Cysteine-string proteins regulate exocytosis of insulin independent from transmembrane ion fluxes

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Abstract Cysteine-string proteins (Csps) are vesicle proteins involved in exocytosis of synaptic vesicles in *Drosophila* and modulation of presynaptic calcium influx. As both the contribution of calcium channel regulation to the role of Csp in exocytosis and a function of Csp outside the nervous system are unknown, we studied its function in endocrine exocytosis from large dense core vesicles (LDCVs) using insulin-secreting pancreatic β-cells. Csps were expressed in primary and derived β-cell lines on insulin-containing LDCVs. Suppression of Csp expression reduced not only depolarisation induced insulin release but also exocytosis in permeabilised cells directly stimulated by Ca^{2+}. Thus, Csp is a secretory granule protein and is required for endocrine exocytosis independent of the modulation of transmembrane calcium fluxes.

Key words: Exocytosis; Vesicle protein; Calcium; Insulin; Cysteine string protein

1. Introduction

Exocytosis constitutes the final step in regulated secretion [1]. This process involves macromolecular complexes and their highly orchestrated assembly, rearrangement and disassembly constitute a key part of the exocytotic process [2]. Despite progress in the identification of components of the exocytotic machinery in recent years, numerous proteins still await assignment of a specific role. The cysteine-string proteins (Csps) were first discovered as presynaptic proteins in *Drosophila* and a densely clustered string of 12–15 cysteine residues gave rise to their name [3]. The fatty acylation of these residues is thought to tether Csps to synaptic vesicle membranes as shown in *Torpedo* [4].

Csps are involved in neurotransmission [5], as deletion of the Csp gene in *Drosophila* causes a temperature sensitive block of synaptic transmission followed by paralysis and premature death [6,7]. Oocyte expression studies indicate that Csps may modulate presynaptic Ca^{2+} channels [8] and thereby interfere with Ca^{2+} influx required for exocytosis. Moreover detailed analysis of Csp mutant *Drosophila* strains revealed a major defect in calcium entry [9–11].

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Abbreviations: Csp, cysteine-string protein; HIT-T15, hamster insulinoma cells; INS-1E, rat insulinoma cells; LDCV, large dense core vesicles; MIN6, mouse insulinoma cells; RT-PCR, reverse transcription-polymerase chain reaction; SLMV, synaptic-like microvesicles; SL-O, streptolysin-O; SV, synaptic vesicles; SVP38, synaptophysin

Similar to neuroexocytosis, insulin release from large dense core vesicles in pancreatic β-cells is triggered by calcium influx through voltage dependent channels [12]. Likewise, insulin exocytosis requires the function of known protein components of the exocytotic machinery such as synaptobrevin/VAMP [13,14], syntaxin [15,16], α-SNAP [17], SNAP-25 [18] and synaptotagmin [19,20]. We have therefore used insulin-secreting cells as a model system to investigate whether Csp is involved in exocytosis of LDCVs and whether its putative function may involve modulation of Ca^{2+}-influx.

2. Materials and methods

2.1. Materials

Streptolysin-O was produced and purified as described [21]. 5-*N*-Ethyl-*N*-isopropyl-amiloride was obtained from Sigma (Wallisellen, Switzerland). Csp1 and Csp2 cDNA sequences were cloned from bovine adrenal chromaffin cells and ligated into the *Bam*HI/*Eco*RI sites of pcDNA3 (Invitrogen, Leek, The Netherlands) [22] and Csp1 antisense cDNA was constructed by ligation of the complete coding sequence of Csp1 into the *Bam*HI/*Hin*dIII sites of pcDNA3.1(−) (Invitrogen). pNHE3, encoding the NHE3 cell specific and amiloride insensitive human Na^+/H^+ antiport, was kindly provided by Dr J. Pouyssegur (Nice, France) [23]. The following monoclonal antibodies were used: anti-synaptophysin/SVP38 (Boehringer, Rotkreuz, Switzerland), anti-VAMP (kindly provided by Dr R. Jahn, Goettingen, Germany), anti-SNAP-25 (Sternberger Monoclonals, Baltimore, MD, USA), anti-syntaxin 1 (Sigma) and anti-insulin (K36aC10, Sigma, Buchs, Switzerland). Polyclonal antibody against rabbit Csp was raised against recombinant Csp1 [22,24]. In immunoblots this polyclonal antibody recognised well 20 ng of recombinant GST-Csp1, but did not detect up to 500 ng of GST-Csp2. Fluorescent second antibodies were purchased from Pierce (Oud Beijerland, The Netherlands).

