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Cysteine String Protein Functions Directly in Regulated Exocytosis

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Cysteine string protein (Csp) is essential for neurotransmitter release in *Drosophila*. It has been suggested that Csp functions by regulating the activity of presynaptic Ca^{2+} channels, thus controlling exocytosis. We have examined the effect of overexpressing Csp1 in PC12 cells, a neuroendocrine cell line. PC12 cell clones overexpressing Csp1 did not show any changes in morphology, granule number or distribution, or in the levels of other key exocytotic proteins. This overexpression did not affect intracellular Ca^{2+} signals after depolarization, suggesting that Csp1 has no gross effect on Ca^{2+} channel activity in PC12 cells. In contrast, we show that Csp1 overexpression enhances the extent of exocytosis from permeabilized cells in response to Ca^{2+} or GTPγS in the absence of Ca^{2+}. Because secretion from permeabilized cells is not influenced by Ca^{2+} channel activity, this represents the first demonstration that Csp has a direct role in regulated exocytosis.

INTRODUCTION

Cysteine string proteins (Csps) are cysteine-rich proteins that belong to the DnaJ family of molecular chaperones (Zinsmaier *et al*., 1990; Gundersen and Umbach, 1992; Braun and Scheller, 1995; Mastrogiacomo and Gundersen, 1995; Chamberlain and Burgoyne, 1996). Csps are localized to synaptic vesicles in neurons (Mastrogiacomo *et al*., 1994; Van de Goor *et al*., 1995), pancreatic zymogen granules (Braun and Scheller, 1995), chromaffin granules in adrenal chromaffin cells (Chamberlain *et al*., 1996), and secretory granules of the neurohypophysis (Pupier *et al*., 1997).

Csps have four domains: 1) an N-terminal "J" domain, which is homologous to a region of the bacterial protein DnaJ and is found in a number of eukaryotic DnaJ-like proteins; the J domain of Csp interacts with, and stimulates the ATPase activity of, the chaperone protein Hsc70 (Braun *et al*, 1996; Chamberlain and Burgoyne, 1997a,b); 2) a central cysteine "string" region, which is heavily palmitoylated and may serve to anchor Csp to vesicle membranes (Gundersen *et al*., 1994), although this idea is controversial (Van de Goor and Kelly, 1996); 3) a linker region, which separates the J domain and cysteine-rich region and is highly conserved; and 4) a C-terminal region, which is less well conserved. The C-terminal region and linker region may be involved in substrate interactions.

The importance of Csp was demonstrated with the study of *Drosophila* Csp null mutants (Umbach *et al*., 1994; Zinsmaier *et al*., 1994). This mutation was partially lethal, and only a small number of flies survived to adulthood, dying soon thereafter. Characterization of these surviving adult mutant flies or mutant larvae revealed that they had a defect in presynaptic neurotransmission. Further analysis revealed that the mutant flies were defective in some aspect of stimulus–release coupling but not vesicle recycling (Heckmann *et al*., 1997; Umbach and Gundersen, 1997; Ranjan *et al*., 1998). Interestingly, this defect was more pronounced at 30°C than at 22°C, suggesting that Csp may be required to stabilize components of the exocytotic machinery at the higher temperature. Deletion of the gene encoding *Escherichia coli* DnaJ also causes a temperature-sensitive phenotype (Ohki *et al*., 1992), suggesting that the J domain function (and Hsc70 interaction) of Csp is important for exocytosis.

An intriguing discovery showed that injection of Csp antisense RNA into *Xenopus* oocytes, engineered to express functional N-type Ca^{2+} channels, inhibited the activity of these channels, whereas Csp mRNA stimulated channel activity (Gundersen and Umbach, 1992). Thus, it was suggested that the function of Csp is to regulate voltage-dependent Ca^{2+} channel activ-

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ity. Beause presynaptic neurotransmission is triggered by Ca^{2+} influx after nerve terminal depolarization, this suggested that Csp plays a key regulatory role in exocytosis. This model of Csp action is also appealing because it dictates that Ca^{2+} entry will be greatest through channels that are physically linked to vesicles. Any effects of Csp on channel activity would have to be indirect, because a direct interaction of Csp with N-type Ca^{2+} channels has not been demonstrated (Pupier *et al*., 1997).

Ca^{2+} channel regulation is unlikely to be the only function of Csp, because this protein is expressed in a wide range of non-neuronal cell types, which either do not have Ca^{2+}-regulated exocytotic pathways or do not express voltage-dependent Ca^{2+} channels (Braun and Scheller, 1995; Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996). Further insight into Csp function was gained with the demonstration that Csp binds to and stabilizes the partially unfolded conformation of heat-denatured firefly luciferase, preventing its aggregation (Chamberlain and Burgoyne, 1997b). Indeed, Csp and Hsc70 function cooperatively to prevent luciferase aggregation. Thus, it may be that the function of Csp (and Hsc70) in regulated exocytosis involves stabilizing or refolding partially unfolded synaptic protein(s), and this idea is consistent with the temperature-sensitive phenotype of *Drosophila* Csp null mutants.

In the present study we have examined the role that Csp1 (the major form of Csp in adrenal chromaffin cells and PC12 cells) plays in exocytosis by generating PC12 cells that overexpress Csp1. We show that Csp1 overexpression does not affect the Ca^{2+} rise in the cytosol after depolarization but, in contrast, directly stimulates exocytosis assayed in permeabilized cells.

