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Mutational analysis of cysteine-string protein function in insulin exocytosis

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SUMMARY

Cysteine-string proteins (Csps) are vesicle proteins involved in neurotransmission. They contain at least four domains: an N-terminal J-domain which can interact with the chaperone Hsc70, an adjacent linker region, the defining cysteine rich domain and a variable C terminus. As the relevance of these domains for the function of Csps in exocytosis is unknown, we have performed a mutational analysis of Csp domains using insulin release by large dense core vesicles (LDCVs) as a model of regulated exocytosis. All mutants were apparently palmitoylated and their subcellular distribution was similar to endogenous Csp. Point mutations within the highly conserved HPD motif of the J-domain abolished activation of Hsc70. However, these mutations altered the effect of Csp on exocytosis only after additional truncation of the extreme C terminus as found in the Csp splice variant Csp2. Furthermore, the strikingly conserved linker region adjacent to the J-domain was important for Csp function in exocytosis, but not for the activation of Hsc70 ATPase. The effects of Csp wild-type or mutants were preserved in permeabilized cells excluding an effect on transmembrane ion fluxes. These observations demonstrate a functional difference between the two isoforms and suggest a role for the J-domain co-chaperone function as well as for the newly defined linker region in LDCV exocytosis.

Key words: Exocytosis, Insulin, Calcium, DnaJ

INTRODUCTION

Neurotransmitters and peptide hormones are released by exocytosis, the final step in regulated secretion. A network of proteins acting in multi-subunit complexes mediates this dynamic event. Their highly orchestrated assembly, rearrangement and disassembly constitute a key part of the events leading to exocytosis (Augustine et al., 1996; Martin, 1997) and may require chaperone function (Morgan and Burgoyne, 1995). The cysteine-string proteins (Csps) belong to the DnaJ family of molecular chaperones and were first discovered as presynaptic proteins in *Drosophila* (Zinsmaier et al., 1990). Csps are involved in neurotransmission as deletion of the Csp gene in *Drosophila* causes a temperature-sensitive block of synaptic transmission (Umbach et al., 1994; Zinsmaier et al., 1994).

The molecular basis for Csp function in exocytosis is unknown. Extensive homology exists between the N terminus of Csps and the J-domain of the *Escherichia coli* DnaJ chaperone/heat shock protein family (Caplan et al., 1993). In bacteria, the prototypical DnaJ acts in conjunction with the heat-shock protein DnaK, an *E. coli* Hsp70, and stimulates hydrolysis of ATP bound to Hsp70/DnaK, while GrpE acts as nucleotide exchanger, promoting continuous cycles of activity (for review see Kelley, 1998). The triad DnaJ/DnaK/GrpE has the capacity to modulate the structure of native proteins by controlling their oligomerisation state or their association with interacting proteins. Indeed, the J-domain of Csps themselves behaves as a chaperone and stimulates the ATPase activity of Hsc70 (Braun et al., 1996; Chamberlain and Burgoyne, 1997a,b). Chaperone function of Csps might be required for the ordered interaction of exocytotic proteins. The J-domain is linked to the cysteine string region by an evolutionarily highly conserved domain. The putative involvement of this linker region in Csp function, however, is unknown. Moreover, two splice variants of Csps have been identified in mammals, Csp1 and the truncated form Csp2, which lacks the 31 carboxy-terminal amino acids (Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996; Mastrogiacomo and Gundersen, 1995). Functional differences between the isoforms have not been described although differential expression of Csp isoforms had been observed in *Drosophila* (Eberle et al., 1998).

Insulin release from large dense core vesicles (LDCVs) of pancreatic β-cells constitutes an established model for peptide hormone exocytosis (Wollheim et al.,1996; Lang, 1999). Csp1 is found on insulin-containing secretory granules and its transient overexpression induces a considerable reduction in insulin secretion from clonal β-cells (Brown et al., 1998; Zhang et al., 1997). Although this effect was not accompanied by appreciable changes in transmembrane Ca^{2+} fluxes (Brown et al., 1998), the use of intact cells does not allow assessment

of a direct effect on exocytosis (Augustine et al., 1996). In order to define now functional domains of Csp in exocytosis, we have performed a mutational analysis using intact and permeabilized insulin-secreting cells as a model system of LDCV exocytosis.

MATERIALS AND METHODS

Materials

Streptolysin-O was produced and purified as described (Weller et al., 1996). Primary antibodies and dilutions were: polyclonal anti-insulin antibody (Linco, 1:400); monoclonal anti-myc antibody 9E10 (1:100 for immunofluorescence, 1:2000 for immunoblots); the generation and specificity of polyclonal rabbit anti-Csp1 has been described (Chamberlain and Burgoyne, 1996; Zhang et al., 1998) (1:200 for immunofluorescence, 1:2000 for immunoblots).

Western blotting and immunofluorescence

Preparation of homogenates from insulin secreting cell lines and SDS/PAGE immunoblots were performed as published (Lang et al., 1997a,c). Membrane attachment was tested as described (van de Goor and Kelly, 1996) with minor modifications. Briefly, 80 μg of homogenates were treated for 2 hours at 4°C with buffer (150 mM NaCl, 10 mM Hepes, 1 mM EGTA and 0.1 mM $MgCl_2$, final pH 7.0) in the absence or presence of 1 M hydroxylamine and analysed by western blotting. Immunofluorescence and confocal laser microscopy were performed with a Zeiss LSM 410 inverse laser scan microscope equipped with an argon and helium-neon laser (Lang et al., 1997a,c; Zhang et al., 1998).

