The synaptic vesicle protein, cysteine-string protein, is associated with the plasma membrane in 3T3-L1 adipocytes and interacts with syntaxin 4

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SUMMARY

Adipocytes and muscle cells play a major role in blood glucose homeostasis. This is dependent upon the expression of Glut4, an insulin-responsive facilitative glucose transporter. Glut4 is localised to specialised intracellular vesicles that fuse with the plasma membrane in response to insulin stimulation. The insulin-induced translocation of Glut4 to the cell surface is essential for the maintenance of optimal blood glucose levels, and defects in this system are associated with insulin resistance and type II diabetes. Therefore, a major focus of recent research has been to identify and characterise proteins that regulate Glut4 translocation. Cysteine-string protein (Csp) is a secretory vesicle protein that functions in presynaptic neurotransmission and also in regulated exocytosis from non-neuronal cells. We show that Csp1 is expressed in 3T3-L1 adipocytes and that cellular levels of this protein are increased following cell differentiation. Combined fractionation and immunofluorescence analyses reveal that Csp1 is not a component of intracellular Glut4-storage vesicles (GSVs), but is associated with the adipocyte plasma membrane. This association is stable, and not affected by either insulin stimulation or chemical depalmitoylation of Csp1. We also demonstrate that Csp1 interacts with the t-SNARE syntaxin 4. As syntaxin 4 is an important mediator of insulin-stimulated GSV fusion with the plasma membrane, this suggests that Csp1 may play a regulatory role in this process. Syntaxin 4 interacts specifically with Csp1, but not with Csp2. In contrast, syntaxin 1A binds to both Csp isoforms, and actually exhibits a higher affinity for the Csp2 protein.

The results described raise a number of interesting questions concerning the intracellular targeting of Csp in different cell types, and suggest that the composition and synthesis of GSVs may be different from synaptic and other secretory vesicles. In addition, the interaction of Csp1 with syntaxin 4 suggests that this Csp isoform may play a role in insulin-stimulated fusion of GSVs with the plasma membrane.

Key words: Cysteine-string protein, Exocytosis, Membrane fusion, Adipocyte, Glut4

INTRODUCTION

Intracellular targeting of proteins and the extracellular secretion of various compounds is mediated by transport vesicles that bud from donor membranes and fuse with the appropriate acceptor membranes. Membrane fusion can occur constitutively or can be tightly regulated, such as the fusion of synaptic vesicles with the presynaptic plasma membrane. A large number of proteins have been identified that are important mediators of membrane fusion reactions (Sudhof, 1995; Benfenati et al., 1999), including the universal 'SNARE' proteins (Sollner et al., 1993). The majority of characterised secretory proteins have homologues that function in diverse membrane fusion events. However, the tissue-specific expression of certain proteins makes it likely that their function is relevant only to specific types of membrane fusion events.

Cysteine-string protein (Csp) has been implicated in a number of regulated exocytotic pathways (for a recent review see Chamberlain and Burgoyne, 2000). This protein is essential for presynaptic neurotransmission, and also functions in regulated exocytosis in PC12 and pancreatic beta cells (Zinsmaier et al., 1994; Umbach et al., 1994; Chamberlain and Burgoyne, 1998a; Brown et al., 1998; Zhang et al., 1998; Zhang et al., 1999). Distribution studies have shown that Csp is associated with a variety of secretory vesicles, including synaptic vesicles, chromaffin granules, insulin-containing granules, pancreatic zymogen granules and secretory granules of the neurohypophysis (Mastrogiacomo et al., 1994b; Van de Goor et al., 1995; Chamberlain et al., 1996; Brown et al., 1998; Zhang et al., 1998; Braun and Scheller, 1995; Pupier et al., 1997). The targeting of Csp to cell membranes is dependent upon palmitoylation, which occurs on a central 'string' of cysteine residues (Chamberlain and Burgoyne, 1998b). In contrast to some other secretory vesicle proteins, there is no *S. cerevisiae* Csp homologue, suggesting that Csp may function specifically in regulated membrane fusion.

Coexpression studies in *Xenopus* oocytes suggested that Csp may regulate the activity of voltage-dependent calcium

channels (Gundersen and Umbach, 1992). Such a function would make Csp an important regulator of calcium-dependent exocytosis. However, it has not been possible to show a direct interaction between Csp and calcium channels in vivo (Pupier et al., 1997), although the proteins interact in vitro (Leveque et al., 1998). Recent genetic and biochemical analyses has revealed a functional interaction between *Drosophila* Csp and the t-SNARE syntaxin 1A (Wu et al., 1999; Nie et al., 1999). Interestingly, syntaxin interacts with voltage-gated calcium channels both in vivo and in vitro (Leveque et al., 1994; Sheng et al., 1994; Martin-Moutot et al., 1996), and may negatively regulate channel activity (Bezprozvanny et al., 1995; Wiser et al., 1996). Because of this, it has been proposed that the interaction of Csp with syntaxin could act to relieve the syntaxin-mediated inhibition of presynaptic calcium channel activity (Wu et al., 1999). Indeed, depolarisation-induced calcium influx into nerve terminals was reported to be suppressed in *Drosophila* Csp null mutants (Umbach et al., 1998).

Nevertheless, calcium channel regulation cannot be the only role of Csp in regulated exocytosis, as Csp has been shown to function in calcium-stimulated exocytosis from permeabilised cells, which is independent of calcium channel activity (Chamberlain and Burgoyne, 1998a; Zhang et al., 1998; Zhang et al., 1999). Overexpression of Csp modified the kinetics of granule release events, consistent with a late function in exocytosis (Graham and Burgoyne, 2000). In addition, Csp overexpression does not affect calcium channel activity in PC12 and pancreatic beta cells (Chamberlain and Burgoyne, 1998a; Brown et al., 1998), and calcium currents are normal in peptidergic nerve terminals of *Drosophila* Csp null mutants (Morales et al., 1999). Furthermore, recent work has shown that stimulus-evoked presynaptic calcium ion influx is not decreased in *Drospophila* Csp null mutants (Dawson-Scully et al., 2000). Thus, these combined findings suggest that Csp does not regulate calcium channel activity.

Other studies have suggested that Csp may function as a chaperone protein in regulated exocytosis. Csp is a member of the eukaryotic DnaJ protein family, which share a common domain with the bacterial chaperone protein, DnaJ (Kelley, 1998). Csp functions as a typical DnaJ protein by interacting with and stimulating the ATPase activity of the molecular chaperone heat-shock cognate protein (70 kDa) Hsc70 (Braun et al., 1996; Chamberlain and Burgoyne, 1997a; Chamberlain and Burgoyne, 1997b; Zhang et al., 1999). In addition, Csp binds to model unfolded protein substrates, and acts as a molecular chaperone by preventing their aggregation (Chamberlain and Burgoyne, 1997b). DnaJ proteins that are involved in protein folding have also been shown to interact with unfolded proteins in vitro (Langer et al., 1992; Minami et al., 1996). This suggests that Csp may function in regulated exocytosis by controlling the conformational status of one or more secretory vesicle proteins, which could explain the temperature-sensitive phenotype of Csp null mutant *Drosophila* (Umbach et al., 1994).

