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The CSPα/G protein complex in PC12 cells

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Abstract

Cysteine string proteinα (CSPα) is a regulated vesicle protein and molecular chaperone that has been found to be critical for continuous synaptic transmission and is implicated in the defense against neurodegeneration. Previous work has revealed links between CSPα and heterotrimeric GTP binding protein (G protein) signal transduction pathways. We have shown that CSPα is a guanine nucleotide exchange factor (GEF) for $G_{\alpha s}$. *In vitro* Hsc70 (70 kDa heat shock cognate protein) and SGT (small glutamine-rich tetratricopeptide repeat domain protein) switch CSPα from an inactive GEF to an active GEF. Here we have examined the cellular distribution of the CSPα system in the PC12 neuroendocrine cell line. CSPα, an established secretory vesicle protein, was found to concentrate in the processes of NGF-differentiated PC12 cells as expected. G_{β} subunits co-localized and $G_{\alpha s}$ subunits partially co-localized with CSPα. However, under the conditions examined, the GEF activity of CSPα is expected to be inactive, in that Hsc70 was not found in PC12 processes. These results indicate that CSPα activity is subject to regulation by factors that alter Hsc70 distribution and translocation within the cell.

Keywords: CSPα; Cysteine string proteinα; G protein; Hsc70; SGT; Chaperone; J protein

Cysteine string proteinα (CSPα) is a member of the evolutionary conserved J protein family also called the DnaJ or Hsp40 (heat shock protein 40) protein family. The cDNA clone for rat CSP (594 bp) contains 181 nucleotides of 5′ untranslated region, 1.2 kb of 3′ untranslated region and encodes a 35 kDa protein with extensive lipid modification [7] that purifies with lipid raft fractions [37]. CSPα is found on synaptic vesicles [40] and clathrin-coated vesicles [4] in neurons as well as exocrine [7,60], endocrine [10,58], and neuroendocrine [15,33] secretory granules. Together with Hsc70 (70 kDa heat shock cognate protein) and SGT (small glutamine-rich tetratricopeptide repeat domain protein), CSPα assembles into an enzymatically active chaperone complex [8,49]. Recent work has demonstrated that the active CSPα chaperone complex regulates signaling through heterotrimeric GTP binding protein signal transduction pathways. Specifically we have shown that in association with Hsc70 and SGT, CSPα regulates heterotrimeric GTP binding protein (G protein) function by preferentially targeting the inactive GDP-bound form of $G_{\alpha s}$ and promoting GDP/GTP exchange which increases cAMP [45]. CSPα is selective for $G_{\alpha s}$, and, as such, is the first identified GEF (guanine nucleotide exchange factor) for $G_{\alpha s}$. Isoproterenol-induced elevation in cAMP is increased dramatically in the presence of CSPα [45]. In addition, CSPα associates with G_{β} and enhances G protein inhibition of N-type calcium channels, through interactions with the synprint (*syn*aptic *pro*tein *int*eraction) region of the N-type calcium channel and G protein βγ subunits [36,41,42]. The downstream effects of the CSPα-induced increase in cAMP and its involvement in degeneration remain to be established. CSPα-modulation of transmembrane calcium flux [19,30,50] as well as exocytosis [10,12,25,28,43,46,57,58] have been reported and signaling through $G_{\alpha s}$ may underlie these events.

In vitro, CSPα's GEF activity is manifested in the presence of Hsc70/SGT. We have previously established that CSPα has two G protein binding sites. Site 1 (residues 83–112) binds G_{α} and $G_{\beta\gamma}$, while site 2 (residues 1–82) binds G_{α}. The CSPα/$G_{\beta\gamma}$ interaction is robust and nucleo-

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tide insensitive [42]. In contrast CSPα preferentially targets the GDP-bound form of G_α and promotes GDP/GTP exchange [45]. Both sites are required for CSPα's GEF activity. *In vitro*, full length CSPα is inactive on its own, but is activated in the presence of SGT/Hsc70. Although full length CSPα requires Hsc70/SGT for activation, the $CSP\alpha_{1-112}$ truncation mutant is active on its own, and activity is not further increased in the presence of SGT/Hsc70. These results suggest that the C terminus is a regulatory (inhibitory) domain of CSPα. The mechanism underlying Hsc70/SGT activation of CSPα is not yet established. *In vitro* studies show that CSPα interacts with Hsc70 and accelerates its ATPase activity [8]. Hsc70ATPase is typically coupled to conformational changes in target proteins. The CSPα/Hsc70 association is ATP-dependent and is mediated by the region of CSPα encoding the J domain ($CSP\alpha_{1-82}$) [8,11,48]. The presence of SGT, in addition to CSPα/Hsc70 further accelerates Hsc70 ATPase [49]. The requirement of Hsc70/SGT for CSPα/GEF activity *in vitro* implies a mechanism for the cellular regulation of CSPα GEF activity.