Site directed mutagenesis

The cDNA fragments encoding Csp1 and Csp2 (Chamberlain and Burgoyne, 1996) were first ligated into the *Bam*HI/*Eco*RI sites of pGEM3Zf(+) (Stratagene). Single strand DNA was produced by mobilisation with phage VCSM13 (Stratagene) in *E. coli* strain CJ236 and mutagenesis performed by the method of Kunkel (1985). The primers used were H43Q: (5′-GGTTCTTGTCAGG-(G/T)TGGTATTTCAAG-3′), D45N: (5′-CAGGGTTCTTGT(C/T)-AGGGTGGTATTTC-3′); and E93V: (5′-GTGTTCACGTTC(T/A)-CCTCCCCGAACTG-3′). All mutations were verified by double strand sequence analysis. The *Bam*HI/*Eco*RI fragments encoding wild-type and mutant Csps were excised from pGEM3Zf(+) and subcloned into the corresponding sites of the expression vector pcDNA3.1 (Invitrogen). To obtain N-terminal myc-tagged Csps, a *Hin*dIII/*Eco*RI myc-rab3A fragment was first subcloned from pGEM-myc-rab3A (kindly provided by Dr L. Johannes, Institut Pasteur, Paris) and ligated with pcDNA3.1 digested with the same enzymes. Finally, the rab3A fragment was excised and replaced by the *Bam*HI/*Eco*RI fragments encoding the Csps.

Cell culture, transient transfection and release studies

Cell cultures and transient cotransfections were performed as published (Lang et al., 1995, 1997a,b,c; Regazzi et al., 1996; Zhang et al., 1998). For expression and secretion experiments, 2.5 μg of preproinsulin encoding plasmid (phINS) were mixed with either 2.5 μg pcDNA3 (control) or with 0.5 μg Csp-constructs and 2.0 μg pcDNA3 per well. Hydroxylamine sensitivity experiments were performed as single transfections using 5 μg of Csp-encoding plasmids per well to ensure optimal detection by the anti-myc antibody. Transient expression of wild-type and mutated Csps were determined in parallel with secretion experiments by scanning of immunoblots using Imagequant v3.3 (Molecular Dynamics). Several autoradiographic exposures were made and scanned to determine the region of maximum linearity of film and scanner response. Those exposures were chosen in which the signals were above the film sensitivity limit and not saturated. The bands were compared using area measurements. Release studies from intact or permeabilized cells were performed as described before and human insulin C-peptide was determined as measure of insulin release from cotransfected cells by a species specific ELISA (DAKO) (Lang et al., 1997a,c; Zhang et al., 1998).

Purification of recombinant proteins and ATPase assays

Csp fragments were subcloned into *Bam*HI/*Eco*RI sites of the vector pGEX-2T (Pharmacia Biotech) and GST tagged recombinant proteins purified according to the instructions of the manufacturer. pHsc70 plasmid (Wang and Lee, 1993) was transformed into *E. coli* BL21 (lDE3) and Hsc70 was prepared according to published methods (Schlossman et al., 1984; Wang and Lee, 1993). Recombinant proteins were over >95% pure as judged by Coomassie Blue stained SDS-PAGE gels. For Hsc70 products, EDTA was added to a final concentration of 4 mM to restore nucleotide dependence. ATPase activity assays were measured as inorganic phosphate release using a spectrophotometric method (Chamberlain and Burgoyne, 1997a).

Statistical analysis

Data are presented as mean ± s.e.m. from experiments performed independently on at least three different cell preparations. Statistical analysis was performed by Student's two-tailed *t*-test for unpaired data (2p).

RESULTS

Csp domains and expression of point mutants

In order to define which domains of Csps were required for their function in hormone release, several point mutants were constructed of the two described isoforms of mammalian Csps, namely Csp1 and Csp2 which differ only in their C-termini (Fig. 1). Csps contain an N-terminal J domain and an HPD tripeptide motif that is highly conserved in all J domain proteins studied. Point mutations in this HPD motif attenuate or abolish J domain function in many systems (for review see Kelley, 1998). Therefore the point mutations H43Q and D45N were engineered. Moreover, the linker region between the J domain and the cysteine-rich string is strikingly conserved in all Csps described (Chamberlain and Burgoyne, 1996; Gundersen and Umbach, 1992; Mastrogiacomo and Gundersen, 1995; Mastrogiacomo et al., 1998b; Wilson et al.,

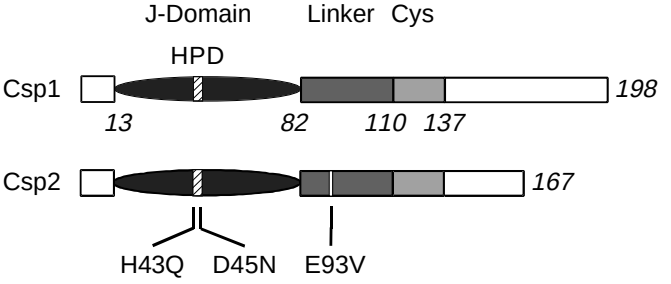

Fig. 1. Schematic representation of the domains of mammalian Csp splice isoforms and engineered mutations. Csp1 and its splice variant Csp2 contain a short N-terminal domain, a J-domain (13-82) with the HPD tri-motif, an adjacent highly conserved linker region, a cysteine string and a variable C terminus. Numbers indicate the approximate boundaries of the domains. Point mutations H43Q and D45N are located in the HPD-motif of the J-domain, whereas the mutation E93V resides in the linker region. The double point mutant Csp2H43Q/E93V was abbreviated as Csp2DM.