2.2. Western blotting and subcellular fractionation

To prepare crude membranes, cells were detached by incubation for 5 min at 37°C with PBS containing 10 mM EDTA, centrifuged for 10 min at 4°C at 14 000×*g*, resuspended in ice-cold PBS-EDTA, sonicated and again centrifuged for 30 min at 4°C at 14 000×*g*. Western blotting using 12% SDS-PAGE minigels, subcellular fractionation, immunofluorescence and confocal laser microscopy were performed as described previously [19,25,26]. Primary antibodies were diluted at 1:100 for immunofluorescence. For immunoblotting the following dilutions were used: anti-Csp1 1:1000; anti-SVP38 1:2000; anti-Na^+/K^+ 1:1000; anti-syntaxin 1:2000; anti-SNAP25 1:2000 and anti-VAMP 1:10 000.

2.3. RT-PCR of Csp isoforms

Total RNA was prepared from whole rat brain, indicated cell lines or primary cells by the use of Trizol (Gibco Life Sciences, Basel, Switzerland) and cDNA was synthesised using AMV reverse transcriptase (Promega, Catalys AG, Wallisellen, Switzerland). The following primers were used: 5′-AAGGATCCATGGCTGACCAGAGGCAGCG (sense, *Bam*HI restriction site underlined), and antisense 5′-CTCAAGCTTTTAGTTGAACCCGTCGGTGTG (*Hin*dIII restriction site underlined). PCR reactions were carried out with

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an initial denaturation cycle at 95°C for 3 min followed by 30 cycles at 95°C (1 min), 52°C (1 min) and 72°C (2 min) using Taq polymerase (Pharmacia Biotech, Däbendorf, Switzerland). The purified PCR fragments were digested with *Bam*HI and *Hin*dIII, inserted into the corresponding sites of pcDNA3.1(−) vectors and used for the transformation of DH5α *E. coli* cells. The identity of the positive clones was verified by sequencing of both strands.

2.4. Cell culture and transient transfection

Cell cultures of INS1-E, HIT-T15, MIN6 and RINm5F cells, preparation of primary β-cells and cotransfections were performed as described [19,25–31]. Briefly, for cotransfections cells were plated in 24 wells at 200 000 cells/well and transfected 2 days later. To this end, cells were washed with Mg^{2+} and Ca^{2+} free PBS. One μl Transfectam (Promega, Wallisellen, Switzerland) was complexed for 15 min with a total of 5 μg cDNA (equal amounts of reporter gene and Csp antisense cDNA or control plasmid) in 400 μl Dulbecco's minimal essential medium. The mixture was added to the cells after aspiration of PBS and exchanged for complete medium after 12 h. Experiments implying co-transfected cells were performed after 8 days.

For release studies, cells were washed twice at 37°C with Krebs-Ringer-bicarbonate-HEPES buffer (KRB, with the following composition in mM: 134 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1 $CaCl_2$, 5 $NaHCO_3$, 25 HEPES, 0.1% bovine serum albumin, pH 7.4). Thereafter cells were incubated for 10 min in KRB (basal) or KRB containing 50 mM KCl (KCl) and subsequently supernatants were removed, centrifuged at 4°C and kept at −20°C until human insulin C-peptide assay by species specific ELISA (Dako, Küssnacht, Switzerland) [15,19,29,31]. For streptolysin-O (SL-O) permeabilisation, cells were washed at 37°C with KG (140 mM potassium glutamate, 5 mM NaCl, 7 mM $MgS0_4$, 5 mM Na_2ATP, 0.4 mM EGTA, 20 mM HEPES, pH 7.0). Permeabilisation was performed with recombinant SL-O in 0.1 μM free Ca^{2+} with KG/EGTA/$CaCl_2$-buffer containing 5 mM ATP during 7 min resulting in at least 95% of cells permeable to trypan blue [19,29,31]. Subsequently cells were exposed to 0.1 or 10 μM free Ca^{2+} in KG/EGTA/$CaCl_2$-buffer for 8 min in the presence of 5 mM ATP.

