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Characterization of cysteine string protein in rat parotid acinar cells

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ABSTRACT

Cysteine string proteins (CSPs) are secretory vesicle chaperone proteins that contain: (i) a heavily palmitoylated cysteine string (comprised of 14 cysteine residues, responsible for the localization of CSP to secretory vesicle membranes), (ii) an N-terminal J-domain (DnaJ domain of Hsc70, 70 kDa heat-shock cognate protein family of co-chaperones), and (iii) a linker domain (important in mediating CSP effects on secretion). In this study, we investigated the localization of CSP1 in rat parotid acinar cells and evaluated the role of CSP1 in parotid secretion. RT-PCR and western blotting revealed that CSP1 was expressed and associated with Hsc70 in rat parotid acinar cells. Further, CSP1 associated with syntaxin 4, but not with syntaxin 3, on the apical plasma membrane. Introduction of anti-CSP1 antibody into SLO-permeabilized acinar cells enhanced isoproterenol (IPR)-induced amylase release. Introduction of GST-CSP1$_{1-112}$, containing both the J-domain and the adjacent linker region, enhanced IPR-induced amylase release, whereas neither GST-CSP1$_{1-82}$, containing the J-domain only, nor GST-CSP1$_{83-112}$, containing the linker region only, did produce detectable enhancement. These results indicated that both the J-domain and the linker domain of CSP1 are necessary to function an important role in acinar cell exocytosis.

Introduction

Cysteine string proteins (CSPs[1]) are evolutionally conserved secretory vesicle chaperone proteins. CSPs contain: (i) a heavily palmitoylated cysteine string containing 14 cysteine residues, which is responsible for the localization of CSP to secretory vesicle membranes, (ii) an N-terminal J-domain, (DnaJ domain of the Hsc70, 70 kDa heat-shock cognate protein, family of co-chaperones), and (iii) a linker domain (important in mediating CSP effects on secretion) [1–6] (Fig. 1). CSPs consist of two protein subtypes, CSP1 and the C-terminal truncated splice variant CSP2. CSP1 was initially identified in Drosophila as a synaptic vesicle protein [7,8]. Many studies have been performed concerning the localization and the function of CSPs, which have shown that CSPs are localized to neuronal synaptic vesicles, pancreatic zymogen granules [9,10], pancreatic endocrine cells [11,12], chromaffin granules in adrenal chromaffin cells [13,14], and secretory granules of neurohypophysis [15]. Additionally, CSPs regulate secretion of dopamine from PC-12 cells [16], exocytosis of chromaffin cells [17], amylase release from pancreatic acinar cells [10] and insulin secretion from β-cell [12]. Furthermore, CSP modulates receptor coupled-G proteins to increase intracellular cAMP levels [18], and G protein of N-type calcium channels [19]. Among other observations, it has been reported that CSPs interact with Hsc70, receptor-coupled trimeric GTP binding proteins (G protein) [19], syntaxin 1A during neurotransmitter exocytosis [20], synaptotagmin 1 in synaptic vesicles [21], and syntaxin 4 in the plasma membrane of 3T3-L1 adipocytes [22]. Recently, Zhang et al. [23] and Rozas et al. [24] reported that CSPα regulates the stability of SNAP-25 and its associated proteins, and that CSPα participates in synaptic vesicle endocytosis through promoting dynamin 1 oligomerization using CSPα knockout mice.

The parotid gland is a typical exocrine gland that releases protein rich saliva and serous saliva by sympathetic and parasympathetic stimulation, respectively. That is exocytosis in salivary glands is mainly evoked by β-adrenergic stimulation through cAMP/kinase A pathway. On the other hand, exocytosis is evoked by calcium-dependent manner in many types of cells such as neuronal synaptic cells, chromaffin cells, pancreatic acinar and endocrine cells so on. The functions of CSPs in those cells have been clarified [3,7–16]. In the parotid gland, the expression and function of CSPs have not been clarified to date.