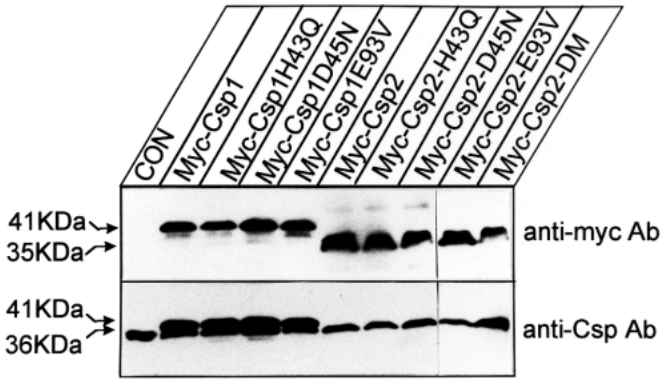

Fig. 2. Expression of wild-type and mutated Csps in transiently cotransfected hamster insulinoma HIT-T15 cells. Wild-type or mutant myc-tagged Csps were transiently expressed in HIT-T15 cells and analysed by western blotting with anti-myc (myc) antibody or anti-Csp1 antibody (anti-Csp). Quantification of blots revealed the following expression levels as compared to myc-Csp1 wild-type (mean + s.e.%): myc-Csp1H43Q 98.7+8.0; myc-Csp1D45N 110.5+7.8; myc-Csp1E93V 98.1+12.6; myc-Csp2 113.3+23.5; myc-Csp2H43Q 110.3+7.3; myc-Csp2D45N 113.0+14.2; myc-Csp2E93V 110.5+13.5; myc-Csp2DM 108.1+16.9.

1994; Zinsmaier et al., 1990) and presented an additional target for mutational analysis (E93V). Finally we constructed a double mutant of the HPD motif and the linker region (Csp2H43Q/E93V, abbreviated as Csp2DM).

We first examined the levels of transiently expressed proteins in hamster insulinoma HIT-T15 cells by immunoblot analysis of homogenates. To permit identification of transiently expressed versus endogenous Csps, constructs were used which express an N-terminal myc-epitope (Fig. 2). Note that tagged and untagged constructs did not differ in functional assays (data not shown). Incubation of blots with an antibody against the myc-tag revealed comparable expression of all constructs used (see legend, Fig. 2). The anti-Csp1 antibody stained two bands in homogenates from cells cotransfected with myc-tagged Csp1 constructs, but not in cells cotransfected with a control plasmid (pcDNA3) or with Csp2 constructs. Whereas the lower band represents endogenous Csp1, the upper band corresponds to transiently expressed myc-Csp1. Quantification of scanned band indicated that myc-tagged wild-type Csp1 was expressed at levels similar to endogenous Csp1 (81.8+15%). Taking into account a transfection rate of 15% transfected cells as determined by control expression of enhanced green fluorescent protein (eGFP), overexpression amounts to an approximately fivefold increase as compared to endogenous levels.

Effect of Csp point mutants on insulin secretion and exocytosis

As transient transfection will result in transgene expression only by a fraction of cells, we employed cotransfections with a plasmid encoding human preproinsulin (phINS). The ensuing expression and release of human insulin C-peptide serves as a reporter for exocytosis in the hamster HIT-T15 cell line and can be reliably distinguished from rodent insulin released from all cells by a species-specific ELISA (Lang et al., 1995, 1997a,c; Regazzi et al., 1996; Zhang et al., 1998). Total cellular contents of transiently expressed human insulin C-peptide did not differ between control and cells overexpressing Csp constructs indicating that Csp expression did not perturb insulin biosynthesis (data not shown). As given in Fig. 3 (upper panel), overexpression of Csp1 largely inhibited human insulin C-peptide release in response to depolarization evoked Ca^{2+} influx as reported previously (Zhang et al., 1997; Brown et al., 1998). A similar degree of inhibition, i.e. approximately 80%, was observed for Csp2. In the case of Csp1, constructs with point mutations in its J domain (Csp1H43Q, Csp1D45N) or in its linker region (Csp1E93V) reduced secretion to the same extent as wild-type Csp1. In contrast, constructs with identical point mutations in the splice variant Csp2 reduced insulin C-peptide release by only 50% (Csp2H43Q, Csp2D45N) and 33% (Csp2E93V), respectively. Similar results were observed for Csp2 and Csp2E93V when the insulin-secreting cells were stimulated by glucose (data not shown). Moreover, double mutation in Csp2 of both the HPD-motif and the linker region (Csp2DM) completely abolished the inhibitory effect. These results suggest that the mutations in the J-domain or linker region of Csp2, but not of Csp1, affect the ability of Csps to interfere with insulin secretion.

As Csp may modulate Ca^{2+} channel activity (Ranjan et al.,

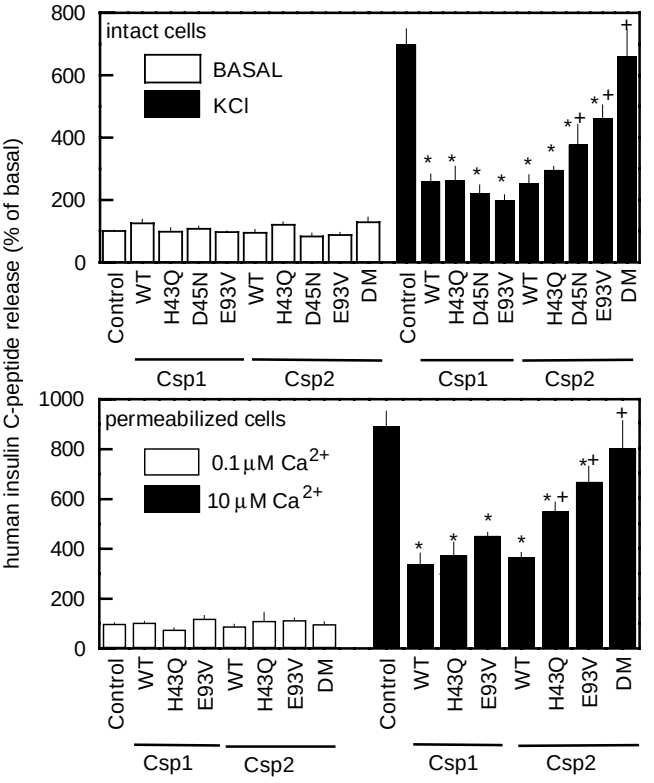

Fig. 3. Effects of Csps on insulin secretion and exocytosis in HIT-T15 cells. Cells were co-transfected with cDNA for human insulin as reporter gene and a control plasmid (pcDNA3) or with cDNA encoding wild-type Csps or mutated Csps. 2 days later, human insulin C-peptide release was measured and normalised to basal release from control cells which was set to 100%. Upper panel: release under basal conditions (KRB, open bars) or after stimulation by 50 mM KCl (KCl, filled bars). n=10-22; *2P<0.05 as compared to control; $^{+}2P$<0.05 as compared to the corresponding wild-type Csp isoform. Lower panel: cotransfected cells were permeabilized with streptolysin O and exposed to 0.1 μM free Ca^{2+} (basal, open bars) or 10 μM free Ca^{2+} (stimulated, filled bars). n=6-24; * and + as above.