MATERIALS AND METHODS

Materials

An ECL kit and [7,8,^{3}H]dopamine were purchased from Amersham (Buckinghamshire, United Kingdom). Fura-2 was from Boehringer Mannheim (Sussex, United Kingdom). RPMI-1640 media, horse serum, FCS, and G418 sulfate were from Life Technologies (Paisley, United Kingdom). pcDNA3 plasmid was from Invitrogen (San Diego, CA), and restriction enzymes and ligase were from Promega (Madison, WI). Secretogranin II antiserum was a gift from Dr. Dan Cutler (Medical Research Council Laboratory of Molecular Cell Biology, University College London, London, United Kingdom). Vesicle-associated membrane protein antiserum, synaptosomal-associated protein of 25 kDa antiserum, and Csp antiserum were as previously described (Roth and Burgoyne, 1994; Chamberlain and Burgoyne, 1996). Anti-Hsc70, anti-syntaxin, and anti-synaptophysin monoclonal antibodies and all other reagents were obtained from Sigma (Poole, United Kingdom).

Buffers

Buffer A consisted of 139 mM potassium glutamate, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM ATP, and 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.5. Krebs buffer was composed of 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 3 mM $CaCl_2$, and 20 mM HEPES, pH 7.4.

PC12 Cell Culture and Transfection

PC12 cells were cultured in suspension in RPMI-1640 media containing 10% horse serum, 5% FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Media for transfected cells were identical but also contained 0.5 mg/ml G418 sulfate. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Transfected PC12 cell lines were generated by electroporation using a ProgenitorII PG200 electroporator (Hoefer, San Francisco, CA) at 1080 μF, 260 V, and three discharges per sample in a 0.4-cm cuvette. Transfection was performed on 5×10^6 freshly trypsinized cells in 1 ml of culture medium in the presence of 10 μg of linearized pcDNA3 plasmid with the Csp1 coding sequence cloned between *Bam*HI (5′) and *Eco*RI (3′) restriction sites (Chamberlain and Burgoyne, 1996). Transfected cells were selected by growth on media containing 0.5 mg/ml G418, and clones were screened by immunoblotting with Csp antiserum.

PC12 Cell Fractionation Analysis

Cells (80–100 million) were pelleted by centrifugation at $800 \times g$ for 3 min and washed twice with ice-cold PBS-protease mix (1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 35 μg/ml PMSF). The cells were then resuspended in 3 ml of homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4, and proteases) and homogenized with a Dounce homogenizer. The cell homogenate was spun at $750 \times g$ for 5 min (4°C), and the postnuclear supernatant (PNS) was removed. The PNS was centrifuged at $100{,}000 \times g$ for 60 min at 4°C and the supernatant (cytosol) and pellet (membranes) fractions separated. The cytosol and membrane fractions from wild-type and clones were added to SDS sample buffer to give a final protein concentration of 1 mg/ml. For sucrose gradient fractionation analysis, 300–350 million control cells and 80–100 million overexpressing cells were used as the starting material. The PNS from the homogenized cells was loaded onto a 0.6–1.8 M linear sucrose gradient and centrifuged at $100{,}000 \times g$ for 6 h at 4°C. Fractions were carefully removed and diluted in SDS sample buffer.

Immunofluorescence

PC12 cells were trypsinized and maintained in culture for 3 d on glass coverslips (0.1×10^6/ml) before fixation in 4% formaldehyde in PBS. The cells were then washed twice in PBS and incubated for 30 min in PBTA (0.1% Triton X-100 and 0.3% BSA in PBS). After this, the cells were incubated in anti-Csp immunoglobulin G (IgG; 1:100 for control cells) or Csp antiserum (1:1000 for clones) in PBTA for 60 min and subsequently washed three times in PBTA. The cells were then incubated with anti-rabbit IgG biotinylated (1:100 with PBTA) for 60 min and washed three times with PBTA. Finally, the cells were incubated with streptavidin-Texas Red (1:50 with PBTA) for 30 min and washed three times with PBTA. The coverslips were blotted, allowed to air dry, and then mounted on antifade glycerol (glycerol/PBS [9:1] containing 0.25% 1,4-diazabicyclo [2,2,2]octane and 0.002% *p*-phenyldiamine).

Calcium Measurements

PC12 cells (0.5×10^6) were plated on collagen-coated coverslips (in 24-well trays) and maintained in culture for 2–3 d. The cells were washed twice with 1 ml of Krebs buffer and 0.5% BSA and then incubated for 30 min at room temperature in Krebs buffer, 0.5% BSA, and 2 μM Fura-2. After this, the cells were washed twice with 1 ml of Krebs buffer and 0.5% BSA and left at room temperature for 30 min in Krebs buffer and 0.5% BSA. The cells were then washed twice with 1 ml of Krebs buffer. The coverslips were placed in a Perkin Elmer (Norwalk, CT) LS-5 fluorimeter, incubated in Krebs buffer, and stimulated by addition of 50 mM KCl. The concentration

of cytosolic Ca^{2+} was followed by monitoring cell fluorescence at 340 and 380 nm as described previously (Graham and Burgoyne, 1995). Cell recordings were performed for 10 min, with 50 mM KCl being added at 3 min. The values for $[Ca^{2+}]_i$ were determined from fluorescence emission ratios using a k_D for Ca^{2+} of 135 nm.