In this study, we begin to address the hypothesis that the GEF activity of CSPα system is regulated (not constitutive) within the cell. While our *in vitro* data are consistent with a direct physical interaction between CSPα and G proteins, co-localization has not previously been documented. As a first step towards testing this hypothesis, we investigated the cellular proximity of CSPα, G proteins, and Hsc70. Our observations support the proposal that the CSPα/GEF system is regulated.

Materials and methods

Immunofluorescence. PC12 cells were obtained from ATCC. PC12 cells were plated on coverslips and grown in Dulbecco's modified Eagle's Medium supplemented with 10% heat-inactivated horse serum and 5.0% fetal calf serum. For differentiation, cells were treated with 50 ng/ml mouse NGF (R&D Systems) for 10 days. Cells were washed in PBS, fixed in PBS containing 4% paraformaldehyde, and permeabilized in PBS containing 0.5% Triton X-100. Incubations of cells with primary antibodies were carried out sequentially for 30 min at room temperature. Following incubation with primary antibody, the cells were washed with blocking solution and incubated with a mixture of goat anti-rabbit secondary antibody conjugated to Cy3 and a goat anti-mouse secondary antibody conjugated to Alexa 488. Following secondary antibody incubation, the cells were washed in PBS, mounted onto glass slides, and photographed using a Leica model DMRXA confocal fluorescence microscope or an Olympus BX60WI confocal microscope. The fluorophores Alexa 488 and Cy3 were excited using a mercury lamp at wavelengths of 470 nm and 535 nm, respectively. Images were collected at wavelengths 525 nm and 610 nm. False coloring and superimposition were performed with Adobe Photoshop 7.0.1.

PC12 transfection and immunoblotting. Myc-tagged rat $CSP\alpha_{1-198}$, $CSP\alpha_{1-82}$, $CSP\alpha_{1-198\Delta CCC}$, $CSP\alpha_{1-112}$, and $CSP\alpha_{113-198}$ DNA constructs were prepared in pCMV vector and the sequences confirmed. PC12 cells were transiently transfected with lipofectamine-2000 (Invitrogen), maintained in culture for 24 h and then harvested in lysis Buffer [20 mM Hepes, pH 7.0, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail (Sigma)]. Cells were pelleted by centrifugation at 15000 rpm for 60 min, and the particulate and cytosolic fractions were collected. Protein concentration was determined by Bio-Rad using bovine serum albumin as the standard. The solubilized cellular proteins were resolved by SDS–PAGE.

Proteins were transferred electrophoretically at 70 V for 45 min from polyacrylamide gels to 0.45 μm nitrocellulose in 20 mM Tris, 150 mM glycine, and 12% methanol [6]. Transferred proteins were visualized with Ponceau S. Nitrocellulose membranes were blocked for non-specific binding using 5% milk, 0.1% Tween 20, and PBS, pH 7.3 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) prior to a 2-h incubation with primary antibody. The membranes were washed three times in the above milk/Tween/PBS solution and incubated with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL, Pierce). Immunoreactive bands were visualized following exposure of the membranes to Amersham Hyperfilm-MP.

Results and discussion

CSPα is a member of the ancient evolutionarily conserved class of proteins called the J protein family. In yeast, it is known that certain J proteins are tethered to specific locations within a cellular compartment, and that this specific localization is important for function [17,24,55]. In mammalian neurons, J proteins are predicted to have specialized functions based on their cellular locale, however with the exception of auxilin and CSPα, the function of most mammalian J proteins remains undefined. All members of the J protein family contain a signature 'J domain' which is a 70 amino acid region of homology composed of four helices with a conserved histidine, proline, and aspartic acid tripeptide located between helices II and III. The J protein family recruits and activates the Hsc70/Hsp70 chaperone family, a class of ATPases that couple energy from ATP hydrolysis to conformation changes in a variety of target proteins. Hsc70-mediated conformational work must be highly regulated in the cell for proper function. For example, clathrin is removed from clathrin-coated vesicles but not from clathrin-coated pits by Hsc70 through the intercession of the J protein, auxilin [35,44,52]. Is cellular CSPα activity regulated? The expression of CSPα is reported to be modified by antidepressants [20–23,56], amphetamines [5], and diabetes [59] which is anticipated to alter CSPα activity. The anchoring of CSPα to secretory vesicles, the assembly of the CSPα/Hsc70/SGT complex, and the association of the CSPα system with G proteins/target proteins are other potential sites of regulation.