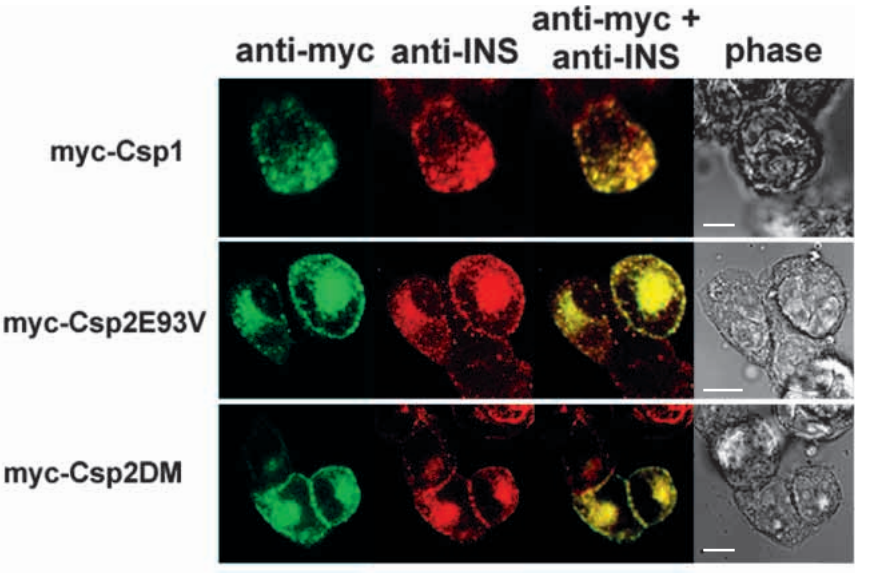

Fig. 4. Subcellular localisation of wild-type or mutant Csps transiently expressed in HIT-T15 cells. Cells were transiently transfected with plasmids encoding for myc-tagged Csp1 wild-type (Csp1), Csp2E93V or CSP2DM. 2 days later, cells were fixed, permeabilised and incubated with a monoclonal antibody against the myc-tag and a polyclonal antibody against insulin staining secretory granules. Myc-Csps were revealed using FITC-conjugated anti-mouse IgG (green) and rhodamine-conjugated anti-rabbit IgG (red) was used as a second antibody to stain insulin.

1998; Umbach et al., 1998), we also tested the effect of transient coexpression in cells subsequently permeabilised by the pore forming toxin streptolysin-O and stimulated by defined concentration of free Ca^{2+}. Under these conditions, transient overexpression of wild-type Csp1 or Csp2 again inhibited Ca^{2+}-stimulated exocytosis by 59% and 64%, respectively (Fig. 3, lower panel). As already observed in intact cells, this inhibition was not altered by the point mutations H43Q or E93V of Csp1 (61% and 64%), but was affected only by the corresponding single point mutations of the splice variant Csp2 (37% and 22%). Again, the double mutant of Csp2 (Csp2DM) was ineffective.

Exocytosis from SL-O permeabilised cells can be stimulated independent of Ca^{2+} by the stable GTP-analogue GTPS (Lang et al., 1997a; Li et al., 1993). GTPγS stimulated insulin exocytosis 3.5-fold in controls. Again, Csp1, Csp1E93V and Csp2 decreased the release by 83.7+8.8%, 81.9+5.8% and 72.2+4.4%, respectively, whereas Csp2E93V inhibited only by 31.5+8.9% (*n*=6-15). These results indicate that Csp action is situated at a late step in exocytosis distal to the regulation of ion fluxes.

Subcellular localisation and hydroxylamine sensitivity of transiently expressed Csps

Endogenous Csps are located on insulin-containing secretory granules (Zhang et al., 1997, 1998; Brown et al., 1998). In order to address the subcellular localisation of transiently expressed Csp constructs, affinity tagged proteins and secretory granules were visualised by confocal laser immunomicroscopy using the corresponding antibodies. As shown in Fig. 4, overexpressed Csp1 wild-type (Csp1), Csp2 mutated in the linker region (Csp2E93V) or in both the HPD-motif and the linker (Csp2DM) colocalised with secretory granules stained with anti-insulin antibodies. Comparable results were obtained for Csp2wt, Csp2H43Q, Csp2D45N, Csp1H43Q, Csp1D45N or Csp1E93V (data not shown).