Dopamine Release Assays

Cells were plated at a density of 0.5×10^6/ml on collagen-coated 24-well trays and maintained in culture for 4 d. The cells were then incubated in 500 μl/well RPMI-1640 media with 0.088 mg/ml ascorbic acid and 0.5 μCi/ml [7,8,^{3}H]dopamine at 37°C for 90 min. After this, the cells were washed three times with 1 ml of Krebs buffer (without $MgCl_2$ or $CaCl_2$) and permeabilized by incubation in 300 μl of buffer A with 20 μM digitonin for 6 min. Release of dopamine from the permeabilized cells was measured in the presence of 0 Ca^{2+}, 10 μM Ca^{2+}, or 100 μM GTPγS (in 300 μl of buffer A) for 20 min at room temperature. The supernatant from this step was removed and centrifuged at 13,000 $\times$ *g* for 2 min, whereas the cells were solubilized in 300 μl of 0.5% Triton-X-100. The [^{3}H]dopamine content in both the cell and supernatant fractions was counted in duplicate in a scintillation counter, and dopamine release was expressed as a percentage of total cell content for each well. Comparisons of dopamine release between control and Csp-overexpressing cells were always performed on the same 24-well tray.

RESULTS

Generation of PC12 Clones Overexpressing Csp1

To study the role of Csp in secretory granule trafficking, PC12 cells were transfected with pcDNA3 plasmid containing DNA encoding Csp1 under the control of the high-level cytomegalovirus promoter, and stably transfected cells selected by growth on G418 sulfate. Three selected clones were analyzed for Csp1 content by immunoblotting with a Csp antiserum (Chamberlain and Burgoyne, 1996). Figure 1, top panel, shows that all three clones had a greatly increased level of Csp1 expression compared with control cells. The amount of Csp1 overexpression was quantified by densitometry of both monomer and dimer forms of Csp and estimated to be ~15-fold.

The expression levels of other proteins was also compared between the three clones and wild-type cells. Figure 1 shows that there was essentially no difference between the clones and control cells in the levels of vesicle-associated membrane protein, syntaxin, and synaptosomal-associated protein of 25 kDa, proteins that are known to be essential for exocytosis. Similarly, the level of expression of the Csp-interacting protein Hsc70 was not affected by Csp1 overexpression. Finally, the amounts of synaptophysin and secretogranin II were similar in the clones and wild-type cells. Synaptophysin is often used as a marker protein for small, synaptic-like vesicles, and secretogranin II is also used for large, dense-core granules, suggesting that the increase in Csp1 expression does not affect the amount of these organelles. Electron microscopic analysis of wild-type cells and overexpressing clones showed that the cells had a similar overall morphology, and there were no consistent differences in the number and distribution of dense-core granules within the cells (our unpublished observations).

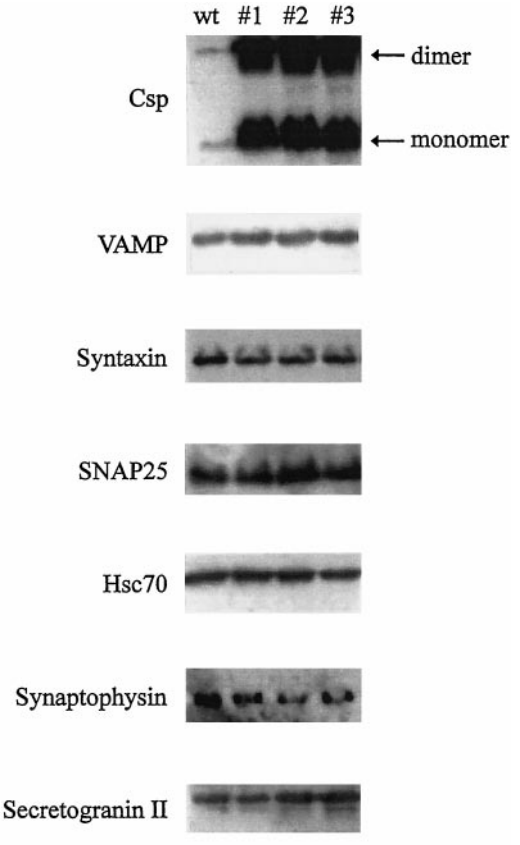

Figure 1. Analysis of protein expression in control cells and overexpressing clones. Cells (1×10^6) were resuspended in 100 μl of SDS-dissociation buffer. The protein samples were separated by SDS-PAGE on 12.5% polyacrylamide gels and blotted onto nitrocellulose. The expression levels of various proteins were examined by immunoblotting with specific antisera as indicated, and wild-type cells (wt) were compared with three selected overexpressing clones (1–3).

Analysis of Csp Distribution in Control Cells and Overexpressing Clones

Csp1 is present on the large, dense-core granules in adrenal chromaffin cells, from which PC12 cells are derived (Chamberlain *et al.*, 1996). The distribution of Csp1 in PC12 cells was studied, and it was found that Csp1 immunoreactivity was associated mainly with the membrane fraction from PC12 cells, although there was a very low level of Csp1 in the cytosolic fraction. Csp1 fractionated between membrane and cytosol fractions in the clones to the same extent as in control cells (our unpublished observations). Thus, overexpressed Csp1 is targeted to membranes.