The fusion of Glut4-containing vesicles with the plasma membrane is an example of regulated membrane fusion. Glut4 is an insulin-responsive, facilitative glucose transporter, whose expression is restricted to adipocytes, cardiomyocytes and muscle cells. Under basal conditions, around 95% of Glut4 is located intracellularly, sequestered within the endosomal system and in specialised Glut4 storage vesicles (GSVs) (Hashiramoto and James, 2000). Insulin increases glucose uptake into fat and muscle by promoting the fusion of Glut4-containing vesicles with the plasma membrane. The major effect of insulin is to stimulate the translocation and fusion of GSVs with the plasma membrane, but endosomal Glut4 also translocates to the cell surface (Hashiramoto and James, 2000). The fusion of GSVs with the plasma membrane is a well-characterised process, and involves protein components implicated in other regulated and constitutive membrane fusion pathways, such as SNARE proteins and a Sec1p homologue (Volchuk et al., 1995; Volchuk et al., 1996; Cheatham et al., 1996; Tellam et al., 1997; Macaulay et al., 1997; Olson et al., 1997; Rea et al., 1998; Martin et al., 1998; Thurmond et al., 1998). There may also be proteins that function specifically in GSV fusion, such as the recently identified protein, Synip (Min et al., 1999).

In the present study, we demonstrate that the secretory vesicle protein Csp1 is expressed in 3T3-L1 adipocytes. Surprisingly, Csp1 is not a component of GSVs, but is associated with the plasma membrane. Our finding that Csp1 interacts with the t-SNARE syntaxin 4 suggests that Csp1 may regulate the insulin-stimulated fusion of GSVs with the plasma membrane.

MATERIALS AND METHODS

Materials

Csp antiserum was as previously described (Chamberlain and Burgoyne, 1996). Dr David James (University of Queensland, Australia) provided antiserum specific for syntaxin 4. Dr Richard Scheller (Howard Hughes Medical Institute, California, USA) donated GST-syntaxin 1A and 4 plasmids. IRAP monoclonal antibody was provided by Drs Luis Garza and Morris Birnbaum (University of Pennsylvania, PA, USA). Syntaxin 4 antibody (used for immunofluorescence analysis) was purchased from Chemicon (Harrow, UK). IPTG and protease inhibitor tablets were obtained from Boehringer Mannheim (East Sussex, UK). Iodixanol (Optiprep) was purchased from Life Technologies (Paisley, UK). Glutathione sepharose was purchased from Amersham Pharmacia Biotechnologies (Buckinghamshire, UK). Ni^{2+}-NTA agarose was obtained from Qiagen (Germany). Hydroxylamine and all other reagents were of an analytical grade from Sigma (Poole, UK).

Cell culture

3T3-L1 fibroblasts were grown in 10% newborn-calf serum in DMEM at 37°C in 10% CO_2 and passaged at about 70% confluence. Cells for use in experiments were grown in the same medium until 4 days post-confluence, then differentiation into adipocytes was achieved as described elsewhere (Frost and Lane, 1988). Cells were used for experiments between 9 and 12 days post-differentiation and between passages 5 and 12. All cells used in experiments were pre-incubated for at least 2 hours in serum-free medium.

Preparation of cell/tissue extracts for immunoblotting analysis

3T3-L1 fibroblasts and adipocytes were homogenised in HES buffer (20 mM Hepes, 1 mM EDTA, 225 mM sucrose, pH 7.4) with 20 strokes of a Dounce homogenizer, and the post-nuclear supernatant (PNS) recovered by centrifugation at 2,000 ***g*** for 5 minutes at 4°C. Rat brain was added to 5 mM Tris-HCl, pH 8.0, and homogenised using a rotor-stator homogenizer. The protein concentration of the samples were calculated, and known amounts separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis.

3T3-L1 cell fractionation

3T3-L1 adipocytes (either untreated or stimulated with 1 μM insulin for 15 minutes) were washed twice in ice-cold HES buffer, and scraped into HES supplemented with a protease-inhibitor cocktail. Cell fractionation was performed as described (Piper et al., 1991). The cells were homogenised with 20 strokes of a Dounce homogenizer, and centrifuged at 16,000 ***g*** for 20 minutes at 4°C. The pellet from this spin was resuspended in 1 ml of HES buffer, layered onto 1 ml of a 1.12 M sucrose cushion, and centrifuged at 40,000 ***g*** for 1 hour at 4°C in a swing-out rotor. The plasma membranes were collected from the top of the sucrose cushion, diluted in 20 mM Hepes, 1 mM EDTA, pH 7.4, and collected by centrifugation at 196,000 ***g*** for 1 hour at 4°C. The supernatant from the initial spin was centrifuged at 46,000 ***g*** for 20 minutes at 4°C. The pellet from this spin represents the high density microsome (HDM) fraction. The supernatant was centrifuged at 196,000 ***g*** for 1 hour at 4°C, which separates the low density microsomes (LDM) (pellet) and cytosol (supernatant) fractions. Equal amounts of the protein fractions were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis.

Chemical treatment of 3T3-L1 membranes

3T3-L1 adipocytes were washed twice in ice-cold HES, scraped into HES supplemented with protease inhibitor cocktail, and homogenised with 20 strokes of a Dounce homogenizer. The PNS was recovered by centrifugation at 2000 ***g*** for 5 minutes at 4°C, and centrifuged at 196,000 ***g*** for 1 hour at 4°C. The recovered membranes were either incubated in 1 M Tris, pH 7.0, or 1 M hydroxylamine, pH 7.0, for 20 hours at room temperature. The membranes were then centrifuged at 196,000 ***g*** for 1 hour at 4°C, and the recovered supernatant and pellet fractions resuspended in SDS-dissociation buffer. The samples were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis.

Plasma membrane lawn assay

After experimental manipulations, adipocytes on coverslips were rapidly washed in ice-cold buffer for the preparation of plasma membrane lawns exactly as described (Martin et al., 1998). Triplicate coverslips were prepared for each experimental condition, and four random images of plasma membrane lawns were collected from each. These were quantified using Metamorph (Universal Imaging, West Chester, PA, USA) software on a DAN personal computer (Noran Instruments, Surrey, UK).

Whole cell immunofluorescence

Adipocytes grown on collagen-coated coverslips were washed three times in PBS, and fixed in 4% formaldehyde for 30 minutes at room temperature. The fixed cells were washed twice in PBS and incubated for 10 minutes in PBTA (0.1% Triton X-100, 0.3% BSA in PBS). The permeabilised cells were then incubated for 10 minutes in PBS with 1% FBS. Following this, the cells were incubated with Csp IgG (5 μg/ml), syntaxin 4 IgG (Chemicon; 5 μg/ml), control IgG (5 μg/ml), or anti-Glut 4 (1:100) for 1 hour, and washed three times in PBS. The cells were then incubated with anti-rabbit FITC (1:100) in PBS for 1 hour, and washed three times in PBS. The coverslips were allowed to air dry and mounted in citifluor.

cDNA cloning

cDNA encoding the cytoplasmic tail of syntaxin 4 (amino acids 1-274) was amplified by PCR from purified 3T3-L1 RNA, and cloned into the pQE-30 plasmid (Qiagen). The cloned DNA was fully sequenced, confirming that there were no errors introduced by PCR. The pQE-30[Csp1] and pQE-30[Csp2] constructs were those previously described (Chamberlain and Burgoyne, 1996). The GST-Syntaxin 1A and 4 plasmids were provided by Dr Richard Scheller (Howard Hughes Medical Institute, California, USA).