Csps are postranslationally modified by palmitoylation of their cysteine residues and chemical deacylation by hydroxylamine induces a shift in the R_f values on SDS/PAGE (Gundersen et al., 1994; van de Goor and Kelly, 1996; Mastrogiacomo et al., 1998a). The increased apparent molecular weight of myc-tagged Csps as compared to endogenous Csps suggests acylation of transiently expressed proteins similar to endogenous protein (see above, Fig. 2). We addressed the question whether transiently expressed Csps were palmitoylated by chemical deacylation of wild-type Csps and those point mutants, which produced the most prominent effects on insulin C-peptide release. To this end, cells were transiently transfected with the corresponding cDNAs and the apparent shifts in SDS/PAGE determined after pretreatment with or without hydroxylamine. As shown in Fig. 5, all constructs exhibited a comparable shift in R_f values after deacylation suggesting similar degrees of postranslational

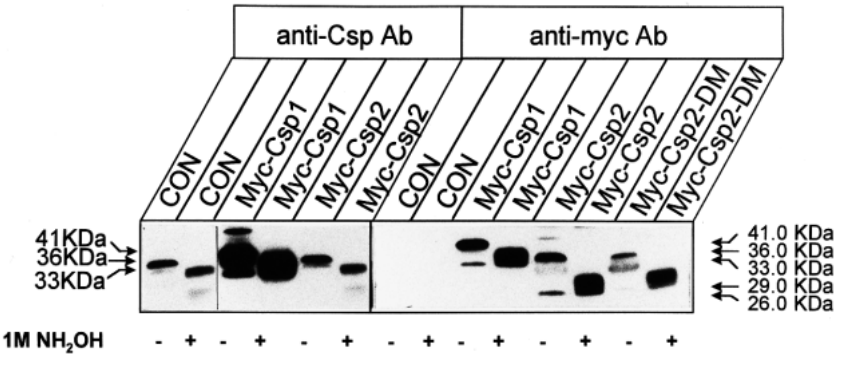

Fig. 5. Hydroxylamine sensitive postranslational modification of endogenous and transiently expressed Csp. Wild-type and mutated myc-tagged Csps were transiently expressed in HIT-T15 cells after single transfection (see Materials and Methods). Cells were homogenized, treated for 2 hours either with buffer (–) or with 1 M hydroxylamine (+) and immunoblots were performed with anti-Csp1 or anti-myc antibody.

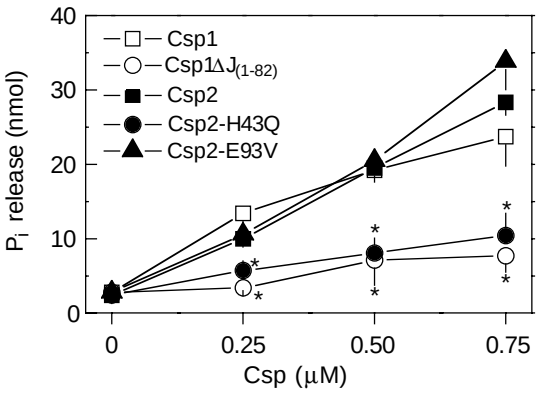

Fig. 6. Effect of recombinant wild-type or mutated Csps on Hsc70 activity. Indicated amounts of purified GST-Csp fusion protein and 0.4 μM of purified recombinant Hsc70 were mixed for 1 hour at 37°C. ATPase activity was determined by a spectrophotometric method and compared with a standard curve using K_2HPO_4. *, $2P<0.05$ as compared to Csp2wt.

palmitoylation. The anti-myc antibody detected a minor second band at 33 and 26 kDa (myc-Csp1 and myc-Csp2, respectively) in the absence of hydroxylamine treatment which may represent a small amount of non-palmitoylated protein.

Mutational analysis of the function of Csps in stimulation of Hsc70 ATPase activity

Csps may exert some of their biological effect by interaction with the Hsc70 heat shock protein family, since the J domain of Csps stimulates Hsc70 ATPase activity (Braun et al., 1996; Chamberlain et al., 1997a,b). Therefore we were also interested to evaluate the effect of some Csp mutations for their capacity to stimulate the ATPase activity of Hsc70. Our results showed that recombinant Csp1 and Csp2 stimulated Hsc70 ATPase in a concentration dependent manner (Fig. 6). Deletion of the J-domain ($Csp1\Delta J_{1\text{-}82}$) or mutation in the HPD-motif of the J-domain abolished the capacity of Csp2 to stimulate the ATPase activity of Hsc70 as predicted from numerous reports (for review see Kelley, 1998). In contrast to the mutations in the J-domain, the point mutation E93V in the linker region of Csp2 did not alter the stimulation of Hsc70 ATPase. Note that this mutation clearly impaired the inhibitory effect of Csp2 on regulated insulin exocytosis as shown above (see Fig. 3).

DISCUSSION

Exocytosis of synaptic or large dense core vesicles is a dynamic process, which is regulated by a large number of distinct gene products. Several of these proteins have been identified and functional sites mapped whereas others still await functional characterisation (Augustine et al., 1996; Martin, 1997). Clearly, a comprehension of their function in molecular terms is required to understand secretory processes. Cysteine string proteins are likely to be a part of the general exocytotic machinery. Suppression of Csp levels in neurons or clonal pancreatic β-cells leads to an impairment of stimulated exocytosis from SVs and LDCVs, respectively (Umbach et al., 1994; Zinsmaier et al., 1994; Eberle et al., 1998; Zhang et al., 1998). Moreover, stable overexpression of Csp1 in neuroendocrine PC12 cells potentiated LDCV exocytosis (Chamberlain and Burgoyne, 1998), whereas transient overexpression of Csp1 in clonal β-cells reduced insulin secretion from intact cells (Zhang et al., 1997; Brown et al., 1998). The difference in effects observed in PC12 and β-cells may be due to the different methods used (stable versus transient transfection) or reflect differences in the levels of endogenous Csp or the organisation of exocytosis in neuroendocrine as compared to endocrine cells.

As demonstrated now, the inhibition of insulin release is observed not only for both isoforms, but also more importantly, the effect persisted in permeabilized cells. This excludes any regulation via soluble second messengers (Augustine et al., 1996; Lang, 1999) and therefore reflects a direct action on exocytosis. Since experiments were conducted in the presence of ATP, the observed effects may relate to several distinct steps during exocytosis such as priming or fusion (Martin, 1997). Only evoked, but not basal exocytosis was altered by deletion or overexpression of Csps (Zhang et al., 1998 and this study) in agreement with studies of Csp null mutants in *Drosophila* at permissive temperature (Eberle et al., 1998). These effects concern most likely a general stimulus-evoked mechanism in exocytosis because they were found for both stimuli, GTPγS and Ca^{2+}, which are known to differ in their requirements for cytosolic factors and the calcium-sensing protein synaptotagmin (Kiraly-Borri et al., 1996; Lang et al., 1997a).