For determination of expression levels of Csp, cotransfected cells were purified by acid-load selection using a vector that encodes the amiloride-resistant Na^+/H^+ exchanger isoform NHE3 [23,32]. HIT-T15 cells were co-transfected with pNHE3 and either pcDNA3 or Csp antisense-cDNA as given above. The selection procedure was performed as described [23] with some modifications using filter sterilized buffers. Briefly, 2 days after cotransfection, cells were washed with PBS and kept for 20 min in loading buffer (70 mM choline chloride, 50 mM NH_4Cl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM HEPES, adjusted to pH 7.5 with Tris-base) at 37°C without CO_2 supply. Subsequently cells were washed rapidly in wash buffer (120 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM MOPS, adjusted to pH 7.0 with Tris-base), kept for 1 h in recovery medium (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM MOPS, adjusted to pH 7.0 with Tris-base, containing 10 μM 5-*N*-ethyl-*N*-isopropyl-amiloride), washed again once with PBS and further incubated in complete medium under normal culture conditions. The following day cells were trypsinised to remove dead cells, reseeded at 0.4×10^6 cells/well and finally cells were harvested 8 days after co-transfection for immunoblots. Control cotransfection experiments using an enhanced green fluorescent protein expressing plasmid (peGFP) indicated that about 90% of the selected cells expressed this cotransfected marker.

2.5. Statistical analysis

Results are presented as mean ± S.E. 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).

3. Results and discussion

3.1. Expression of Csps in insulin secreting cells

We first examined whether Csps are expressed in the insulin-secreting cell lines RINm5F, HIT-T15, INS-1E and MIN6 using a polyclonal anti-Csp antibody raised against recombinant Csp1 (Fig. 1A). This antibody recognized a 35-kDa protein, as expected for Csp1, in immunoblots of crude membranes from all insulin-secreting cell lines tested. As the antibody recognizes only Csp1, but not the C-terminally truncated splice variant Csp2 (see Section 2), we employed RT-PCR to test for the expression of Csp2. As shown in Fig. 1B, we obtained two amplicons of sizes expected for Csp1 and Csp2 using RNA from rat brain, primary rat islet cells, hamster HIT-T15 and rat INS-1E cells. Subcloning of the two amplicons from rat islets into pcDNA3 and full length sequencing revealed them to be identical to the published sequences of rat Csp1 and Csp2 [22], respectively.

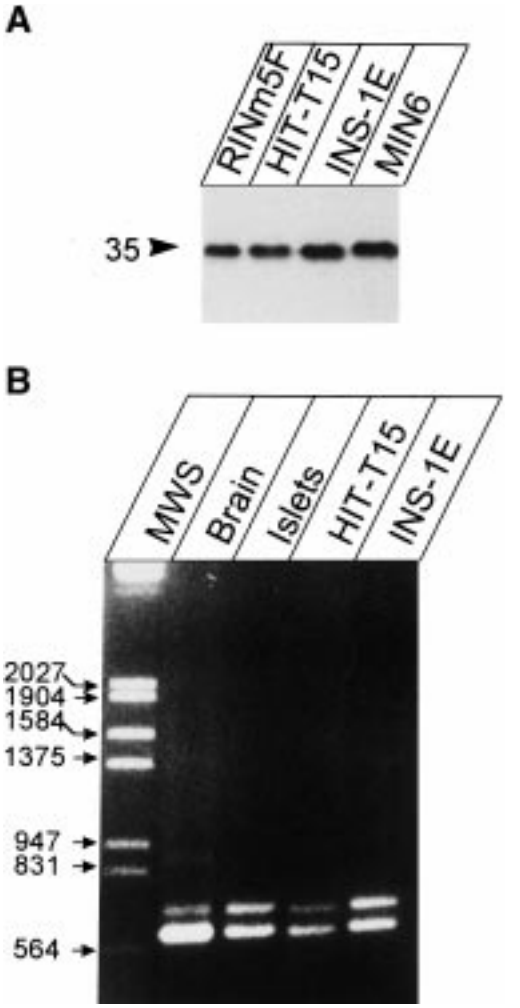

Fig. 1. Expression of Csps in insulin secreting cells. A: Crude membranes (20 μg) from the indicated insulin-secreting cell lines RINm5F, HIT-T15, INS-1E and MIN6 were separated by 12% SDS-PAGE, transferred to a PVDF membrane and probed with a polyclonal anti-Csp antibody. B: PCR amplification of Csp cDNA from rat brain, rat islets and the derived clonal cell lines HIT-T15 and INS1-E. PCR was performed using primers corresponding to the 5′ and 3′ ends of the Csp1 coding sequence. The size of the two amplicons generated is compatible with Csp1 (598 bp) and Csp2 (666 bp). Their identity was further confirmed by subcloning and sequencing of both strands (see Section 3).