Protein purification

E. coli M15[pREP4] cells (Qiagen) were transformed with the above constructs, and protein expression was induced by growing the cells in 0.5 mM IPTG for 5 hours at 37°C. Cells were washed and resuspended in breaking buffer (100 mM Hepes, 5 mM ATP, 5 mM $MgCl_2$, 500 mM KCl, 2 mM 2-mercaptoethanol, pH 7.0), containing a protease inhibitor cocktail and 1 mM phenylmethylsulphonyl fluoride. The cells were lysed by a combination of freeze-thawing, lysozyme treatment and ultrasonication (see Chamberlain and Burgoyne, 1997a). Cell debris was pelleted by centrifugation at 100,000 ***g*** for 1 hour at 4°C, and the supernatants containing recombinant proteins recovered.

His_6-tagged proteins were purified by Ni^{2+} affinity chromatography. Briefly, bacterial cell lysates were loaded onto a 3 ml Ni^{2+}-NTA agarose column, and washed in a buffer containing 50 mM imidazole (50 mM imidazole, 20 mM Hepes, 200 mM KCl, 2 mM 2-mercaptoethanol, 0.5 mM ATP, 2 mM $MgCl_2$, 10% glycerol, pH 7.0). Proteins were eluted from the column by applying a step gradient of increasing concentrations of imidazole. Peak fractions containing recombinant proteins were identified by SDS-PAGE.

GST-syntaxin 4-containing lysate was loaded onto a 3 ml glutathione-sepharose column, and washed with PBS to remove non-bound proteins. GST-Syntaxin 4 was eluted from the column with 10 mM reduced glutathione in 50 mM Tris, pH 8.0.

Binding studies with His_6-Syntaxin 4 and a Triton-extract from adipocyte membranes

3T3-L1 adipocytes were washed twice in ice-cold HES buffer, and scraped into HES supplemented with a protease-inhibitor cocktail. The cells were homogenised with 20 strokes of a Dounce homogenizer, and the membranes recovered by centrifugation at 196,000 ***g*** for 1 hour at 4°C. The membranes were incubated in 1 M NaCl (in HES) for 2 hours at 4°C with end-over-end rotation, and then centrifuged at 196,000 ***g*** for 1 hour at 4°C. The recovered membranes were incubated in 1% Triton-X-100 (in HES) for 2 hours at 4°C with end-over-end rotation. The Triton-extracted membranes were removed by centrifugation at 196,000 ***g*** for 1 hour at 4°C, and the recovered supernatant dialysed against buffer A (20 mM Hepes, 100 mM NaCl, 1 mM DTT, 1% Triton X-100, pH 7.4), divided into portions and stored at –20°C.

His_6-tagged syntaxin 4 was incubated with Ni^{2+}-NTA agarose (1 μg/μl agarose) for 3 hours at 4°C with end-over-end rotation. The coupled beads were washed three times in buffer A, and 10 μl of the beads were incubated with 100 μl of the Triton-extract at 4°C overnight with end-over-end rotation. The beads were then washed three times with buffer A + 50 mM imidazole, and bound proteins eluted by boiling the beads in SDS-dissociation buffer. The eluted proteins were separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting analysis.

Binding studies with GST-Syntaxin and His_6-Csp

GST-Sepharose beads were washed three times in buffer B (150 mM potassium acetate, 1 mM $MgCl_2$, 0.05% Tween-20, 20 mM Hepes, pH 7.4) and incubated with a protein extract from *E. coli* (approx. 0.2 μg per μl of bead slurry) for 1 hour at 4°C with end-over-end rotation. After washing the beads three times with buffer B, either 2 μM GST, GST-syntaxin 1A or GST-syntaxin 4 were added and incubated for 30 minutes at 4°C with end-over-end rotation. An equivalent volume of recombinant His_6-tagged Csp protein was added to make a final concentration of 1 μM for Csp in a final reaction volume of 100 μl, and incubated for 2 hours at 4°C with end-over-end rotation. Beads were washed twice with buffer B containing 1 mg/ml gelatin and three times with buffer B containing 5% glycerol. Bound proteins were eluted by boiling the beads in SDS-dissociation buffer. Eluted proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis.

The chemiluminescence antibody signal was quantified using the image analysis software program Image Quant.

Iodixanol and sucrose gradient fractionations

Purified LDMs (in HES) were mixed with Iodixanol (OptiPrep) to a final concentration of 14%, and centrifuged for 1 hour at 295,000 ***g*** (4°C) in a Beckman TLN100 rotor. 300 μl fractions were collected from the bottom of the gradient, and equal volumes of the recovered fractions were separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting analysis.

For sucrose gradient fractionation, purified LDMs were made up to 60% sucrose (in 20 mM Hepes, 1 mM EDTA, pH 7.4), and placed at the bottom of a centrifuge tube. This was overlayed successively with 50%, 30%, 10% and 5% sucrose, and centrifuged in a Beckman SW40 rotor at 175,000 ***g*** for 18 hours (4°C). 1 ml fractions were collected from the top of the gradient, and equal volumes were separated by SDS-PAGE, and transferred to nitrocellulose for immunoblotting analysis.

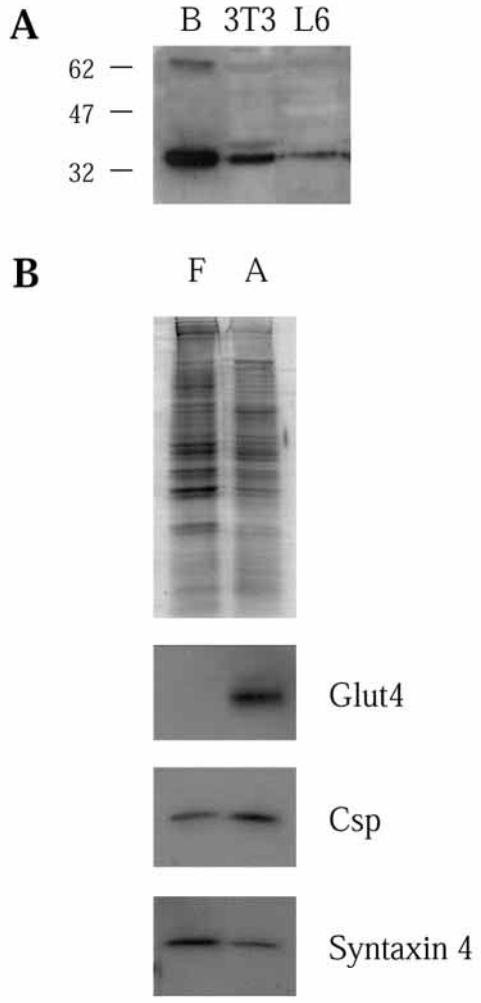

Fig. 1. Expression of Csp1 in 3T3-L1 adipocytes and L6 skeletal muscle cells. (A) 5 μg of a rat brain sample (B) and 20 μg of post-nuclear supernatant (PNS) prepared from 3T3-L1 (3T3) or L6 cells were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis with Csp antiserum. (B) 20 μg of PNS prepared from 3T3-L1 fibroblasts (F) and 3T3-L1 adipocytes (A) was separated by SDS-PAGE and stained with Coomassie Blue (top). Samples were also transferred to nitrocellulose and probed with antibodies specific for Glut4, Csp and syntaxin 4.