Similar to the situation for Csp, inhibition of exocytosis by both deletion and by overexpression has been reported for rop, the *Drosophila* homologue of sec1/unc-18 (Schulze et al., 1994; Gengyoando et al., 1996). As both rop and Csps are required for normal exocytosis, the observed inhibition upon overexpression may result from the formation of complexes with interacting proteins. They may be located up- or downstream from Csps within the sequence of events leading to ATP-dependent exocytosis. Excessive binding of Csps to Hsc70 is probably not involved. Indeed, inhibition of insulin exocytosis was also induced by Csp1 mutated in the HPD motif, which no longer binds to Hsc70 (Chamberlain and Burgoyne, 1997b). As shown here, overexpression does not alter targeting and seemingly does not change postranslational acylation. We have therefore used overexpression combined with point mutations to investigate which domains of the protein are necessary for function and may thereby be relevant in interactions with other components of the exocytotic machinery.

We have focused on: (i) the N-terminal J-domain, (ii) the adjacent linker region, and (iii) the variant extreme C terminus. Within the J-domain, the canonical HPD motif is required to stimulate Hsc70 ATPase by Csp1 (Chamberlain and Burgoyne, 1997b; Kelley, 1998) and by Csp2, as shown here. However, only mutations in Csp2 reduced the interference of Csps with LDCV exocytosis. As the two splice variants, Csp1 and Csp2, differ only in their C-terminal 31 amino acids (Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996), our results suggest a functional interaction between the C terminus and the J-domain in the regulation of exocytosis, but not in the stimulation of Hsc70 ATPase in-vitro. To our knowledge, this constitutes the first evidence that the two isoforms differ in their function. Interestingly the C terminus varies also widely among Csp isoforms in *Drosophila* (Zinsmaier et al., 1990).

Prediction of structural features (Lupas, 1996) indicates that the C terminus as found in Csp1 has a high probability to confer a coiled-coil region. Such a motif may participate in protein-protein interactions as previously shown for other proteins involved in exocytosis (Lin and Scheller, 1997).

The importance of the C terminus of Csps is further underscored by the effects of the point mutant E93V. This construct stimulated the ATPase of Hsc70 to the same extent as wild-type Csp. However, in the C-terminally truncated splice variant Csp2 this mutation largely alleviated the effect of wild-type Csp2 on stimulated insulin release. The amino acid E93 is located between the J-domain and the cysteine-string in a region, which has been strikingly conserved during evolution. Indeed, this 'linker region' (Csp83-110) displays 68%, 96% and 100% identity in *Drosophila*, *Torpedo* and *Xenopus* Csps as compared to the mammalian protein (Zinsmaier et al., 1990; Gundersen and Umbach, 1992; Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996; Mastrogiacomo et al., 1998b) and can still be found in a *C. elegans* Csp homologue (Wilson et al., 1994). Bacterial DnaJ proteins contain a glycine/phenylalanine rich linker region (G/F linker) next to their J-domain and this linker is known to be important for the efficient stimulation of DnaK ATPase (Wall et al., 1995). A homologous domain cannot be found in Csps. However, the residues adjacent to CspE93 are predicted to form an exposed hydrophilic α-helix, which might engage the specific substrate of Hsc70 to combine a Csp/Hsc70 substrate complex. Similarly, the clathrin-binding domain of auxilin has been mapped adjacent to the auxilin J-domain (Holstein et al., 1996). The mutant Csp2E93V clearly defines a new domain necessary for the action of Csps in exocytosis and again stresses the functional importance of the extreme C terminus. Interestingly, the effects of the point mutations H43Q and E93V were additive in Csp2. This suggests that both, activation of Hsc70 and the linker region are required for normal function of Csps in exocytosis.

The molecular actions of Csps in neuroexocytosis have been linked to the sites of Ca^{2+}-entry rather than to a failure of Ca^{2+} action on the exocytotic machinery (Ranjan et al., 1998; Umbach et al., 1998). In contrast, major alterations in Ca^{2+} influx have not been detected following overexpression of Csp1 in PC12 cells or clonal β-cells (Brown et al., 1998; Chamberlain and Burgoyne, 1998). Moreover, as shown in the current study, the effects of Csps on insulin release are fully expressed in streptolysin-O permeabilised cells and they are even apparent in GTPγS-stimulated exocytosis, which proceeds independently of Ca^{2+} (Li et al., 1993; Lang, 1999). Although overexpression of Csps cannot be directly compared to the situation in *Drosophila* null mutants, we have shown previously that reduction of Csp levels impedes Ca^{2+} stimulated exocytosis in permeabilised cells (Zhang et al., 1998). Consequently, Csps must exert a part of their action on exocytosis independent of the regulation of ion fluxes. Indeed, presynaptic P/Q- and N-type Ca^{2+} channels may also organise the physical site of exocytosis in addition to the mediation of Ca^{2+} influx (Stanley, 1997). Csp1 interacts in-vitro with presynaptic P/Q-type Ca^{2+} channels (Leveque et al., 1998) and this effect may provide the molecular basis for the initially proposed link between synaptic vesicles and presynaptic Ca^{2+} channels (Mastrogiacomo et al., 1994). In pancreatic β-cells mainly L-, but also P/Q-type Ca^{2+} channels are implicated in excitation-secretion coupling (Bokvist et al., 1995; Ligon et al., 1998). It is currently not known whether L-type channels bind Csps but a situation similar to neurons may apply for the different types of Ca^{2+} channels linked to secretion in endocrine β-cells.