Distribution of transiently expressed CSPα in PC12 cells

CSPα is reported to be localized to secretory vesicles as a result of extensive palmitoylation of cysteine residues [13,29]. In contrast, G proteins are localized to the plasma membrane as a result of fatty acid modifications, but undergo activity-dependent internalization. Furthermore, G proteins have also been found on regulated secretory vesicles [1,4,34,54], although the physiological function of vesicle-associated heterotrimeric G proteins is unknown. First, we examined the biochemical fractionation of native and transiently expressed myc-$CSP\alpha_{1-198}$ in PC12 cells (Fig. 1). Native CSPα localized to the particulate fraction

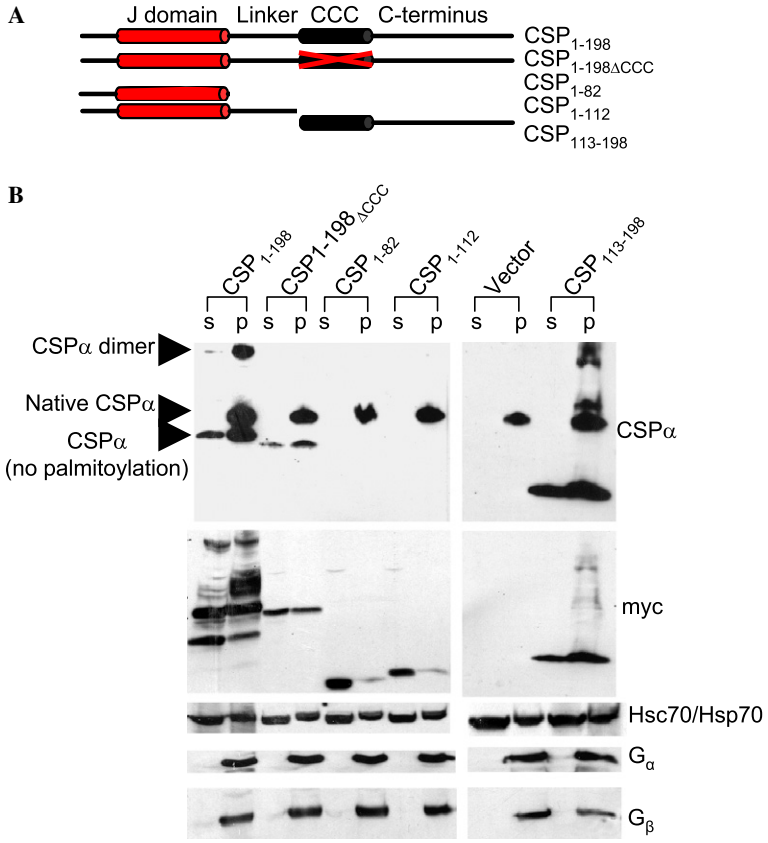

Fig. 1. Expression of CSPα constructs in PC12 cells. (A) Schematic representation of CSPα truncation constructs. (B) The particulate and cytosolic fractions of PC12 cells transiently transfected with plasmids encoding the indicated myc-tagged constructs were separated on 12% SDS–PAGE gels, subsequently transferred to nitrocellulose membrane, and stained with anti-CSPα antibody, anti-myc antibody, anti-Hsp70/Hsc70 antibody (Sigma), anti-G_{α} antibody (Santa Cruz), and anti-G_{β} antibody (Transduction Labs).

consistent with its presence on synaptic vesicles. Fig. 1 shows that PC12 cells transfected with CSPα_{1-198} display cytosolic and particulate CSPα_{1-198}. A fraction of the membrane associated CSPα and all of the cytosolic CSPα has an apparent molecular weight lower than that of the native CSPα reflecting a lack of palmitoylation. CSPα can form dimers [7] and the CSPα-dimer was more abundant after transient expression of CSPα. Our results indicate that the native secretory vesicle distribution of the CSPα system can be changed experimentally perhaps because CSPα palmitoylation becomes rate limiting. Changes in CSPα's cellular location may block native activity as a dominant negative or alter other cellular J protein/Hsc70 systems.