In this study, we investigated the localization of CSP1 in parotid acinar cells and evaluated the role of CSP1 in parotid secretion.

Materials and methods

Materials

Rabbit anti-CSP1 and rabbit anti-syntaxin 3 antibodies were obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA) and

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[1] *Abbreviations used:* CSP, cysteine string protein; IPR, isoproterenol; Hsp70, 70 kDa heat shock protein; Hsc70, 70 kDa heat shock cognate protein; ICM, intracellular membrane; Cyto, cytosol fraction; BLM, basolateral plasma membrane; APM, apical plasma membrane; SG, secretory granule; SGM, secretory granule membrane; SLO, streptolysin O; GST, glutathione S-transferase.

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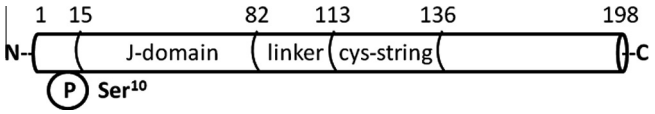

Fig. 1. Schematic representation of SCP1 structure. Ser^{10}: Ser phosphorylated by protein kinase A.

Chemicon (Billerica, MA, USA), respectively. Mouse anti-Hsc70 was purchased from StressMarq Biosciences Inc. (Victoria, BC, Can.). Rabbit anti-syntaxin 4 was purchased from Alomone Labs (Jerusalem, Israel). Isoproterenol (IPR), ATP and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Preparation of subcellular fractions from rat parotid glands

All procedures were approved by the laboratory animal care committee of The Nippon Dental University, School of Life Dentistry at Niigata. Subcellular fractions were prepared from a homogenate of acinar cells, as described previously [25]. Specifically, parotid acinar cells were homogenized in a 20-fold volume of buffer A (5 mM HEPES-NaOH buffer (pH 7.2) containing 50 mM mannitol, 0.25 mM $MgCl_2$, 25 mM β-mercaptoethanol, 0.1 mM ethylene glycol-bis (β-aminoethylether) *N,N,N,N*-tetraacetic acid (EGTA), 2 μM leupeptin, 2.5 μg/ml of trypsin inhibitor, 0.1 mM 4-amidinophenylmethanesulphonyl fluoride hydrochloride (p-APMSF), 5 mM benzamidine, and 2 mg/ml of aprotinin) using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 9750*g* for 10 min at 4 °C. The supernatant was recentrifuged at 35,000*g* for 30 min at 4 °C, and the resultant supernatant was further centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was used as the intracellular membrane (ICM) fraction and the supernatant was used as the cytosol fraction (Cyto). The pellet obtained by centrifugation at 35,000*g* was suspended in buffer A containing 10 mM $MgCl_2$ and left on ice for 30 min. This suspension was then centrifuged at 3000*g* for 15 min and the resultant pellet was used as the basolateral plasma membrane (BLM) fraction. The resultant supernatant was recentrifuged at 100,000*g* for 1 h and the resultant pellet was recovered and used as the apical plasma membrane (APM) fraction. The APM and BLM fractions were characterized based on estimation of the enzyme activity of γ-glutamyl transpeptidase and alkaline phosphatase, respectively. Secretory granules (SG) were prepared by centrifugation through a 40% RediGrad gradient (GE Healthcare Japan, Tokyo, Japan). Preparation of the secretory granule membranes (SGM) was performed as previously described [26], by treatment of SG with a hypotonic medium (10 mM Tris–HCl, pH 7.2, containing the above described protease inhibitors). Protein assays were performed using a protein assay kit (BioRad, Hercules, CA, USA).

Immunoblot analysis

Samples were solubilized in Laemmli sample buffer and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using pre-made 5–20% polyacrylamide gel plates (e-PAGEL, Atto, Tokyo, Japan). After PAGE, the separated proteins were electrotransferred to PVDF membranes (Immuno-Blot, Bio-Rad). The blots were probed with one of the following primary antibodies: anti-CSP (1:1000), anti-Hsc70 (1:1000), anti-syntaxin 3 (1:250), and syntaxin 4 (1:600). The immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (GE) or Pierce Western Blotting Substrate Plus (Thermo Scientific, IL, USA.).