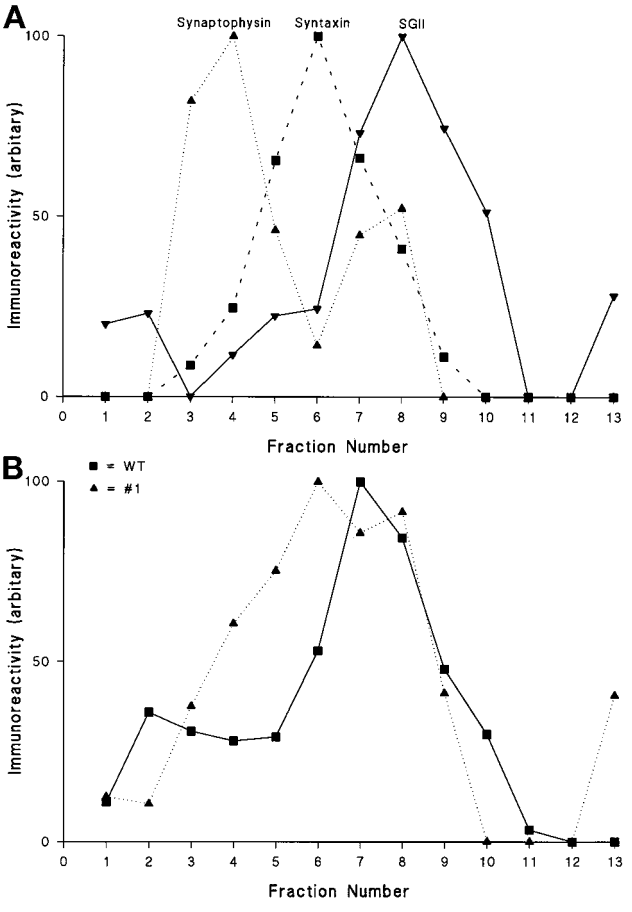

Figure 2. Sucrose gradient fractionation analysis of PC12 cells. Postnuclear supernatants from control cells and Csp1 overexpressing clone 1 were loaded onto a 0.6–1.8 M linear sucrose gradient and centrifuged at 100,000 × *g* for 6 h at 4°C. Fractions were collected, diluted in SDS sample buffer, separated by SDS-PAGE on 12.5% gels, and blotted onto nitrocellulose. The blots were then probed with specific antisera. Immunoreactivity of fractions was quantified by densitometry and plotted as a percentage of the most immunoreactive fraction. (A) Characterization of synaptophysin, syntaxin, and secretogranin II (SGII) in fractions from control cells. (B) Csp distribution in control cells and overexpressing clone 1.

The subcellular distribution of Csp1 in PC12 cells was examined further by fractionating postnuclear supernatants from control cells and clones on sucrose gradients. The fractions were initially characterized by probing with antibodies against synaptophysin, which is enriched on the small, synaptic-like vesicles, syntaxin, which is enriched on the plasma membrane, and secretogranin II, which is enriched on large, dense-core granules (Figure 2A). Csp1 immunoreactivity in control cells codistributes with secretogranin II on sucrose gradients (Figure 2, compare A and B), suggesting that Csp1 is mainly associated with the large, dense-core granules in PC12 cells. Some Csp1 was found in addition in fractions 2–5. Csp1 immunoreactivity has a broader distribution of activity in the clones (Figure 2B shows clone 1) and appears to be present both on the granules and plasma membrane (i.e., codistribution with secretogranin II and syntaxin). Thus, a certain fraction of the overexpressed Csp1 may be targeted to the plasma membrane, implying that the granules in the three clones have a maximal amount of Csp1 associated with them.

The distribution of Csp1 in PC12 cells was further examined by immunofluorescence, which showed that Csp1 has a diffuse distribution, with some enrichment close to the plasma membrane (Figure 3A). This was similar for the clones (Figure 3B, shown is clone 1), although the plasma membrane staining was more prominent than in control cells, consistent with a higher fraction of overexpressed Csp1 being localized to the plasma membrane.

Csp1 Overexpression Has No Measurable Effect on Intracellular Ca^{2+} signals

It has been suggested that Csp functions by controlling the activity of presynaptic voltage-dependent Ca^{2+} channels (Gundersen and Umbach, 1992) to regulate synaptic vesicle fusion. We examined whether Csp1 overexpression had any gross effects on Ca^{2+} entry into the cytosol of PC12 cells after depolarization. Cells were loaded with the Ca^{2+}-sensitive dye Fura-2, and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored after depolarization of the cells with high K^+. Figure 4A shows that depolarization resulted in a transient peak rise in $[Ca^{2+}]_i$ followed by a sustained plateau. There was essentially no change in the basal $[Ca^{2+}]_i$, the peak rise, or the sustained increase in intracellular Ca^{2+} after K^+-induced depolarization in cells overexpressing Csp1. Figure 4A shows representative traces, whereas Figure 4B is averaged data from 12 independent experiments. From these experiments it is clear that Csp1 does not have any gross effects on the activity of Ca^{2+} channels or Ca^{2+} signals generated in PC12 cells; however, we cannot rule out the possibility that Csp1 has more subtle effects on Ca^{2+} channel activity.

Csp1 Has a Direct Effect on Regulated Exocytosis

Csp could act in neurotransmitter release because of control of Ca^{2+} channel activity or by a more direct role in exocytosis. We examined the latter possibility by analyzing the effect of Csp1 overexpression on the release of [^{3}H]dopamine from permeabilized PC12 cells. Cells were permeabilized with digitonin, which creates pores in the plasma membrane, allowing Ca^{2+} to be added directly to the cell cytosol. Thus, exocytosis from permeabilized cells is not dependent on plasma membrane depolarization and is independent of Ca^{2+} channels.