We then proceeded to examine the biochemical fractionation of several CSPα truncation mutants after transfection into PC12 cells. Myc-CSP$\alpha_{1-198\Delta CCC}$ (lacking the cysteine string residues 113–136) and myc-CSPα113–198 (cysteine string and C-terminus) were found in both soluble and particulate fractions. Myc-CSPα1–82 (J domain) and myc-CSPα1–112 (J domain and L region) were found primarily in soluble fractions consistent with other reports [29]. The redistribution of CSPα or CSPα truncation mutants to the cytosolic fraction did not alter the fractionation of Hsc70, Gα or Gβ (Fig. 1). Upon cellular relocalization to the cytosol or in the absence of CSPα, it is predicted that Hsc70 will be unable to localize to secretory vesicles, thereby rendering Hsc70 folding activity mechanically ineffective at this cellular locale. Numerous studies have introduced CSPα or CSPα truncation constructs into a wide range of cell lines to examine CSP's role in either exocytosis or calcium signaling (reviewed in [14,61]). Our results indicate that the cellular distribution is most likely altered in several of these strategies. For example, *Drosophila* CSP is not lipid-anchored to membranes when expressed in PC12 cells [53]. Other studies have shown that deletion of CSPα in mice causes progressive neurodegeneration [16,27,47]. *Drosophila* CSPα-null mutants die as larvae or within days of adulthood. The small number of flies that survive to adulthood are characterized by uncoordinated sluggish movements, shaking, and temperature sensitive paralysis [2,51,62]. These deletion studies indicate that other neural J proteins (e.g., auxilin or Hsp40) do not effectively compensate for CSPα's activity.

Distribution of CSPα, Gαβγ, and Hsc70 in PC12 cells

Next, we investigated the subcellular localization of CSPα and G proteins in NGF differentiated PC12 cells using confocal microscopy. Upon differentiation, CSPα was found to concentrate in the processes, consistent with

its being a synaptic vesicle protein. However, an additional small intracellular CSPα pool was also observed. Fig. 2 demonstrates that in PC12 cells G_β and CSPα localize to processes but that G_β does not co-localize with the intracellular compartment of CSPα. Consistent with previous reports [12], in undifferentiated PC12 cells we observed intracellular punctuate CSPα staining with some enrichment close to the plasma membrane (Fig. 2). PC12 cells are known to express two isoforms, CSPα1 and CSPα2 [13]. In agreement with this previous work, Western blot analysis often reveals a CSPα1/CSPα2 doublet (Fig. 2, panel C). CSPα2 is identical to CSPα1 but lacks the C-terminal 31 amino acids. Fig. 2, panel C also shows immunoblot analysis of another J protein, Hsp40 (heat shock protein 40), and actin as controls. The distribution of CSPα and G_β in PC12 cells is consistent with the robust interaction between CSPα and G_β observed *in vitro* [36,42].

The immunostaining in Fig. 2 indicates a significant fraction of CSPα with intracellular, perhaps, endosomal localization. Therefore, we further examined the co-localization of CSPα in differentiated PC12 cells with the secretory granule protein, synaptophysin. Like CSPα, synaptophysin was observed to concentrate in the processes. However, CSPα in the intracellular compartment did not co-localize with synaptophysin (Fig. 3). Consistent with our findings, a recent report has identified a novel pool of CSPα that does not co-localize with synaptic markers in rat brain [26]. The functional role of this intracellular pool remains to be determined.

Next, we investigated the localization of actin, another abundant protein of the processes (Fig. 4A). In contrast to CSPα/G_β immunolocalization, double labeling with fluorescently labeled phalloidin, an established actin marker, shows that actin does not have the same distribution as CSPα (Fig. 4). Widespread intracellular distribution of $G_{\alpha s}$ in NGF-treated PC12 cells was observed (Fig. 4B), including PC12 processes. Widespread distribution in undifferentiated PC12 cells has previously been reported [32]. The distribution of CSPα suggests that it may modulate specific pools of $G_{\alpha s}$ in PC12 cells.