Reverse transcriptase-PCR (RT-PCR)

Parotid acinar cells were soaked in 0.5 ml RNAlater (Ambion Inc., Austin, TX, USA). Total RNA was prepared using an RNeasy Plus Mini kit (QIAGEN, Chatsworth, CA, USA) according to the manufacturer's instructions. Reverse transcription of total RNA from parotid acinar cells was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). cDNA was synthesized from 3 μg of total RNA, and 1/20th of the reverse transcription reaction mix was used for PCR or real-time quantitative PCR analysis. PCR was performed using KOD-plus DNA polymerase (TOYOBO, Osaka, Japan) and specific primers. The primers used for RT-PCR corresponded to CSP1 (forward: CTCACCTGCTGCTACTGCTG; reverse: CCTCCCTCTCATCAGACTGC) or Hsc70 (forward: CAGAATCCCCAAGATCCAGA; reverse: GTGACATCCAAGAGCAGCAA). Identity of the RT-PCR products was confirmed by sequencing with an ABI PRISM 310NT (Applied Biosystems, Foster City, CA, USA).

Immunoprecipitation

A homogenate of parotid acinar cells was prepared with ice-cold homogenization buffer. The homogenate (0.5 or 1 mg of protein) was solubilized with 1% Triton X-100 at 4 °C for 1 h. Insoluble materials were removed by centrifugation at 15,000*g* for 10 min. The supernatant was incubated at 4 °C for 1 h with one of anti-CSP1, anti-Hsc70, anti-syntaxin 3 or anti-syntaxin 4 antibodies (10 μg/ml), and subsequently incubated for 1 h at 4 °C with Dynabeads M-280 sheep anti-rabbit IgG or sheep anti-mouse IgG (Dynal Biotech, Oslo, Norway). The beads were washed five times with buffer (composed of 50 mM Hepes-KOH (pH 7.2), 150 mM NaCl, and 0.05% Tween 20), and proteins bound to the beads were eluted by boiling in Laemmli SDS-sample buffer.

Preparation of glutathione S-transferase (GST) fusion proteins

Preparation of GST fusion proteins of CSP1 truncated constructs was performed as previously described [27]. Fusion proteins were affinity-purified using glutathione Sepharose 4B (GE, Tokyo, Japan).

Amylase release from streptolysin O (SLO)-permeabilized parotid acinar cells

Amylase released from SLO-permeabilized acinar cells was measured as described previously [28]. Lyophilized SLO powder (Sigma–Aldrich) was dissolved in 10 mM PBS (pH 7.0) and activated with 10 mM dithiothreitol for 1 h on ice. Parotid acinar cells were washed twice with incubation medium (20 mM Hepes-NaOH (pH 7.2), 140 mM KCl, 1 mM $MgSO_4$, 1 mM Mg-ATP, 0.1 mg/ml trypsin inhibitor, and 0.1% BSA). The effects of anti-CSP1 antibody and truncated CSP proteins on IPR-induced amylase release were investigated as follows. Cell suspensions ($5–10 \times 10^5$ cells per 100 μl) were pipetted into a tube containing 2 μl SLO (2500 units/ml) together with rabbit anti-CSP IgG or the truncated CSP protein, and incubated at 37 °C for 5 min. The suspension was then stimulated with 1 μM IPR for 20 min. Incubation medium (900 μl) was added to the reaction mixture, and the mixture was immediately filtered through glass filter paper to remove the acinar cells. The filtrate, containing released amylase, was used for the amylase assay. Total amylase activity was assayed in the centrifuged supernatant of acinar cells following their homogenization in 0.1% Triton X-100. Amylase activity was measured as described by Bernfeld [29].

Protein assay

Protein assays were performed using a protein assay kit (Bio-Rad).