3.2. Subcellular distribution of Csps

In order to investigate the subcellular localisation of Csp1, we performed subcellular fractionation of INS-1E cells on a continuous sucrose density gradient (Fig. 2). This method

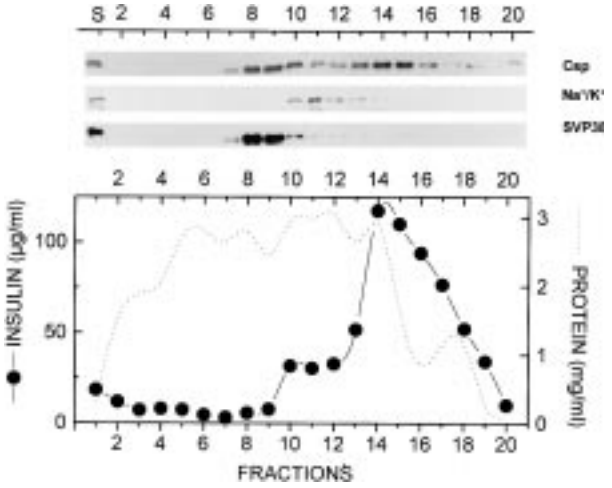

Fig. 2. Subcellular localisation of Csp1. A postnuclear supernatant from INS-1E cells was separated on a continuous sucrose density gradient (0.4–2.0 M). Samples were taken from the top to the bottom of the tube and analysed (S, postnuclear supernatant; 1–20, fraction numbers). Upper panel: Distribution of Csp (Csp) as well as Na^+/K^+-ATPase (Na^+/K^+), and synaptophysin (SVP38), markers for plasma membrane or synaptic-like microvesicles/endocytotic compartments, respectively. Lower panel: Distribution of insulin (closed circles) as determined by RIA and total protein (dashed line).

permits the separation of two vesicle populations in β-cells: the small synaptic vesicle-like microvesicles (SLMVs), carrying GABA, and LDCVs containing insulin, the secretory granules [33]. In endocrine cells the major part of synaptophysin immunoreactivity (SVP38) corresponds to early endocytotic compartments, whereas a smaller amount reflects the presence of SLMVs [33,34]. SVP38 was restricted to fractions 8 and 9 (Fig. 2, upper panel), whereas insulin was detected mainly in fractions 13–16 (Fig. 2, lower panel), indicating the presence of secretory granules. Na^+/K^+ ATPase, a marker protein for plasma membranes, was found in fractions 11–13. Csps were enriched mainly in fractions 8–10 and 13–16.

These findings were extended to primary pancreatic β-cells and derived cell lines by confocal immunomicroscopy. In primary cells, in INS-1E cells and in HIT-T15 cells a granular pattern of immunoreactivity for Csps was observed (Fig. 3A). Double-staining with anti-Csp1 and with anti-insulin antibodies revealed that insulin immunoreactivity largely colocalised with immunostaining for Csp. In addition, a certain amount of Csp immunoreactivity was localised to other cellular compartments and this was most prominent in primary β-cells. Therefore we also investigated whether Csp and synaptophysin immunoreactivity coincide. As shown in Fig. 3B, synaptophysin typically yields a mainly perinuclear stain in accordance with its predominant location on early endosomal compartments [33,34]. Csp immunoreactivity hardly coincided with the perinuclear synaptophysin immunoreactivity.

It may therefore be hypothesized that the comigration of Csp with SVP38 positive vesicles in sucrose gradients might reflect the presence of Csp on SLMVs, but not on early endocytotic compartments, in analogy to its expression on exocytotic vesicles in neurons [3,35]. More importantly, these data indicate that Csp is present on insulin-containing secretory granules and therefore may play a role in insulin exocytosis. The expression of Csps on secretory granules observed here is consistent with their previous localisation on large dense core vesicles in neuroendocrine cells [24,36] and exocrine pancreas [37]. As our antibody recognises only the isoform Csp1, we cannot draw any direct conclusions on the distribution of Csp2. However, transient expression of myc epitope-tagged Csp1 and Csp2 yields a comparable subcellular distribution of the two isoforms (H. Zhang and J. Lang, manuscript in preparation).