Finally, we investigated whether the cytosolic chaperone, Hsc70, also was present in the processes in NGF-differentiated PC12 cells. In contrast to CSPα and G_β, the bulk of Hsc70 immunostaining was nuclear and concentrated away from the processes (Fig. 4C), suggesting that the CSPα/GEF chaperone system is not active in differentiated PC12 cells. In undifferentiated PC12 cells, Hsc70 immunostaining was widespread, cytosolic, and partially overlapped with CSPα immunostaining, suggesting that the CSPα/GEF activity is active in these cells (data not shown). Several conditions have been reported to trigger translocation/availability of Hsc70 or its stress-inducible homologue, Hsp70. ATPase activity of both Hsc70 and Hsp70 is stimulated by CSPα [8,11]. Hsc70 recruitment to

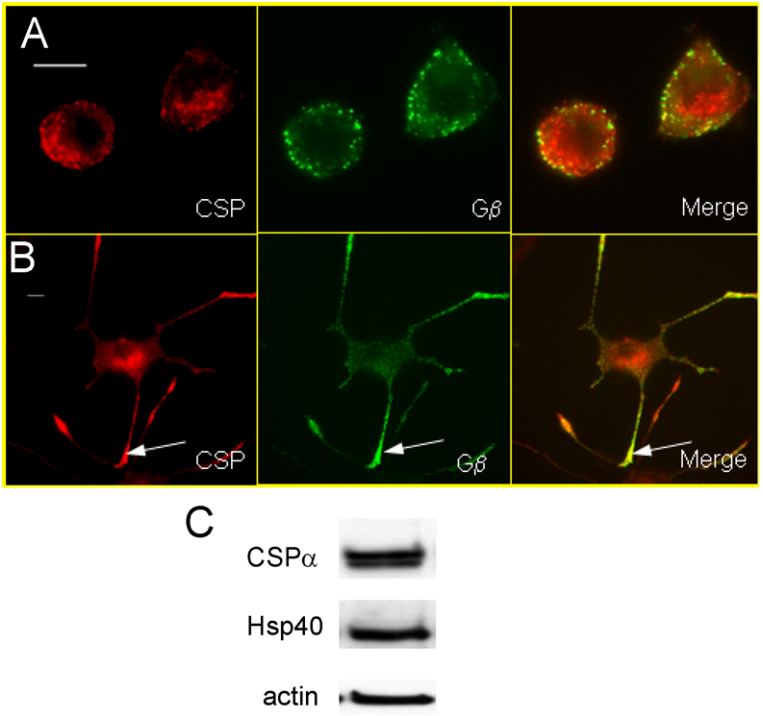

Fig. 2. Co-localization of CSPα and Gβ in PC12 cells. These images were obtained from the same focal plane of the cell. Bar = 10 μM. (A) Undifferentiated PC12 cells immunostained with CSPα polyclonal and G_β monoclonal. Digital superimposition of the G_β and CSPα images demonstrates co-localization of CSPα and G_β. (B) NGF differentiated PC12 cells were immunostained with CSP polyclonal and G_β monoclonal (Transduction Labs). CSPα staining was observed as punctuate, intracellular, and concentrated in the processes. (C) Western blot analysis of CSPα, Hsp40 (Stressgen), and actin (Sigma) in PC12 cells.

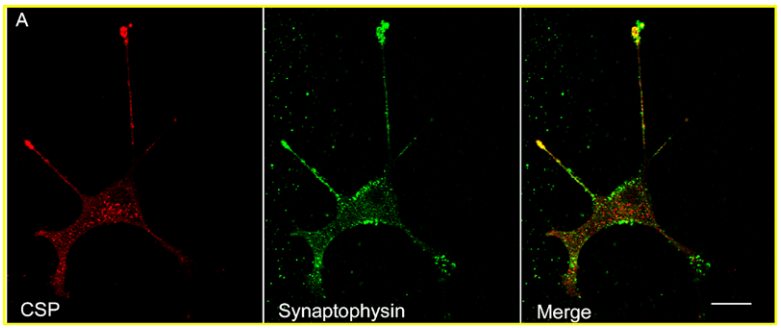

Fig. 3. Co-localization of CSPα and synaptophysin. NGF-differentiated PC12 cells were immunostained with anti-CSPα polyclonal and anti-synaptophysin monoclonal (Sigma). CSPα and synaptophysin were found to concentrate in the processes.