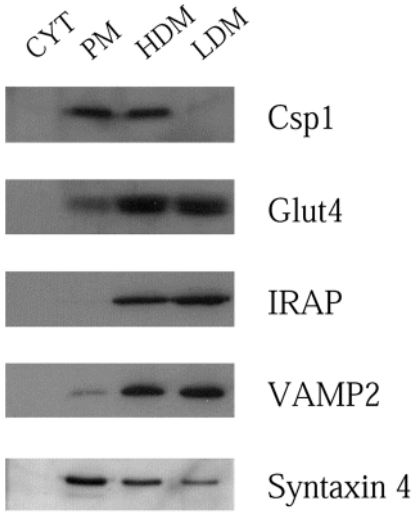

Fig. 2. Distribution of Csp1 in subcellular fractions isolated from 3T3-L1 adipocytes. Fractions containing cytosol (CYT), plasma membranes (PM), high density microsomes (HDM) and low density microsomes (LDM) were prepared by differential centrifugation of homogenized 3T3-L1 adipocytes as described in Materials and Methods. 10 μg of each fraction were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis using antibodies specific for Csp, Glut4, IRAP, VAMP2 and syntaxin 4.

RESULTS

Csp expression in 3T3-L1 adipocytes

The insulin-stimulated fusion of Glut4-containing vesicles with the plasma membrane is a well-defined system of regulated membrane fusion, and requires the participation of SNARE proteins. To further characterise the molecular machinery involved in Glut4 trafficking, we examined the expression of Csp in 3T3-L1 adipocytes. Two mammalian Csp isoforms have been identified (Chamberlain and Burgoyne, 1996; Coppola and Gundersen, 1996). Csp1 has a widespread distribution, whereas the truncated Csp2 isoform may have a more restricted distribution.

Fig. 1A shows a comparison of Csp expression in brain, 3T3-L1 adipocytes and L6 skeletal muscle cells. An immunoreactive band corresponding to Csp1 (approx. 35 kDa) is clearly detectable in both 3T3-L1 and L6 cells, whereas we did not detect expression of Csp2 in these cells. A higher molecular weight immunoreactive band (approx. 65 kDa) is also detected in the samples, which results from dimerisation of Csp1 (Mastrogiacomo et al., 1994a; Mastrogiacomo and Gundersen, 1995; Chamberlain and Burgoyne, 1996). In subsequent figures we have chosen to show only Csp1 monomers, but in all experiments we observed no difference in the behaviour of monomeric and dimeric Csp1.

To examine whether the level of Csp1 expression is altered upon differentiation of 3T3-L1 cells, we prepared protein extracts from fibroblasts and adipocytes. The top panel of Fig. 1B shows a Coomassie Blue stained gel of the extracts, demonstrating that the protein concentration of the samples is similar. As a control we examined Glut4 protein levels in the fractions, which showed induction of Glut4 expression following differentiation (Fig. 1B). In contrast to Glut4, Csp1

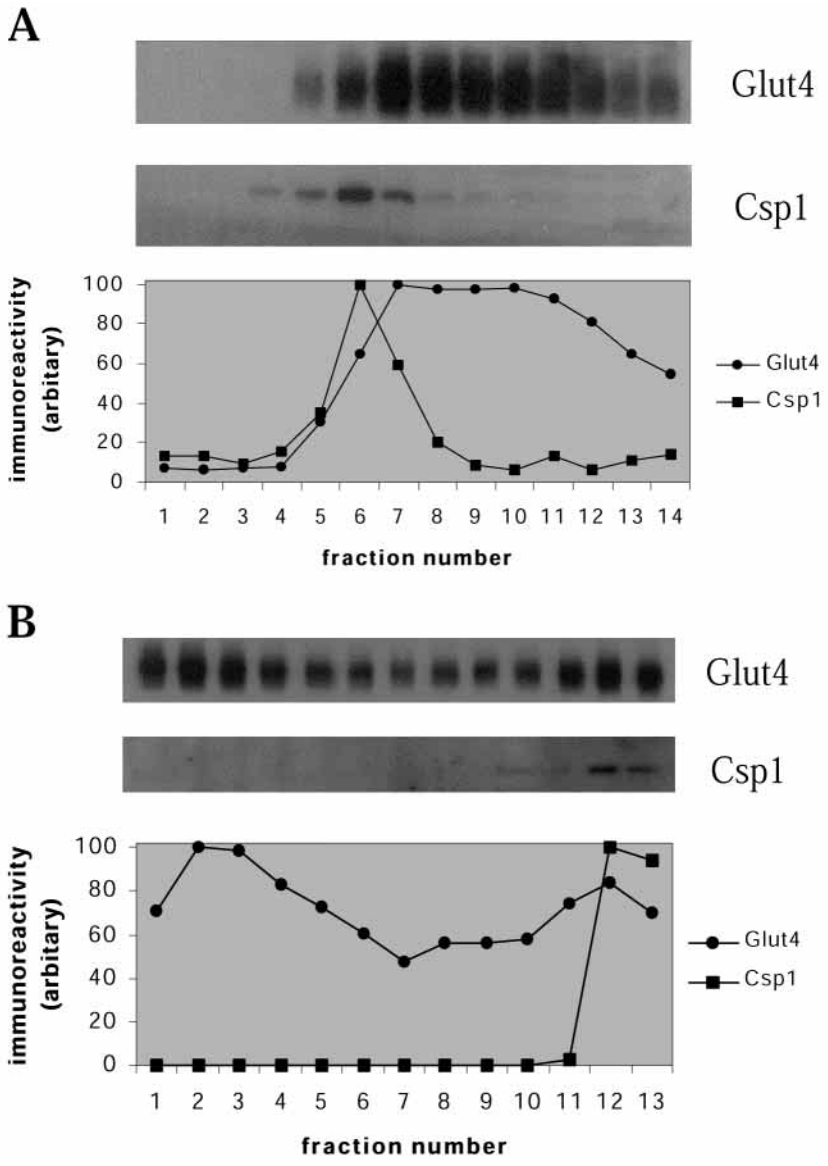

Fig. 3. Analysis of LDM-associated Csp1 and Glut4 by gradient fractionation. (A) LDMs were made up to 60% sucrose, overlayed successively with 50%, 30%, 10% and 5% sucrose layers, and centrifuged at 175,000 ***g*** for 18 hours at 4°C. 1 ml fractions were collected from the top of the gradient (1-13) and the pellet (14) was also recovered. (B) LDMs were mixed with iodixanol to a final concentration of 14% and centrifuged at 295,000 ***g*** for 1 hour (4°C) in a TLN100 rotor. 300 μl fractions were collected from the bottom of the gradient (1-13). Equal volumes of the fractions were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis using antibodies specific for Csp and Glut4. The immunoreactivity of Csp and Glut4 in each fraction from both gradients was quantified and is shown in graph form.

was detected in both fibroblast and adipocyte samples, but there was an increase in Csp1 levels following differentiation (Fig. 1B). For comparison, the expression levels of the t-SNARE syntaxin 4 were examined (Fig. 1B), revealing that the amount of this protein as a percentage of total protein actually decreases upon differentiation. Quantification of the blots shown in Fig. 1B revealed that there was an approximately 1.8-fold increase in Csp1 levels following cell differentiation, whereas the levels of syntaxin 4 decreased by 1.8-fold (data not shown). It should be noted that Fig. 1B compares equal amounts of fibroblast and adipocyte protein. As cell differentiation was accompanied by an increase in total cellular protein levels (data not shown), the relative amount of Csp1 and syntaxin 4 in adipocytes on a per cell basis is probably greater than that shown in Fig. 1B.