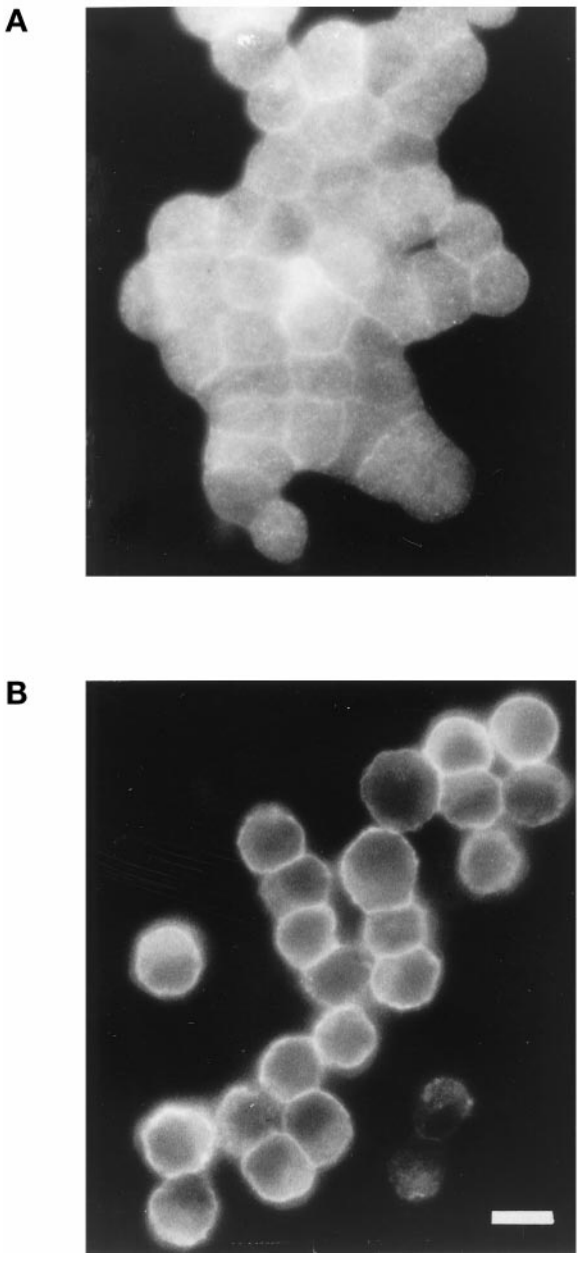

Figure 3. Analysis of Csp distribution in PC12 cells by immunofluorescence. Cells were incubated with anti-Csp antiserum and then with biotinylated anti-rabbit IgG and streptavidin-Texas Red. (A) Control cells using anti-Csp at 1:100. (B) Clone 1 using anti-Csp at 1:1000. Bar, 10 μm.

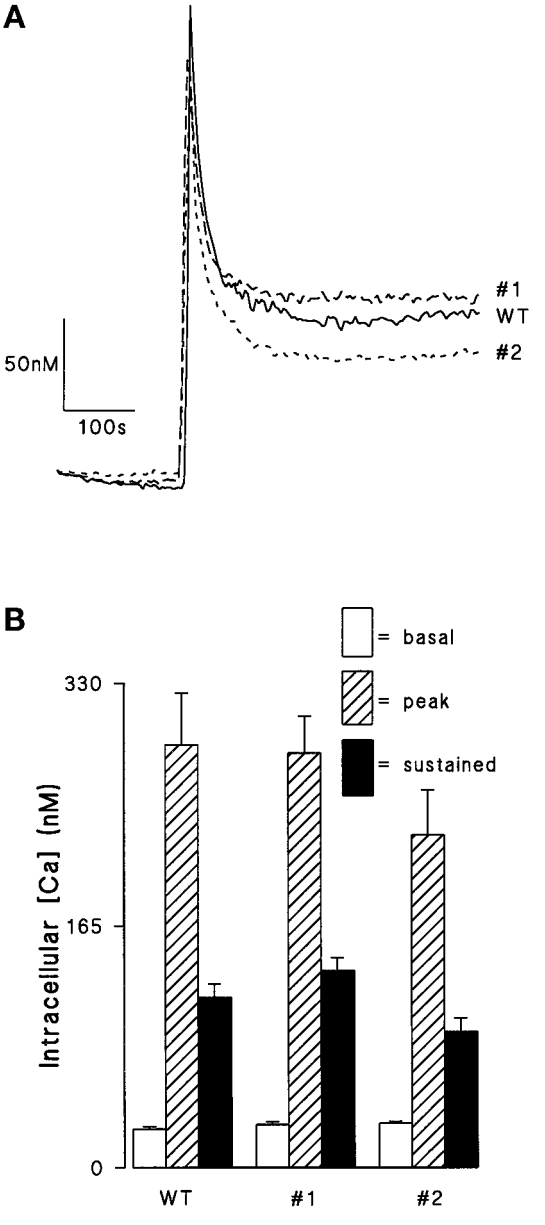

Figure 4. Csp overexpression has no observable effect on intracellular Ca^{2+} signals. Cells were loaded with the Ca^{2+}-sensitive dye Fura-2 and depolarized by addition of 50 mM KCl while monitoring cell fluorescence at 340 and 380 nm. The basal $[Ca^{2+}]_i$ and the peak and sustained increases in Ca^{2+} concentration after depolarization were calculated. (A) Representative traces from control cells and overexpressing clones 1 and 2. (B) Averaged data from 12 experiments, expressed as mean ± SEM.