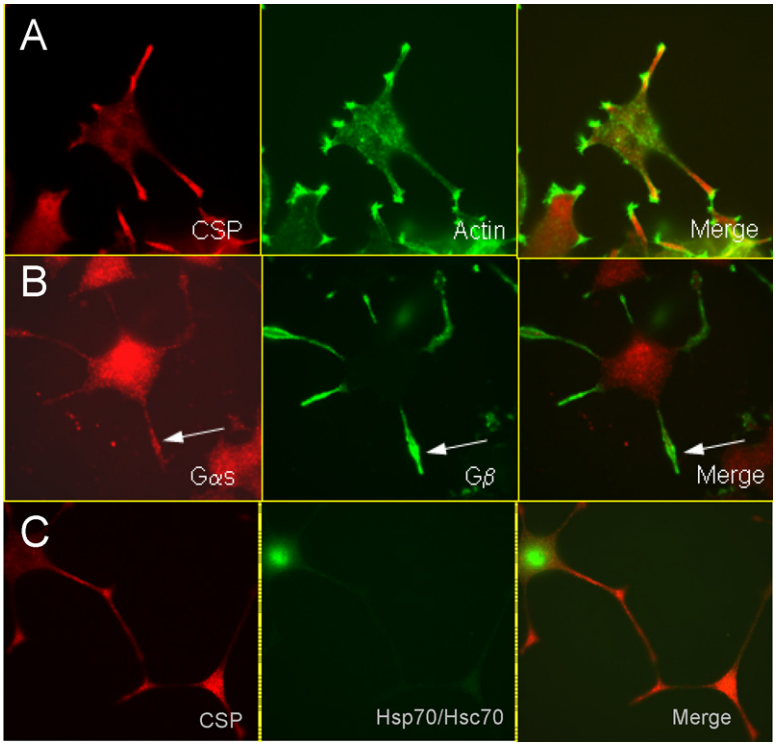

Fig. 4. Localization of actin, $G_{\alpha s}$, and Hsc70/Hsp70 in PC12 cells. NGF-differentiated PC12 cells were immunostained with (A) CSPα polyclonal and rodamine-phalloidin (Molecular Probes), (B) $G_{\alpha s}$ polyclonal (Santa Cruz) and G_{β} monoclonal (Transduction Labs) or (C) anti-CSPα polyclonal and anti-Hsp70/Hsc70 monoclonal (Sigma). CSPα was observed to have a distinct staining pattern in differentiated PC12 cells compared to actin. Hsp70/Hsc70 staining did not concentrate to the processes.

the synapse [3], nucleus [39], lipid rafts [18], and protein aggregates [31] has clearly been demonstrated. Our observations that CSPα activity is inhibited when levels of misfolded/aggregated proteins increase [41] support studies by others showing that Hsc70 is sequestered by protein aggregates [31]. In response to a range of stressful stimuli including hyperthermia and ischemia, the nervous system activates a cellular program called the heat-shock response in which the expression of chaperones, including Hsp70, is induced. Following stress, Hsp70 is rapidly and strongly induced in the nervous system and is translocated to synapses [3]. The severity of the stress required to trigger the heat shock response varies among neuronal populations [38]. A conditioning stress that is sufficient to induce Hsp70 increases neural cell survival following subsequent insults. Furthermore, small molecule modulators have been identified [9], emphasizing Hsc70/Hsp70 potential as a regulatory site. Our *in vitro* work predicts that translocation of Hsc70/Hsp70 to and from the synapse will critically impact CSPα cellular activity.

Summary

Our results suggest that CSPα's activity is regulated by the availability of Hsc70. In the presence of NGF, Hsc70 is translocated away from CSPα in PC12 cell processes. Other reports have documented translocation of Hsc70/Hsp70 to the synapse where it would be expected to acti-

vate CSPα. While G_β is found to be co-localized with CSPα in PC12 cells, only a specific pool of $G_{\alpha s}$ co-localizes with CSPα suggesting that CSPα/G_α association also has cellular regulatory features. Finally, we demonstrate after introduction of CSPα into a cell, its association with the vesicle membranes and palmitoylation does not proceed at the same rate as myc-CSPα expression. Given that CSPα is tethered to secretory vesicles by this process, palmitoylation could well be an important regulatory process for CSPα action.

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References

[1] G. AhnertHilger, T. Schafer, K. Spicher, C. Grund, G. Schultz, B. Wiedenmann, Detection of G-protein heterotrimers on large dense core and small synaptic vesicles of neuroendocrine and neuronal cells, Eur. J. Cell Biol. 65 (1994) 26–38.