Subcellular distribution of Csp1 in adipocytes

In all cell types studied thus far, Csp has been identified as a component of secretory vesicles or granules (Mastrogiacomo et al., 1994b; Braun and Scheller, 1995; Chamberlain et al., 1996; Pupier et al., 1997; Brown et al., 1998; Zhang et al., 1998). To examine the subcellular distribution of Csp1 in 3T3-L1 adipocytes, we isolated cytosol (CYT), plasma membrane (PM), high density microsome (HDM) and low density microsome (LDM) fractions by differential centrifugation. Fig. 2 shows that Glut4, and other GSV proteins such as insulin-responsive aminopeptidase (IRAP) and vesicle-associated membrane protein 2 (VAMP2), are mainly present in the HDM and LDM fractions. It is known that the LDM fraction contains the insulin-responsive GSVs and endosomal Glut4. Surprisingly, Csp1 does not colocalise with the Glut4 vesicle proteins in these fractions, but is most abundant in the PM and HDM fractions (Fig. 2). Indeed, the distribution of Csp1 within the purified fractions is more akin to that of syntaxin 4 (Fig. 2), a protein that is mainly found at the plasma membrane in adipocytes (Volchuk et al., 1996; Tellam et al., 1997). The syntaxin 4 detected in the HDM fraction probably represents plasma membrane contamination (see Gould et al., 1989). Note that syntaxin 4 appears to be more abundant in the LDM fraction than Csp1, consistent with a small pool of syntaxin 4 being found on GSVs (Volchuk et al., 1996).

We detected a small amount of Csp1 in isolated LDM fractions in some experiments, and also upon longer exposure of the blot in Fig. 2. Therefore, it is possible that a small pool of Csp1 is associated with GSVs. To examine this in more detail, we fractionated the purified LDMs by sucrose gradient centrifugation. Fig. 3A shows that the distribution of the small amount of Csp1 in LDMs in the gradient fractions is significantly different from Glut4, with a narrower peak and displacement towards lighter fractions of the gradient.

A method has recently been developed (Hashiramoto and James, 2000) for separating endosomal Glut4 and GSVs. To further characterise the Csp1 present in the LDM fraction, we fractionated purified LDMs on an iodixanol gradient, as described in Materials and Methods. Fig. 3B shows that Glut4 is present in all fractions recovered from this gradient, but that there are clearly two peaks of Glut4 centred around fractions 2 and 12. It has been suggested that the heavier pool of Glut4

(fraction 2) represents GSVs, whereas the lighter fractions (fraction 12) contain endosomal Glut4 (Hashiramoto and James, 2000). Fig. 3B shows that Csp is present only in the lighter fractions of the gradient (fractions 12 and 13), suggesting that the small amount of Csp present in LDMs isolated from 3T3-L1 adipocytes is not associated with GSVs but may be localised to recycling endosomes.

The fractionation data presented in Fig. 2 suggests that Csp1 may be associated with the plasma membrane in 3T3-L1 adipocytes. However, it also possible that Csp1 is associated with other cellular membranes that have a similar size to the plasma membrane fragments formed during homogenisation. To analyse the distribution of Csp1 in more detail, adipocytes were examined by immunofluorescence labelling with Csp IgG. Fig. 4A shows that specific Csp immunofluorescence was detected around the adipocyte plasma membrane. The distribution of Csp was similar to that of syntaxin 4 (Fig. 4D), but clearly different to Glut4 (Fig. 4E), which exhibited a distinct perinuclear localisation. Thus, the combined data of Figs 2 and 4 provide strong evidence that Csp1 is associated with the plasma membrane in 3T3-L1 adipocytes. This localisation of Csp1 is marked in its contrast to other studied cells types, in which Csp has been localised exclusively to secretory vesicles. To investigate the membrane orientation of Csp in the adipocyte plasma membrane, we examined Csp immunofluorescence in cells that had not been permeabilised prior to addition of antibody. As shown in Fig. 4C, no staining was observed in the absence of cell permeabilisation, implying that Csp is associated with the inner surface of the plasma membrane.

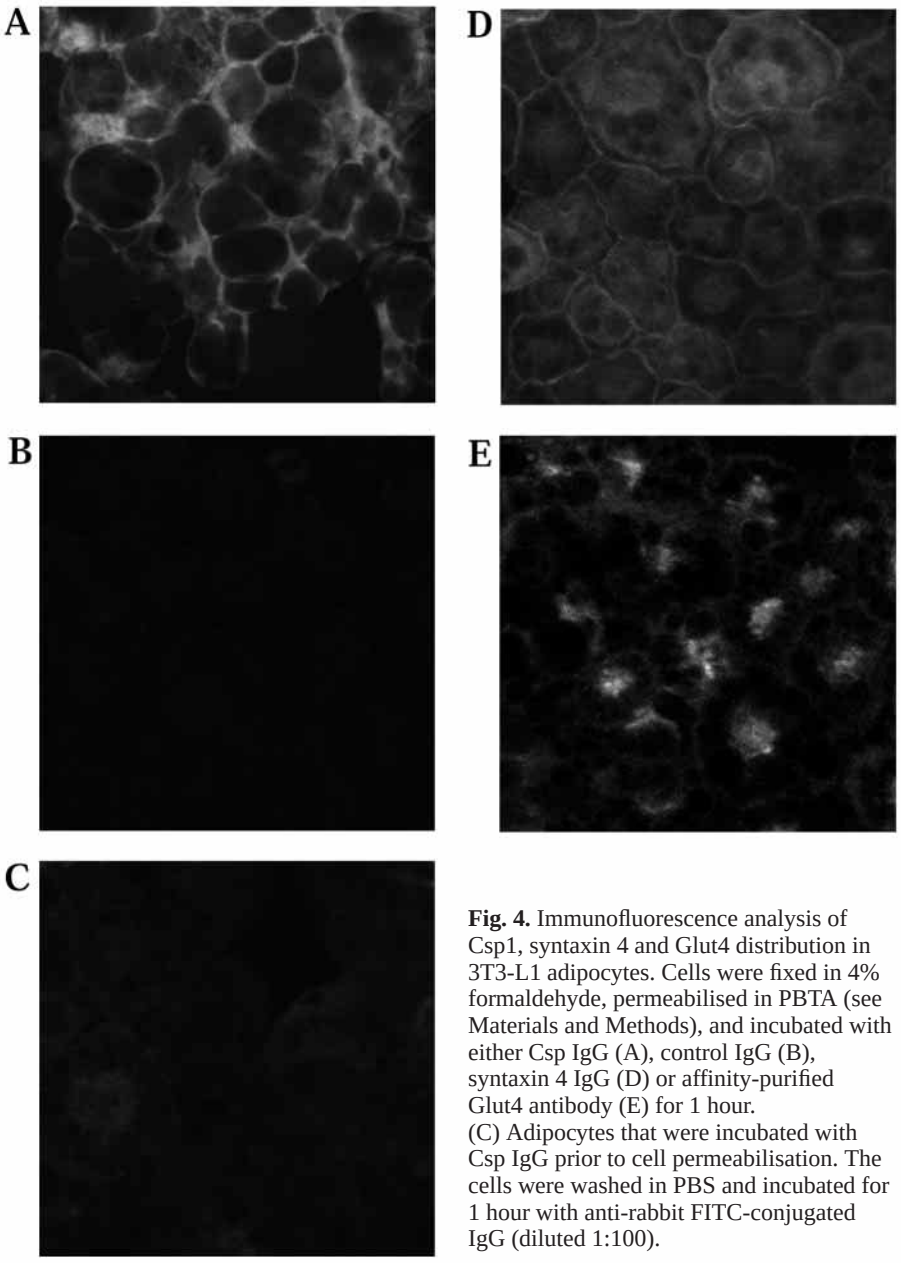

Fig. 4. Immunofluorescence analysis of Csp1, syntaxin 4 and Glut4 distribution in 3T3-L1 adipocytes. Cells were fixed in 4% formaldehyde, permeabilised in PBTA (see Materials and Methods), and incubated with either Csp IgG (A), control IgG (B), syntaxin 4 IgG (D) or affinity-purified Glut4 antibody (E) for 1 hour. (C) Adipocytes that were incubated with Csp IgG prior to cell permeabilisation. The cells were washed in PBS and incubated for 1 hour with anti-rabbit FITC-conjugated IgG (diluted 1:100).