No consistent differences in [^{3}H]dopamine uptake were seen in Csp1-overexpressing cells compared with control cells (our unpublished observations). Exocytosis from permeabilized cells, preloaded with [^{3}H]dopamine, was stimulated by addition of 10 μM free Ca^{2+}, and Figure 5A shows that there was a consistent increase (~50%) in Ca^{2+}-stimulated secretion from all three clones in comparison with control cells. There was a similar increase over wild-type cells when the cells were stimulated to secrete with 100 μM GTPγS (Figure 5B). Poorly hydrolyzable GTP analogues are not as effective as Ca^{2+} but can activate Ca^{2+}-independent exocytosis in these cells in the pres-

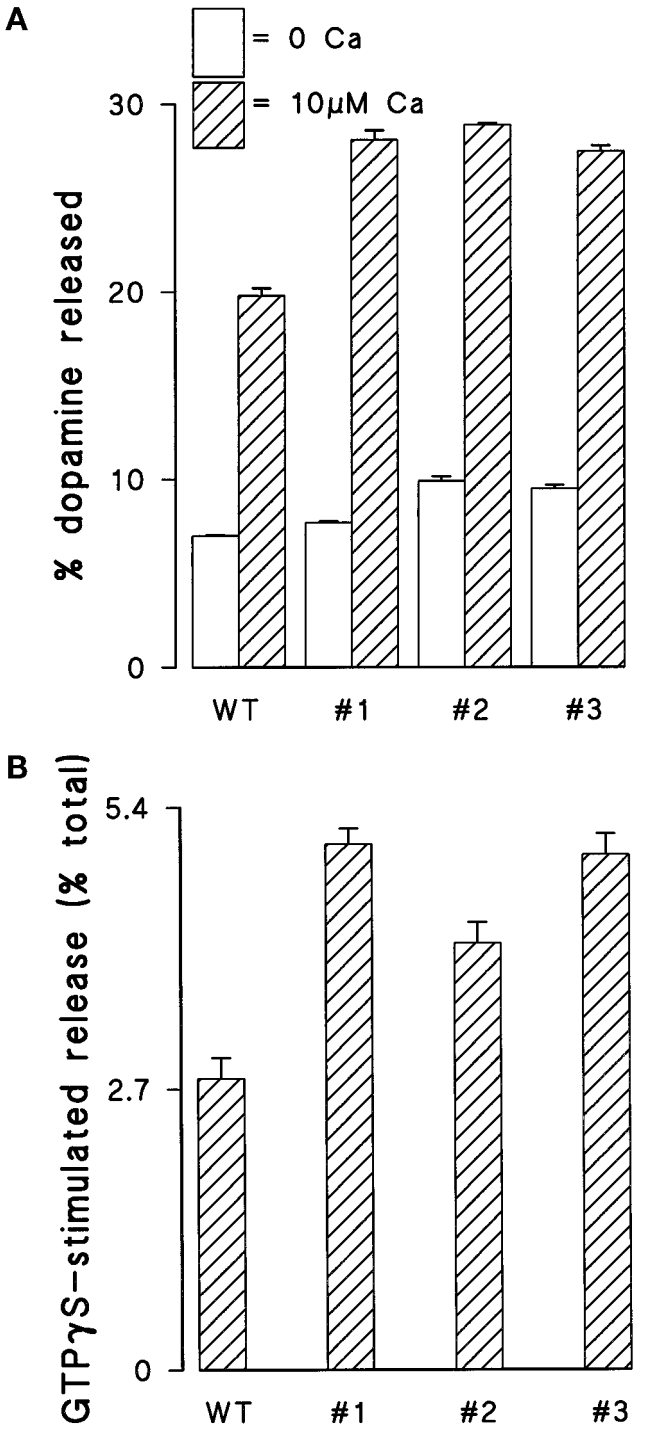

Figure 5. Regulated exocytosis is enhanced from cells overexpressing Csp. Cells in 24-well trays (0.5×10^6/well) were incubated with [^{3}H]dopamine for 90 min at 37°C. The cells were washed, permeabilized for 6 min with 20 μM digitonin, and challenged by addition of 0 or 10 μM Ca^{2+} (A) or 100 μM GTPγS in the absence of Ca^{2+} (B), and release of [^{3}H]dopamine was determined. Data are expressed as mean ± SEM of the percentage of total dopamine cell content (n = 6). For GTPγS the data are shown with basal release at 0 Ca^{2+} subtracted.

ence of 5 mM EGTA but no added Ca^{2+}. These experiments clearly show that overexpression of Csp1 increases the extent of exocytosis, suggesting that it functions directly in both Ca^{2+}-dependent and Ca^{2+}-independent regulated exocytosis.

The time-course of secretion from clone 1 and control cells was compared, and Figure 6A shows that the initial rate of secretion (over the initial 10 min) from overexpressing cells was greater than from wild-type cells. At later time points the rate of secretion was no different from wild-type cells. Figure 6B shows that overexpression of Csp1 does not significantly affect the Ca^{2+} dependency of exocytosis, and that the maximal increase in exocytosis above control levels is achieved at 10 μM free Ca^{2+}. There was also no significant change in the Ca^{2+} cooperativity of release (a log release versus log [Ca^{2+}] plot gave slopes of 0.61 for wild-type cells and 0.70 for overexpressing cells in the linear region over 1–10 μM Ca^{2+}). These experiments were repeated on clone 2 with similar results (our unpublished observations). The possibility that increased exocytosis could be attributed to growth of the clones on G418 was ruled out by comparison with previously selected clones (Graham *et al.*, 1997), which showed no difference from control cells.