3.3. *Effect of transient expression of Csp-antisense DNA on insulin secretion and exocytosis*

In order to investigate the role of Csps in the exocytosis of insulin, we employed transient antisense transfection in the insulin-secreting hamster β-cell line HIT-T15. To test the effect of transient antisense transfection on the expression of Csp, we purified cotransfected cells using coexpression of an amiloride-insensitive Na^+/H^+ antiport as selection marker followed by a challenge with acid [23,32] (see Section 2). As shown in Fig. 4A, cotransfection with Csp antisense cDNA specifically reduced the expression of Csp. In contrast, no alteration was found in the levels of vesicular or plasma membrane SNARE proteins known to be involved in insulin exocytosis, such as syntaxin 1, SNAP-25 or VAMP [13,15,18].

To monitor the effect of Csp anti-sense cDNA on secretion and exocytosis, we exploited our cotransfection system using a plasmid encoding human preproinsulin (phINS). The ensuing release of the gene product human insulin C-peptide is used as a reporter for insulin release from the cotransfected rodent cells [15,19,25,29,31]. Intact cells were either exposed to Krebs-Ringer buffer alone or stimulated by the addition of 50 mM KCl (Fig. 4B, upper panel), which leads to depolarization-induced Ca^{2+}-influx through voltage-dependent Ca^{2+} channels and secretion of the reporter gene product insulin C-peptide. We observed that co-transfection with pcDNA3asCsp (antisense) inhibited the release of human C-peptide induced by 50 mM KCl by 52% as compared to co-transfection with the control vector pcDNA3 (control).

The effect of Csp suppression on insulin secretion in intact cells does not permit defining the steps at which the protein normally interacts with the secretory pathway. The observed inhibition of insulin secretion may be attributed to a variety of mechanisms including differences in Ca^{2+} influx through voltage-dependent Ca^{2+} channels. To bypass this step, we also studied the effects of antisense cDNA in streptolysin-O (SL-O) permeabilised cells. In these permeabilised cells, ionic gradients are abolished and the cytosolic Ca^{2+} is clamped to defined values by the use of Ca^{2+}/EGTA buffers [19,26,31,38]. Furthermore, soluble second messengers such as cAMP or IP_3 no longer contribute to the observed response [29]. As shown in Fig. 4B (lower panel), raising the concentration of free Ca^{2+} from basal levels of 0.1 μM to the maximally stimulatory levels of 10 μM [38] induced a threefold increase in human insulin C-peptide release. Again, in cells transiently transfected with Csp antisense cDNA (antisense), Ca^{2+} stimulated exocytosis was decreased to a similar extent as in intact cells. These observations demonstrate an important role for the secretory granule protein Csp in insulin exocytosis independent from the modulation of ion fluxes through plasma membrane Ca^{2+} channels. Similarly, stable overexpression of Csp1 in neuroendocrine PC12 resulted in an increase in LDCV exocytosis which was not accompanied by macroscopic changes in Ca^{2+} influx and was still observed in permeabilised cells [39].