In conclusion, Csps regulate insulin exocytosis by a combined functional interaction of their J-domain and their linker region with the C terminus. A precedent for chaperones and ATPase function in exocytosis is given by the role of NSF and α-SNAP (Morgan and Burgoyne, 1995). It is not surprising that the complex interactions between multimeric protein assemblages necessary for exocytosis require chaperones for correct folding, assembly and disassembly (Hanson et al., 1997). As the role of Csps in insulin exocytosis has now been established and functional domains mapped, future research should address the precise nature of effector(s) and regulators of Csps.

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REFERENCES

Augustine, G. J., Burns, M. E., Debello, W. M., Pettit, D. L. and Schweizer, F. E. (1996). Exocytosis: proteins and perturbations. *Annu. Rev. Pharmacol. Toxicol.* **36**, 659-701.

Bokvist, K., Eliasson, L., Ammala, C., Renstrom, E. and Rorsman, P. (1995). Co-localization of L-type Ca^{2+} channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic B-cells. *EMBO J.* **14**, 50-57.

Braun, J. E. A., Wilbanks, S. M. and Scheller, R. H. (1996). The cysteine string secretory vesicle protein activates Hsc70 ATPase. *J. Biol. Chem.* **271**, 25989-25993.

Brown, H., Larsson, O., Bränström, R., Yang, S. N., Leibiger, B., Leibiger, I., Fried, G., Moede, T., Deeney, J. T., Brown, G. R., et al. (1998). Cysteine string protein (CSP) is an insulin secretory granule-associated protein regulating beta-cell exocytosis. *EMBO J.* **17**, 5048-5058.

Caplan, A. J., Cyr, D. M. and Douglas, M. G. (1993). Eukaryotic homologues of Escherichia coli dnaJ: a diverse protein family that functions with hsp70 stress proteins. *Mol. Biol. Cell* **4**, 555-563.

Chamberlain, L. H. and Burgoyne, R. D. (1996). Identification of a novel cysteine string protein variant and expression of cysteine string proteins in non-neuronal cells. *J. Biol. Chem.* **271**, 7320-7323.

Chamberlain, L. H. and Burgoyne, R. D. (1997a). Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. *Biochem. J.* **322**, 853-858.

Chamberlain, L. H. and Burgoyne, R. D. (1997b). The molecular chaperone function of the secretory vesicle cysteine string proteins. *J. Biol. Chem.* **272**, 31420-31426.

Chamberlain, L. H. and Burgoyne, R. D. (1998). Cysteine string protein functions directly in regulated exocytosis. *Mol. Biol. Cell* **9**, 2259-2267.

Coppola, T. and Gundersen, C. (1996). Widespread expression of human cysteine string proteins. *FEBS Lett.* **391**, 269-272.

Eberle, K. K., Zinsmaier, K. E., Buchner, S., Gruhn, M., Jenni, M., Arnold, C., Leibold, C., Reisch, D., Walter, N., Hafen, E., et al. (1998). Wide distribution of the cysteine string proteins in *Drosophila* tissues revealed by targeted mutagenesis. *Cell Tissue Res.* **294**, 203-217.

Gengyoando, K., Kitayama, H., Mukaida, M. and Ikawa, Y. (1996). A murine neural-specific homolog corrects cholinergic defects in Caenorhabditis elegans unc-18 mutants. *J. Neurosci.* **16**, 6695-6702.

Gundersen, C. B. and Umbach, J. A. (1992). Suppression cloning of the cDNA for a candidate subunit of a presynaptic calcium channel. *Neuron* **9**, 527-537.

Gundersen, C. B., Mastrogiacomo, A., Faull, K. and Umbach, J. A. (1994). Extensive lipidation of a Torpedo cysteine string protein. *J. Biol. Chem.* **269**, 19197-19199.

Hanson, P. I., Heuser, J. E. and Jahn, R. (1997). Neurotransmitter release – four years of SNARE complexes. *Curr. Opin. Neurobiol.* **7**, 310-315.

Holstein, S. E., Ungewickell, H. and Ungewickell, E. (1996). Mechanism of clathrin basket dissociation: separate functions of protein domains of the DnaJ homologue auxilin. *J. Cell Biol.* **135**, 925-937.

Kelley, W. L. (1998). The J-domain family and the recruitment of chaperone power. *Trends Biochem. Sci.* **23**, 222-227.

Kiraly-Borri, C., Morgan, A., Burgoyne, R., Weller, U., Wollheim, C.B. and Lang, J. (1996). Soluble N-ethylmaleimide sensitive factor attachment protein and N-ethylmaleimide insensitive factors are required for Ca^{2+} stimulated exocytosis of insulin. *Biochem. J.* **314**, 199-203.

Kunkel, T. A. (1985). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Nat. Acad. Sci. USA* **82**, 477-492.

Lang, J., Nishimoto, I., Okamoto, T., Regazzi, R., Kiraly, C., Weller, U. and Wollheim, C. B. (1995). Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases G_i and G_o or by the expression of their active Gα subunits. *EMBO J.* **14**, 3635-3644.

Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K. and Wollheim, C. B. (1997a). The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic β-cells: Action of synaptotagmin at low micromolar calcium. *EMBO J.* **16**, 5837-5846.

Lang, J., Regazzi, R. and Wollheim, C. B. (1997b). Clostridial toxins and endocrine secretion: Their use in insulin-secreting cells. In *Bacterial Toxins: Tools in Cell Biology* (ed. K. Aktories), pp. 217-240. Weinheim: Chapman & Hall.

Lang, J., Zhang, H., Vaidyanathan, V. V., Sadoul, K., Niemann, H. and Wollheim., C. B. (1997c). Transient expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose induced insulin secretion in clonal β-cells. *FEBS Lett.* **419**, 13-17.

Lang, J. (1999). Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. *Eur. J. Biochem.* **259**, 1-16.