[2] J.W. Barclay, H.L. Atwood, R.M. Robertson, Impairment of central pattern generation in *Drosophila* cysteine string protein mutants, J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 188 (2002) 71–78.

[3] D.A. Bechtold, S.J. Rush, I.R. Brown, Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain, J. Neurochem. 74 (2000) 641–646.

[4] F. Blondeau, B. Ritter, P.D. Allaire, S. Wasiak, M. Girard, N.K. Hussain, A. Angers, V. Legendre-Guillemin, L. Roy, D. Boismenu, R.E. Kearney, A.W. Bell, J.J. Bergeron, P.S. McPherson, Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling, Proc. Natl. Acad. Sci. USA 101 (2004) 3833–3838.

[5] J.F. Bowyer, D.L. Davies, Changes in mRNA levels for heat-shock/stress proteins (Hsp) and a secretory vesicle associated cysteine-string protein (Csp1) after amphetamine (AMPH) exposure, Ann. NY Acad. Sci. 890 (1999) 314–329.

[6] J.E. Braun, B.E. Fritz, S.M.E. Wong, A.W. Lowe, Identification of a vesicle-associated membrane protein(VAMP)-like membrane protein in zymogen granules of the rat exocrine pancreas, J. Biol. Chem. 269 (1994) 5328–5335.

[7] J.E. Braun, R.H. Scheller, Cysteine string protein, a DnaJ family member, is present on diverse secretory vesicles, Neuropharmacology 34 (1995) 1361–1369.

[8] J.E. Braun, S.M. Wilbanks, R.H. Scheller, The cysteine string secretory vesicle protein activates Hsc70 ATPase, J. Biol. Chem. 271 (1996) 25989–25993.

[9] J.L. Brodsky, G. Chiosis, Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators, Curr. Top. Med. Chem. 6 (2006) 1215–1225.

[10] H. Brown, O. Larsson, R. Branstrom, S. Yang, B. Leibiger, I. Leibiger, G. Fried, T. Moede, J.T. Deeney, G.R. Brown, G. Jacobsson, C.J. Rhodes, J.E. Braun, R.H. Scheller, B.E. Corkey, P. Berggren, B. Meister, Cysteine string protein (CSP) is an insulin secretory granule-associated protein regulating beta-cell exocytosis, EMBO J. 17 (1998) 5048–5058.

[11] L.H. Chamberlain, R.D. Burgoyne, Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein, Biochem. J. 322 (Pt 3) (1997) 853–858.

[12] L.H. Chamberlain, R.D. Burgoyne, Cysteine string protein functions directly in regulated exocytosis, Mol. Cell Biol. 9 (1998) 2259–2267.

[13] L.H. Chamberlain, R.D. Burgoyne, The cysteine-string domain of the secretory vesicle cysteine-string protein is required for membrane targeting, Biochem. J. 335 (Pt 2) (1998) 205–209.

[14] L.H. Chamberlain, R.D. Burgoyne, Cysteine string protein: the chaperone at the synapse, J. Neurochem. 74 (2000) 1781–1789.

[15] L.H. Chamberlain, J. Henry, R.D. Burgoyne, Cysteine string proteins are associated with chromaffin granules, J. Biol. Chem. 271 (1996) 19514–19517.

[16] S. Chandra, G. Gallardo, R. Fernandez-Chacon, O.M. Schluter, T.C. Sudhof, Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration, Cell 123 (2005) 383–396.

[17] M.E. Cheetham, A.J. Caplan, Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function, Cell Stress Chaperon. 3 (1998) 28–36.

[18] S. Chen, D. Bawa, S. Besshoh, J.W. Gurd, I.R. Brown, Association of heat shock proteins and neuronal membrane components with lipid rafts from the rat brain, J. Neurosci. Res. 81 (2005) 522–529.

[19] S. Chen, X. Zheng, K.L. Schulze, T. Morris, H. Bellen, E.F. Stanley, Enhancement of presynaptic calcium current by cysteine string protein, J. Physiol. 538 (2002) 383–389.

[20] M.L. Cordeiro, C.B. Gundersen, J.A. Umbach, Dietary lithium induces regional increases of mRNA encoding cysteine string protein in rat brain, J. Neurosci. Res. 73 (2003) 865–869.

[21] M.L. Cordeiro, C.B. Gundersen, J.A. Umbach, Convergent effects of lithium and valproate on the expression of proteins associated with large dense core vesicles in NGF-differentiated PC12 cells, Neuropsychopharmacology 29 (2004) 39–44.