Regulated exocytosis can occur in the absence of MgATP but is increased in its presence because of reactions that prime the granules for fusion (Holz *et al.*, 1989; Hay and Martin, 1992). Preincubation with certain protein factors in priming incubations in the presence of MgATP can increase subsequent Ca^{2+}-triggered exocytosis in the absence of MgATP (Hay and Martin, 1992; Chamberlain *et al.*, 1995). We examined Ca^{2+}-stimulated secretion from wild-type cells and clone 1 in the presence or absence of MgATP, after previous incubation with MgATP. Secretion from both control and Csp1-overexpressing cells was reduced in the absence of MgATP but was still greater in overexpressing than from control cells (Figure 6C), consistent with a previous increase in priming due to overexpression.

DISCUSSION

It is now clear that synaptic vesicle and secretory granule exocytosis are highly complex processes requiring the sequential and specific interactions of a large number of cellular proteins. Many of these proteins have been identified, and the roles played by some of these are beginning to be understood, whereas other proteins that have been implicated in exocytosis have not been ascribed specific functions (for review see Sudhof, 1995). For a complete molecular understanding of secretory processes it is essential to determine the exact functions of proteins in exocytosis.

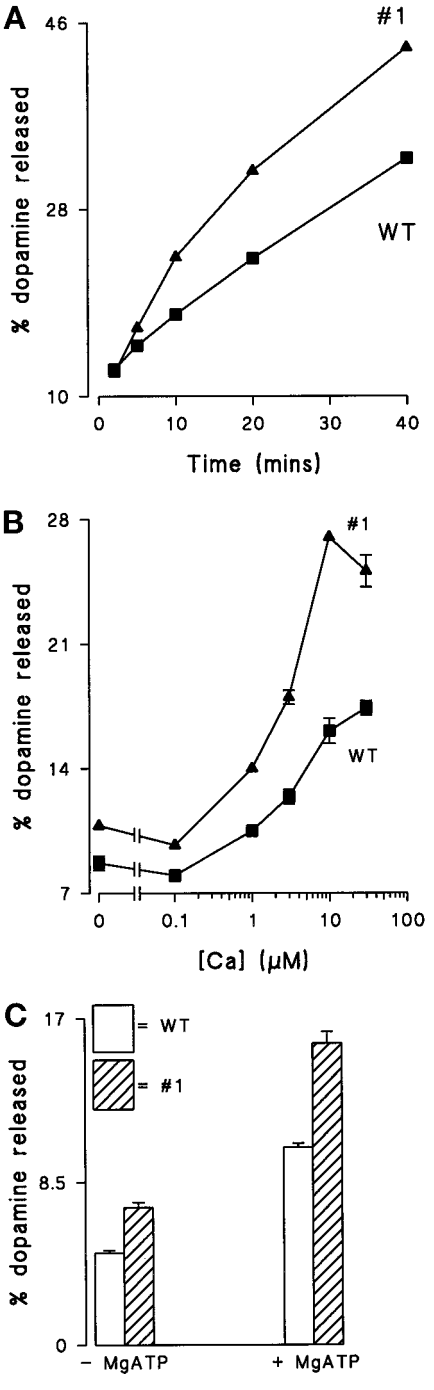

Figure 6. Characteristics of Ca^{2+}-evoked dopamine release from Csp-overexpressing cells. Cells in 24-well trays (0.5×10^6/well) were loaded with [^{3}H]dopamine for 90 min at 37°C. The cells were washed and permeabilized by incubation in 20 μM digitonin for 6 min. (A) Control and overexpressing cells (clone 1) were stimulated to secrete by addition of 10 μM Ca^{2+}, and release was measured at various time points. (B) Cells were stimulated by addition of increasing Ca^{2+} concentrations for 20 min. (C) Dopamine release was analyzed after 20 min in the presence or absence of 2 mM MgATP and with 0 or 10 μM Ca^{2+}, and Ca^{2+}-dependent release is shown. All data are expressed as mean ± SEM of the percentage of total dopamine cell content per each well (n = 6).

There is clear evidence that Csp is involved in neurotransmission, and this has been well documented in *Drosophila* (Umbach *et al*, 1994; Zinsmaier *et al*., 1994; Heckmann *et al*., 1997; Umbach and Gundersen, 1997; Ranjan *et al*., 1998). However, these studies have mainly focused on the idea that the function of Csp is to regulate presynaptic Ca^{2+} channel activity (Umbach and Gundersen, 1997; Ranjan *et al*., 1998), and a direct effect of Csp on exocytosis has not been demonstrated. We were interested in whether Csp has a direct function in exocytosis in addition to Ca^{2+} channel modulation, as suggested by the widespread tissue distribution of Csp (Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996) and its potentially general chaperone function (Chamberlain and Burgoyne, 1997b).

Permeabilized cell studies have previously been used to demonstrate direct effects of proteins on regulated exocytosis and in some cases has allowed an analysis of the step in secretion at which these proteins act (e.g., Hay and Martin, 1992; Chamberlain *et al*., 1995). This model system is appealing because it measures direct effects on exocytosis, independent of membrane depolarization and channel activity. To examine whether Csp1 has direct effects on regulated exocytosis, we generated PC12 cell lines that overexpress this protein. This approach was used because the introduction of recombinant Csp into permeabilized cells had no effect on secretion, presumably because the recombinant protein is not palmitoylated (our unpublished observations). Introduction of Csp IgG into PC12 cells also did not significantly affect stimulated secretion (our unpublished observations). Control and overexpressing cells were permeabilized, and secretion was measured in response to Ca^{2+} or GTPγS. Overexpression of Csp1 enhanced the extent of evoked exocytosis by either stimulus by ~50%. This represents the first demonstration that Csp has a direct role in exocytosis and also demonstrates that it does not not necessarily interact only with Ca^{2+}-sensitive components. Despite the lower extent of release due to GTPγS, this GTP analogue is clearly able to act while Ca^{2+} is clamped to very low levels in the presence of 5 mM EGTA; therefore, Csp can stimulate exocytosis independent of Ca^{2+}.