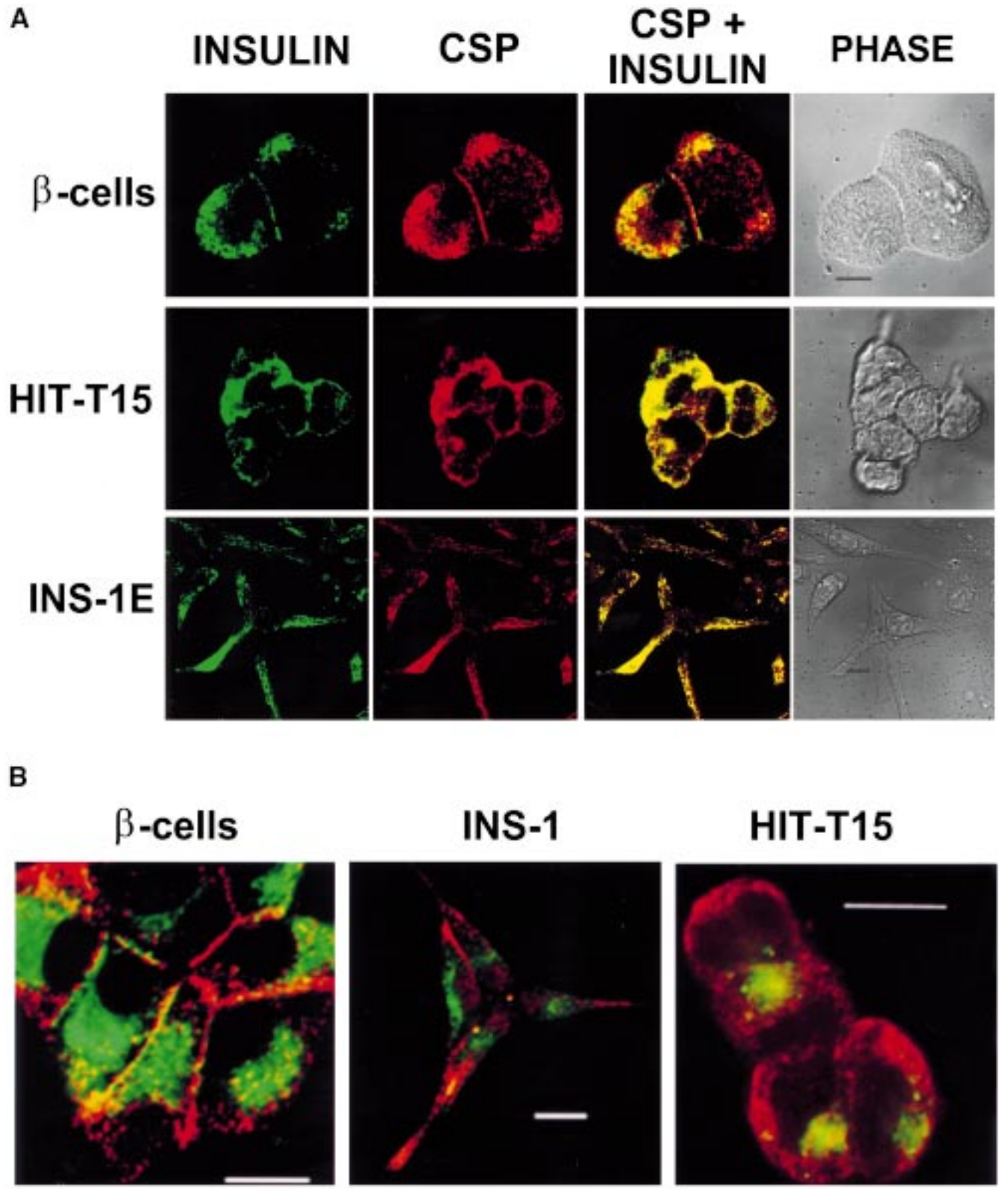

Fig. 3. Localisation of Csp1 in pancreatic β-cells and derived cell lines. A: Primary islet cells, INS-1E cells or HIT-T15 cells were stained with a rabbit polyclonal anti-Csps antibody (CSP) and mouse monoclonal anti-insulin antibody (INSULIN). Immunostaining of Csps was revealed by using rhodamine-conjugated anti-rabbit IgG (red) and insulin staining was detected with a fluorescein-coupled anti-mouse IgG (green). Co-localization results in yellow. Bar size indicated in the phase contrast equals 10 μm. B: Primary islet cells, INS-1E cells or HIT-T15 cells were stained with a polyclonal anti-Csp antibody and a monoclonal anti-synaptophysin antibody. Immunostaining of Csps was revealed by using rhodamine-conjugated anti-rabbit IgG (red) and synaptophysin staining was detected with a fluorescein-coupled anti-mouse IgG (green).

