSP has a broad tissue distribution and functions in a variety of cell types from endocrine cells to neurons, CSP phosphorylation by PKA could be a ubiquitous mechanism for the regulation of exocytosis.

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