The effect of Csp1 overexpression on exocytosis appeared to be a specific effect. No consistent changes in the expression of a number of key exocytotic proteins were seen, and no changes in cell morphology, dopamine uptake, or number and distribution of dense-core granules were detected. In addition, no effect of Csp1 overexpression on fluid phase endocytosis was found (our unpublished observations).

Before this study, Csp had been suggested to regulate the activity of presynaptic Ca^{2+} channels (Gundersen and Umbach, 1992). We analyzed the effect of Csp1 overexpression on K^+-induced Ca^{2+} influx into cells loaded with the Ca^{2+}-sensitive dye Fura-2. No detectable differences in depolarization-induced intracellular Ca^{2+} signals were seen between control cells and Csp1-overexpressing clones. Thus, this work suggests that Csp1 overexpression does not grossly modify Ca^{2+} channel activity or Ca^{2+} signaling in PC12 cells.

It has previously been shown that the main Ca^{2+} channels responsible for depolarization-induced exocytosis in PC12 cells are the L-type channels (Takahashi *et al*., 1985; Kongsamut and Miller, 1986), whereas the Ca^{2+} channels mediating fast neurotransmitter release are typically N- and P/Q-type channels. The original work that demonstrated Ca^{2+} channel regulation by Csp examined N-type channels (Gundersen and Umbach, 1992). It is, therefore, possible that Csp only regulates Ca^{2+} channels involved in fast neurotransmitter secretion and not slower exocytosis from neuroendocrine cells. However, we cannot exclude the possibility that Csp1 has a more subtle regulation of Ca^{2+} channel activity in PC12 cells.

In view of the current results, it is tempting to reexamine previous results obtained with *Drosophila* Csp null mutants (Umbach *et al*., 1994; Zinsmaier *et al*., 1994). Inactivation of Csp is essentially lethal in *Drosophila*, although a small number of flies do survive into adulthood but die soon after. These surviving flies have been studied electrophysiologically, and it has been shown that presynaptic neurotransmission in these mutants is decreased by ~50% at the permissive temperature (22°C) and fails completely at the restrictive temperature (30°C). It is interesting that these flies have a temperature-sensitive phenotype, and this implies that, in the absence of Csp, a protein component of the exocytotic machinery is unstable at high temperatures. It follows that one function of Csp is to stabilize one or more components of the exocytotic machinery using its chaperone activity, as previously suggested (Zinsmaier *et al*., 1994; Chamberlain and Burgoyne, 1997b). These proteins could be involved in Ca^{2+} channel regulation or have a more direct function in exocytosis.

We believe that the temperature-sensitive phenotype displayed by Csp null mutant survivors may be related to the direct effect of Csp on exocytosis, rather than an absence of Ca^{2+} channel regulation. This suggestion is based on a comparison of the Csp mutant phenotype with the phenotype of other mutant flies with temperature-sensitive defects in neurotransmission. When Csp mutant preparations are exposed to the restrictive temperature (30°C), evoked transmission takes several minutes to become inhibited and 10–20 min to recover when the temperature is reduced to 22°C (Umbach *et al*., 1994). These time courses are consistent with a role for Csp in a slow early step of exocytosis, such as vesicle priming (Morgan, 1996).

If the temperature-sensitive phenotype of Csp mutant flies was caused solely by a loss of direct Ca^{2+} channel regulation, then the flies would be expected to have a faster block and recovery of evoked responses than that exhibited. For example, flies with a temperature-sensitive mutation in a voltage-dependent Na^+ channel (*para*) display very rapid paralysis and recovery when exposed to the restrictive and permissive temperatures, respectively (Siddiqi and Benzer, 1976; Loughrey *et al*., 1989). On the other hand, *comatose* flies, which have a temperature-sensitive mutation in *N*-ethylmaleimide-sensitive factor, have similar inhibition and recovery of neurotransmission time courses to Csp mutant preparations (Siddiqi and Benzer, 1976; Pallanck *et al*., 1995). *N*-ethylmaleimide-sensitive factor probably functions in an MgATP-dependent manner to prime vesicles for subsequent fusion (Morgan and Burgoyne, 1995; Mayer *et al*., 1996), and Csp may, therefore, exert its function in a priming step of exocytosis. In support of this, we found that in the absence of MgATP, secretion was reduced in both control and overexpressing cells, but an increase due to Csp overexpression over wild-type cells persisted, showing that its previous expression enhanced MgATP-independent secretion. This suggests that Csp overexpression increased the priming of the exocytotic machinery. Consistent with this interpretation is the increase in the initial rate of exocytosis in Csp1-overexpressing compared with control cells. A role for Csp in ATP-dependent priming is also consistent with the demonstration that Csp interacts with the ATPase Hsc70 (Braun *et al*., 1996; Chamberlain and Burgoyne, 1997a). With the demonstration that Csp functions directly in regulated exocytosis, it will now be important to determine the nature of the multiple or specific substrates for the chaperone action of Csp.

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