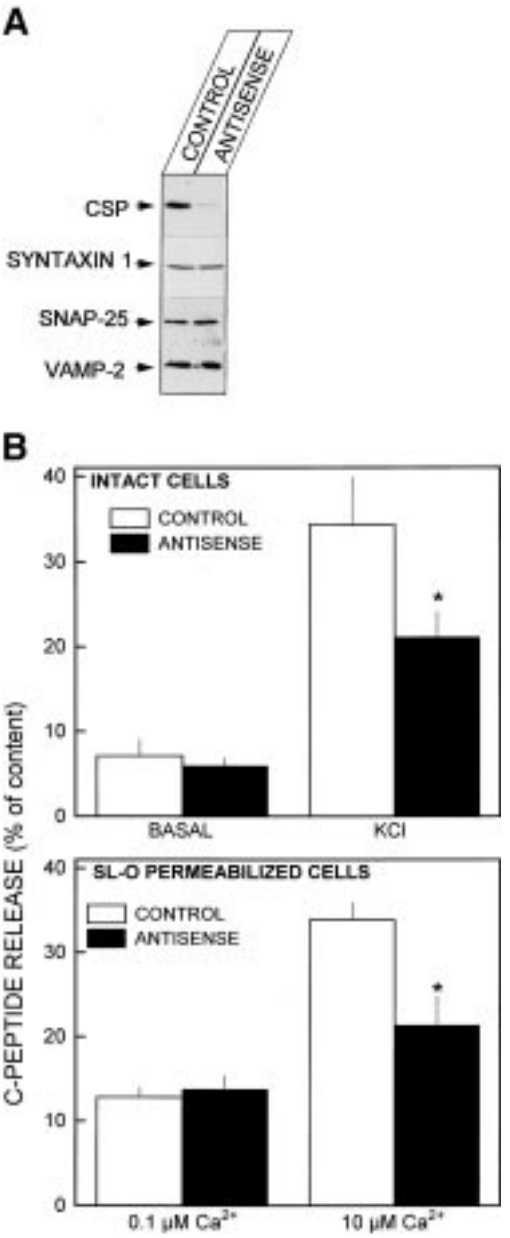

Fig. 4. Suppression of Csp1 expression inhibits exocytosis of insulin. A: Cells transiently co-transfected with pNHE3 and either pcDNA3 (control) or pcDNA3asCsp encoding Csp antisense cDNA (antisense) were purified as described in Section 2. Subsequently cell homogenates were used for the determination of Csp, syntaxin 1, VAMP and SNAP-25 expression levels by immunoblots. B: HIT-T15 cells were transiently co-transfected with a plasmid encoding human preproinsulin as reporter gene and with either control vector pcDNA3 (control, open bars) or pcDNA3asCsp encoding Csp antisense (antisense, filled bars). Eight days later human insulin C-peptide secretion was quantitated as a measure of insulin exocytosis. Upper panel: Secretion from intact cells under basal conditions (KRB, Krebs-Ringer buffer) or after stimulation with 50 mM KCl for 8 min. $N=6$ from 3 independent experiments; * $2P<0.05$. Lower panel: Cells were permeabilised with streptolysin-O and exposed to basal (0.1 μM) or maximally stimulatory (10 μM) levels of free Ca^{2+}. $N=6$ from 3 independent experiments; * $2P<0.05$.

3.4. Conclusions

The molecular actions of Csps in neuroexocytosis of SVs have been linked to the sites of Ca^{2+} entry [6,7,34]. Deletion of the Csp gene in *Drosophila* interrupts only depolarisation-induced neurotransmitter release, but not exocytosis induced independent of ion fluxes through presynaptic Ca^{2+} channels [9,10]. This excludes a failure of Ca^{2+} action itself on the exocytotic machinery. The apparent contradiction between these observations and our results could be reconciled when considering that presynaptic Ca^{2+} channels may not only mediate Ca^{2+} influx. They may, in addition, organise the physical site of exocytosis at regions of maximal calcium concentrations [40]. Indeed, the intracellular loop $L_{II/III}$ in the α1 subunit of presynaptic N- or P/Q-type Ca^{2+} channels associates with the SNARE proteins syntaxin/SNAP-25/synaptobrevin and with synaptotagmin and is thereby linked to exocytotic vesicles [40–43]. Interestingly, it has recently been shown by Leveque et al. [44] that Csp1 also interacts in vitro with the same loop of presynaptic P/Q-type Ca^{2+} channel. These observations lead to the proposal that Csp acts as a chaperone [45,46] and assists the assembly, rearrangement or disassembly of protein complexes at this specific site of exocytosis [44]. Such a function of Csp may provide the molecular basis for the initially proposed link between synaptic vesicles and presynaptic Ca^{2+} channels [35]. In contrast to neurons, L-type Ca^{2+} channels are mainly responsible for Ca^{2+}-influx causing secretion in the endocrine β-cells [47] and L-type Ca^{2+} channels functionally interact with SNARE proteins upon co-expression in *Xenopus* oocytes [48].

Collectively these observations and our results might predict that not only neuroexocytosis of SVs, but also exocytosis of LDCVs in endocrine cells is physically linked to Ca^{2+} entry sites. Such a linkage was suggested by the polarised distribution of secretion and Ca^{2+} influx in mouse β-cells [49]. As the role of Csps in endocrine exocytosis of LDCVs has now been established, future research should address the precise nature of effector(s) and regulators of Csps.

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