Leveque, C., Pupier, S., Marqueze, B., Geslin, L., Kataoka, M., Takahashi, M., DeWaard, M. and Seagar, M. (1998). Interaction of cysteine string proteins with the α_1A subunit of the P/Q-type calcium channel. *J. Biol. Chem.* **273**, 13488-13492.

Li, G., Regazzi, R., Balch, W. E. and Wollheim, C. B. (1993). Stimulation of insulin release from permeabilized HIT-T15 cells by a synthetic peptide corresponding to the effector domain of the small GTP-binding protein rab3. *FEBS Lett.* **327**, 145-149.

Ligon, B., Boyd, A. E. and Dunlap, K. (1998). Class A calcium channel variants in pancreatic islets and their role in insulin secretion. *J. Biol. Chem.* **273**, 13905-13911.

Lin, R. C. and Scheller, R. H. (1997). Structural organization of the synaptic exocytosis core complex. *Neuron* **19**, 1087-1094.

Lupas, A. (1996). Prediction and analysis of coiled-coiled structures. *Meth. Enzymol.* **266**, 513-525.

Martin, T. F. J. (1997). Stages of regulated exocytosis. *Trends Cell Biol.* **7**, 271-276.

Mastrogiacomo, A., Parsons, S. M., Zampighi, G. A., Jenden, D. J., Umbach, J. A. and Gundersen, C. B. (1994). Cysteine string proteins: a potential link between synaptic vesicles and presynaptic Ca^{2+} channels. *Science* **263**, 981-982.

Mastrogiacomo, A. and Gundersen, C. B. (1995). The nucleotide and deduced amino acid sequence of a rat cysteine string protein. *Brain Res. Mol. Brain Res.* **28**, 1218.

Mastrogiacomo, A., Kohan, S. A., Whitelegge, J. P. and Gundersen, C. B. (1998a). Intrinsic membrane association of *Drosophila* cysteine string proteins. *FEBS Lett.* **436**, 85-91.

Mastrogiacomo, A., Kornblum, H. I., Umbach, J. A. and Gundersen, C. B. (1998b). A Xenopus cysteine string protein with a cysteine residue in the J domain. *Biochim. Biophys. Acta* **1401**, 239-241.

Morgan, A. and Burgoyne, R. D. (1995). Is NSF a fusion protein? *Trends Cell Biol.* **5**, 335-339.

Ranjan, R., Bronk, P. and Zinsmaier, K. E. (1998). Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in *Drosophila* but not for vesicle recycling. *J. Neurosci.* **18**, 956-964.

Regazzi, R., Ravazzola, R., Iezzi, M., Lang, J., Zahraoui, A. andereggen, E., Morel, P., Takai, Y. and Wollheim, C. B. (1996). Expression, localization and functional role of small GTPases of the Rab3 family in insulin-secreting cells. *J. Cell Sci.* **109**, 2265-2273.

Schlossman, D. M., Schmid, S. L., Braell, W. A. and Rothman, J. R. (1984). An enzyme that remove clathrin coats: purification of an uncoating ATPase. *J. Cell Biol.* **99**, 723-733.

Schulze, K. L., Littleton, J. T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z. and Bellen, H. J. (1994). rop, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron* **13**, 1099-1108.

Stanley, E. F. (1997). The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci.* **20**, 404-409.

Umbach, J. A., Zinsmaier, K. E., Eberle, K. K., Buchner, E., Benzer, S. and Gundersen, C. B. (1994). Presynaptic dysfunction in *Drosophila* csp mutants. *Neuron* **13**, 899-907.

Umbach, J. A., Saitoe, M., Kidokoro, Y. and Gundersen, C. B. (1998). Attenuated influx of calcium ions at nerve endings of csp and shibire mutant *Drosophila*. *J. Neurosci.* **18**, 3233-3240.

van de Goor, J. and Kelly, R. B. (1996). Association of *Drosophila* cysteine string proteins with membranes. *FEBS Lett.* **380**, 251-256.

Wall, D., Zylicz, M. and Georgopoulos, C. (1995). The conserved G/F motif of the DnaJ chaperone is necessary for the activation of the substrate binding properties of the DnaK chaperone. *J. Biol. Chem.* **270**, 2139-2144.

Wang, C. and Lee, M. R. (1993). High-level expression of soluble rat hsc70 in Escherichia coli: purification and characterization of the cloned enzyme. *Biochem. J.* **294**, 69-77.

Weller, U., Muller, L., Messner, M., Palmer, M., Valeva, A., Tranum Jensen, J., Agrawal, P., Biermann, C., Dobereiner, A., Kehoe, M. A., et al. (1996). Expression of active streptolysin O in Escherichia coli as a maltose-binding-protein-streptolysin-O fusion protein. The N-terminal 70 amino acids are not required for hemolytic activity. *Eur. J. Biochem.* **236**, 34-39.

Wilson, R., Ainscough, R. anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., et al. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans. *Nature* **368**, 32-38.

Wollheim, C. B., Lang, J. and Regazzi, R. (1996). Regulation of exocytosis by calcium and G-proteins. *Diabetes Rev.* **4**, 277-297.

Zhang, H., Kelley, W., Chamberlain, L., Bourgoyne, R., Wollheim, C. B. and Lang, J. (1997). Role of the cysteine string protein in insulin secretion from pancreatic β-cells. *Diabetologia* **40** Suppl. 1, A113:439.

Zhang, H., Kelley, W. L., Chamberlain, L. H., Burgoyne, R. D., Wollheim, C. B. and Lang, J. (1998). Cysteine-string proteins regulate exocytosis of insulin independent from transmembrane ion fluxes. FEBS Lett. **437**, 267-272.

Zinsmaier, K. E., Hofbauer, A., Heimbeck, G., Pflugfelder, G. O., Buchner, S. and Buchner, E. (1990). A cysteine-string protein is expressed in retina and brain of *Drosophila*. *J. Neurogenet.* **7**, 15-29.

Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science* **263**, 977-980.