Effect of insulin stimulation on Csp1 distribution

Insulin action promotes the translocation of intracellular Glut4 to the cell surface. A number of other proteins redistribute in response to insulin stimulation, including IRAP and VAMP2 (GSV proteins), insulin receptor substrate-1 (IRS-1) and PI3-kinase. To examine whether insulin stimulation causes a redistribution of Csp1, we prepared plasma membrane (PM) lawns from basal cells and from cells incubated with insulin. PM lawns are generated by ultrasonication of cells attached to glass coverslips. This procedure disrupts the cells, removing intracellular membranes but leaving plasma membranes firmly attached to the coverslips. Immunofluorescence labelling of PM lawns showed that although there was a large increase in Glut4 PM staining in response to insulin stimulation, there was no redistribution of Csp1 (Fig. 5A,B). Fig. 5A shows representative images, whereas Fig. 5B is averaged data from a number of separate images. In addition to showing that insulin does not alter the level of Csp1 associated with the plasma membrane, this experiment provides further, independent evidence that Csp1 is tightly associated with the plasma membrane in 3T3-L1 adipocytes.

We also examined the distribution of Csp1 in the presence or absence of insulin by a standard fractionation procedure. Fig. 5C shows that while insulin stimulation promoted a redistribution of Glut4 from the LDM to the PM fraction, there was no significant movement of Csp1. Thus, the combined results of the fractionation and PM lawn analyses demonstrate that insulin action does not cause redistribution of Csp1.

Effect of depalmitoylation on Csp membrane attachment

Csps are extensively palmitoylated (Gundersen et al., 1994), and it was originally suggested that this palmitoylation anchored Csp

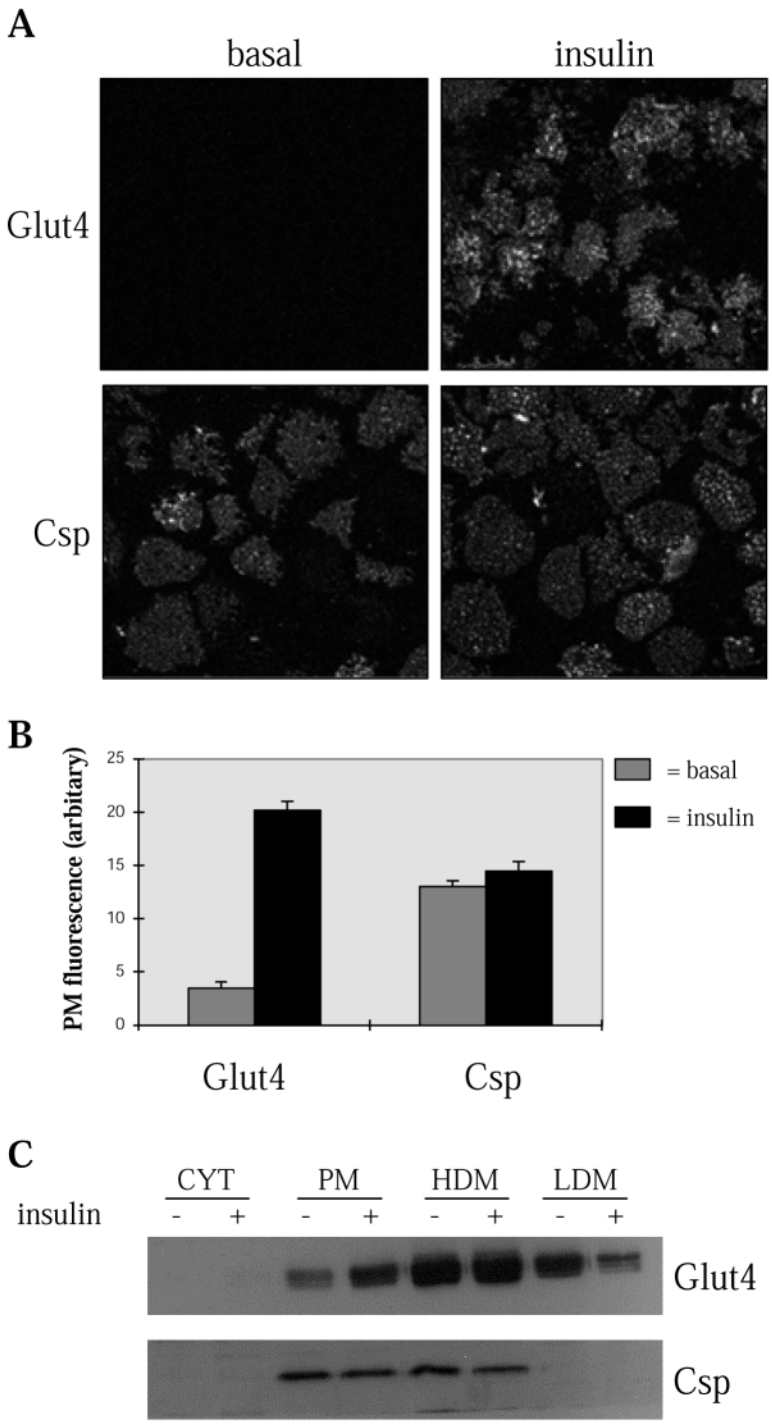

Fig. 5. Effect of insulin stimulation on Csp1 distribution in 3T3-L1 adipocytes. Plasma membrane (PM) lawns were prepared from basal or insulin-stimulated (1 μM for 15 minutes) cells as described in Materials and Methods, and incubated with affinity-purified Glut4 antibody (diluted 1:100) or Csp IgG (5 μg/ml) for 1 hour. Lawns were washed and incubated with anti-rabbit FITC-conjugated IgG (diluted 1:200) for 1 hour. (A) Representative images, (B) averaged data from a number of different images. (C) 3T3-L1 adipocytes were either untreated or stimulated with 1 μM insulin for 15 minutes. Cytosol (CYT), plasma membrane (PM), high density microsomal (HDM) and low density microsomal (LDM) fractions were prepared by differential centrifugation as detailed in Materials and Methods section. 5 μg of each fraction were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis using antibodies specific for Glut4 and Csp.

to vesicle membranes. However, subsequent work showed that palmitoylation is not required for membrane association of Csp (Van de Goor and Kelly, 1996; Chamberlain and Burgoyne, 1998b), although it is essential for the targeting of Csp to secretory vesicles (Chamberlain and Burgoyne, 1998b). One possibility is that the continued association of Csp with secretory vesicle membranes in the absence of palmitoylation may be mediated by the specific association of Csp with a secretory vesicle protein. If this were the case, then depalmitoylation should release Csp from the plasma membrane of 3T3-L1 adipocytes. To examine this, membranes were incubated in either 1 M hydroxylamine (pH 7), which cleaves thioester linkages, or 1 M Tris (pH 7) as a negative control. Fig. 6 shows that hydroxylamine treatment caused a molecular mass shift in Csp1, consistent with depalmitoylation. However, Fig. 6 also shows that this depalmitoylated Csp1 remained associated with membranes, and that no depalmitoylated Csp1 was released into the supernatant. Note that depalmitoylation of Csp1 is accompanied by an increase in dimer formation (upper arrowheads), probably as a consequence of disulphide bond formation between depalmitoylated cysteine residues. Thus, these results suggest that Csp1 may interact directly with membrane phospholipids rather than with specific membrane proteins, and agrees with the proposal that Csp is embedded in the lipid bilayer (Mastrogiacomo et al., 1998).

Interaction of Csp1 with syntaxin 4

Recent work has shown that *Drosophila* Csp interacts with the t-SNARE syntaxin 1A (Wu et al., 1999; Nie et al., 1999). Syntaxin 4 is expressed in adipocytes and functions in insulin-stimulated Glut4 translocation (Volchuk et al., 1996; Cheatham et al., 1996; Tellam et al., 1997). Therefore, we examined whether Csp1 from a 3T3-L1 membrane extract could interact with immobilised His_6-tagged syntaxin 4. We chose to examine the binding of cellular Csp1 to recombinant syntaxin 4, because cellular Csp is extensively palmitoylated, a post-translational modification that is not reproduced when recombinant proteins are expressed in bacteria. Immobilised syntaxin 4 was incubated with a Triton X-100 membrane extract from 3T3-L1 adipocytes, and bound proteins examined by SDS-PAGE and immunoblotting. Fig. 7 shows that Csp1 present in adipocyte membranes bound to immobilised syntaxin 4, whereas there was no significant binding of Csp1 to control beads (minus syntaxin 4).

To confirm that the observed interaction between Csp1 and syntaxin 4 is a result of direct binding, we analysed the association of recombinant Csp1 and recombinant syntaxin 4 in the absence of other proteins. In addition, we compared the binding of syntaxin 1A and syntaxin 4 to Csp1 and Csp2. Csp1 bound to both GST-syntaxin 1A and GST-syntaxin 4 (Fig. 8A).

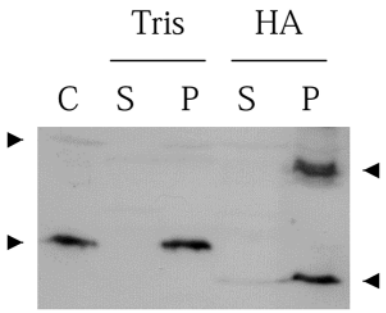

Fig. 6. Effect of hydroxylamine treatment on Csp membrane attachment. A PNS prepared from 3T3-L1 adipocytes was centrifuged at 196,000 ***g*** for 1 hour and the recovered membranes resuspended in either 1 M Tris, pH 7 (Tris) or 1 M hydroxylamine, pH 7 (HA), and incubated at room temperature for 20 hours. The samples were centrifuged at 196,000 ***g*** for 1 hour and supernatant (S) and pellet (P) fractions collected. Equal volumes of all fractions were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis with Csp-specific antiserum. The samples were compared to untreated control (C) membranes. Arrowheads indicate the positions of Csp1 in control and depalmitoylated samples. See text for details.

This result confirms that Csp1 and syntaxin 4 interact directly, and also suggests that the interaction between Csp1 and syntaxin 1A is important for regulated exocytosis in other mammalian cell types. Intriguingly, we found that syntaxin 1A bound Csp2 more tightly than Csp1, whereas there was no detectable interaction between syntaxin 4 and Csp2 (Fig. 8A). These results are the first to identify a functional difference between Csp1 and Csp2. Fig. 8B,C compares the relative affinities of Csp1 for syntaxin 1A and syntaxin 4, demonstrating that syntaxin 1A interacts with Csp1 more strongly than syntaxin 4 does. The different affinities of Csp1 for the two syntaxin isoforms is likely to be important for the fidelity of exocytosis in different cell types.

DISCUSSION

Adipocytes contain two distinct, insulin-responsive intracellular pools of Glut4. One pool is localised to the endosomal system, whereas the other is found in a specialised post-endosomal compartment, referred to as GSVs (Glut4-storage vesicles). GSVs have been suggested to be equivalent to synaptic vesicles, and the fusion of GSVs with the plasma membrane is largely responsible for the increased surface levels of Glut4 seen in response to insulin stimulation. Although GSVs and the endosomal Glut4 compartment contain similar proteins, these compartments have also been characterised based on differences in their protein content (Martin et al., 1996). For example, the endosomal compartment contains the v-SNARE cellubrevin, whereas GSVs contain VAMP2 (Martin et al., 1996). The interaction of VAMP2 with the t-SNAREs syntaxin 4 and syndet is believed to be essential for GSV fusion with the plasma membrane, and functional roles have been demonstrated for all three proteins in Glut4 translocation. In this study we have demonstrated the presence of Csp1 in 3T3-L1 adipocytes and its interaction with the SNARE protein syntaxin 4.

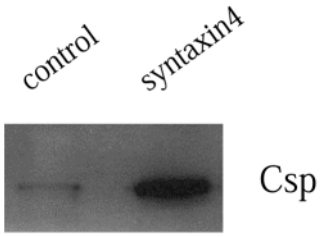

Fig. 7. Interaction of Csp1 with syntaxin 4. A Triton X-100 extract of 3T3-L1 membranes was incubated with either His_6-syntaxin 4 immobilised on Ni^{2+}-NTA agarose (syntaxin 4) or with uncoupled Ni^{2+}-NTA agarose (control) at 4°C overnight. The agarose was then washed in a buffer containing 50 mM imidazole, and bound proteins eluted by boiling the beads in SDS-dissociation buffer. Equal volumes of the eluates were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting analysis using Csp-specific antiserum.

The finding that Csp1 interacts with syntaxin 4 suggests that this protein may perform an important role in GSV trafficking. It has been suggested that Csp functions as a chaperone or 'foldase' in regulated exocytosis. Therefore, Csp1 may act as a chaperone for syntaxin 4, regulating the conformational status of this t-SNARE. This need not necessarily be an acute function during GSV fusion but this effect of Csp1 could allow efficient interaction of syntaxin 4 with other components of the secretory machinery, or may promote the displacement of an inhibitory molecule such as Synip or munc18c. Alternatively, Csp1 may function to help refold syntaxin 4 following GSV fusion. Although Csp has been suggested to modulate calcium channel activity, we believe that such a function is unlikely in adipocytes as it is not clear what role (if any) calcium plays in insulin-stimulated GSV fusion.

Csp has been found to be associated with secretory vesicles in all cell types studied to date. We have shown that Csp1 is not associated with GSVs in 3T3-L1 adipocytes but instead is mainly, if not entirely, plasma membrane associated. The demonstration that Csp1 is associated with the plasma membrane in 3T3-L1 adipocytes suggests that Csp does not function in exocytosis as a docking or tethering agent, because this would necessitate vesicle association in all cell types. Recent work has shown that Csp1 overexpression in chromaffin cells affects both the extent of fusion and individual fusion kinetics (Graham and Burgoyne, 2000). This was in contrast to an α-SNAP mutant protein, which inhibited only the extent of fusion, consistent with an early priming role for this protein in exocytosis (Chamberlain et al., 1995; Xu et al., 1999). The demonstration that Csp alters fusion kinetics in chromaffin cells suggests that this protein may regulate a late step in exocytosis, such as the remodelling of SNARE proteins. Thus, the interaction of Csp1 with syntaxin 4 may be important for the assembly or disassembly of protein complexes essential for membrane fusion.

Recent work has revealed a functional interaction between *Drosophila* Csp and syntaxin 1A (Wu et al., 1999; Nie et al., 1999), and this study is the first to demonstrate an interaction between mammalian Csps and syntaxins 1A and 4. A further interesting result of this study is the difference in relative affinities between syntaxin 1A/4 and Csp 1/2, demonstrating specific interactions between these proteins. Whereas syntaxin 4 did not bind to Csp2, syntaxin 1A actually had a higher affinity for Csp2 than Csp1. This finding is the first to identify a functional difference between the two Csp isoforms. The N-terminal 164 amino acids of Csp1 and Csp2 are identical, but the proteins vary in their C-terminal regions. Whereas Csp1 has

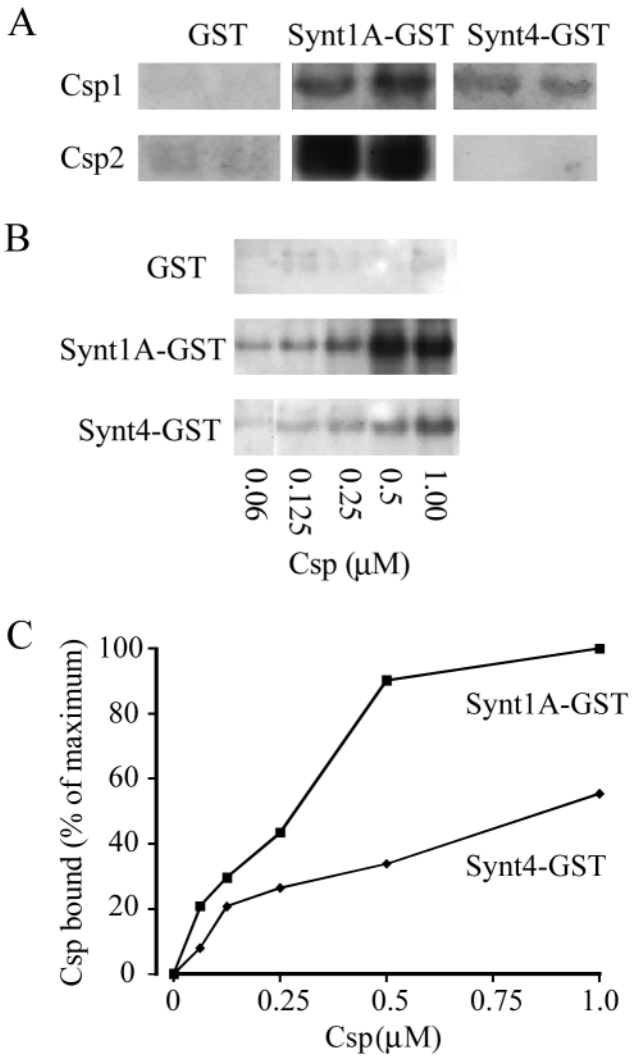

Fig. 8. Comparison of the interaction of syntaxin 1A and syntaxin 4 with Csp1 and Csp2. (A) 1 μM His_6-tagged Csp1 or Csp2 were incubated with an equivalent molarity of GST, GST-syntaxin 1A or GST-syntaxin 4 immobilised to GST-sepharose beads for 2 hours at 4°C. The beads were washed and bound protein eluted by boiling the beads in SDS-dissociation buffer. Eluted protein was electrophoresed and transferred to nitrocellulose for immunoblotting analysis using a His-tag antibody to detect Csp. The antibody gave equivalent signals with the input levels of Csp1 and Csp2. (B) Immobilised GST, GST-syntaxin 1A or GST-syntaxin 4 were incubated with various concentrations of Csp1. Proteins were eluted and analysed as in A. (C) Quantification of Csp1 binding to syntaxins. The antibody signal from B, was scanned into an image analysis program and expressed as amount of Csp bound (percentage of the maximum).

an additional 34 amino acids at its C terminus, Csp2 only has an extra three amino acids. The simplest explanation of the binding experiments is that syntaxin 4 binds to the C-terminal region of Csp1 (precluding an interaction with Csp2), but that syntaxin 1A binds to a common motif present in both Csp1 and Csp2. Although it is possible that syntaxin 1A and syntaxin 4 bind to different sites on Csp1, another possibility is that they recognise different conformational states of the Csps. The different C termini of the two Csp isoforms may have subtle effects on protein folding, which allow syntaxin 1A to bind more tightly to Csp2, while preventing association of syntaxin 4 with Csp2. It has previously been shown that immunoprecipitation of Csp from rat brain coprecipitates VAMP but not syntaxin (Leveque et al., 1998). Thus, the interaction with syntaxin may be transient in vivo, or the epitopes recognised by the Csp antibody may be masked when Csp is complexed with syntaxin. Although it is not known whether Csp and VAMP interact directly, the co-immunoprecipitation of these two proteins suggest that through interactions with VAMP and syntaxin, Csp may regulate SNARE complex dynamics.

The expression of Csp2 protein has been difficult to demonstrate convincingly, probably due to a lack of suitable antisera. However, Csp2 mRNA levels have been shown to be developmentally regulated, and Csp2 mRNA expression in the adult brain is restricted to selected areas such as the cerebellum and olfactory bulb (Kwon et al, 1996). The expression levels of Csp1 and Csp2 mRNA are differentially regulated (Kwon et al., 1996), implying that the two Csp isoforms have specific roles in regulated exocytosis. The different affinities of the two Csp isoforms for syntaxin proteins probably underly the specialised roles performed by Csp1 and Csp2 in regulated membrane fusion.

Why is Csp associated with the plasma membrane in adipocytes? The most striking difference between insulin-stimulated GSV fusion and exocytosis of neuronal and neuroendocrine secretory vesicles is the rate at which fusion occurs. While exocytosis of synaptic vesicles can occur within 100 microseconds of nerve terminal depolarisation and Ca^{2+} entry (Burgoyne and Morgan, 1995), insulin-stimulated GSV translocation has a half-time of around 5 minutes (Satoh et al., 1993; Yang et al., 1992). Therefore, the much greater lag time between stimulation and fusion may change the distribution requirements for Csp1 in adipocytes. We have shown that Csp1 has a lower affinity for syntaxin 4 than syntaxin 1A, which further suggests that exocytosis from neuronal and from neuroendocrine cells are highly tuned processes requiring high affinity interactions, whereas GSV fusion may not have such strict requirements as a result of the slower fusion rate.

There is little known about how Csps or other proteins are targeted to secretory vesicles, and there appears to be no consensus motif mediating vesicle association. Although palmitoylation of Csp is required for initial membrane attachment, it is unlikely that this modification is sufficient to target Csp to secretory vesicles as a number of non-vesicle proteins are also palmitoylated. Targeting of the t-SNARE SNAP-25 to the plasma membrane also requires palmitoylation (Gonzalo and Linder, 1998). Recent work has demonstrated that the initial association of newly synthesized SNAP-25 with the plasma membrane is dependent upon a cytosolic interaction with syntaxin (Vogel et al., 2000). By analogy, membrane targeting of Csp may also be mediated by a carrier protein, which promotes the plasma membrane association of Csp1 in adipocytes. Another possibility is that GSVs have a different lipid composition or mode of synthesis from other regulated secretory vesicles, which prevents Csp association. Whatever the adipocyte-specific factors or signals are that dictate plasma membrane targeting of Csp1, the presence of Csp1 at the cell surface is likely to be of functional importance to adipocyte physiology.

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