[22] M.L. Cordeiro, J.A. Umbach, C.B. Gundersen, Lithium ions enhance cysteine string protein gene expression in vivo and in vitro, J. Neurochem. 74 (2000) 2365–2372.

[23] M.L. Cordeiro, J.A. Umbach, C.B. Gundersen, Lithium ions up-regulate mRNAs encoding dense-core vesicle proteins in nerve growth factor-differentiated PC12 cells, J. Neurochem. (2000) 2622–2625.

[24] E.A. Craig, P. Huang, R. Aron, A. Andrew, The diverse roles of J-proteins, the obligate Hsp70 co-chaperone, Rev. Physiol. Biochem. Pharmacol. 156 (2006) 1–21.

[25] K. Dawson-Scully, P. Bronk, H.L. Atwood, K.E. Zinsmaier, Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in *Drosophila*, J. Neurosci. 20 (2000) 6039–6047.

[26] G.J. Evans, A. Morgan, Phosphorylation of cysteine string protein in the brain: developmental, regional and synaptic specificity, Eur. J. Neurosci. 21 (2005) 2671–2680.

[27] R. Fernandez-Chacon, M. Wolfel, H. Nishimune, L. Tabares, F. Schmitz, M. Castellano-Munoz, C. Rosenmund, M.L. Montesinos, J.R. Sanes, R. Schneggenburger, T.C. Sudhof, The synaptic vesicle protein CSP alpha prevents presynaptic degeneration, Neuron 42 (2004) 237–251.

[28] M.E. Graham, R.D. Burgoyne, Comparison of cysteine string protein (CSP) and mutant alpha-SNAP overexpression reveals a role for CSP in late steps of membrane fusion in dense-core granule exocytosis in adrenal chromaffin cells, J. Neurosci. 20 (2000) 1281–1289.

[29] J. Greaves, L.H. Chamberlain, Dual role of the cysteine-string domain in membrane binding and palmitoylation-dependent sorting of the molecular chaperone cysteine-string protein, Mol. Biol. Cell (2006).

[30] C.B. Gundersen, J.A. Umbach, Suppression cloning of the cDNA for a candidate subunit of a presynaptic calcium channel, Neuron 9 (1992) 527–537.

[31] N.R. Jana, M. Tanaka, G. Wang, N. Nukina, Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity, Hum. Mol. Genet. 9 (2000) 2009–2018.

[32] C. Klattenhoff, M. Montecino, X. Soto, L. Guzman, X. Romo, M.A. Garcia, B. Mellstrom, J.R. Naranjo, M.V. Hinrichs, J. Olate, Human brain synembryn interacts with Gsalpha and Gqalpha and is translocated to the plasma membrane in response to isoproterenol and carbachol, J. Cell Physiol. 195 (2003) 151–157.
[33] S.A. Kohan, M. Pescatori, N.C. Brecha, A. Mastrogiacomo, J.A. Umbach, C.B. Gundersen, Cysteine string protein immunoreactivity in the nervous system and adrenal gland of rat, J. Neurosci. 15 (1995) 6230–6238.
[34] R.J. Konrad, R.A. Young, R.M. Smith, L. Jarett, B.A. Wolf, The heterotrimeric G-protein Gi Is localized to the insulin secretory granules of beta-cells and is involved in insulin exocytosis, FASEB J. 9 (1995) A1411.
[35] D.W. Lee, X. Wu, E. Eisenberg, L.E. Greene, Recruitment dynamics of GAK and auxilin to clathrin-coated pits during endocytosis, J. Cell Sci. 119 (2006) 3502–3512.
[36] J.M. Magga, S.E. Jarvis, M.I. Arnot, G.W. Zamponi, J.E. Braun, Cysteine string protein regulates G-protein modulation of N-type calcium channels, Neuron 28 (2000) 195–204.
[37] J.M. Magga, J.G. Kay, A. Davy, N.P. Poulton, S.M. Robbins, J.E.A. Braun, ATP dependence of the SNARE/caveolin1 interaction in the hippocampus, Biochem. Biophys. Res. Commun. 291 (2002) 1232–1238.
[38] P. Manzerra, I.R. Brown, The neuronal stress response: nuclear translocation of heat shock proteins as an indicator of hyperthermic stress, Exp. Cell Res. 229 (1996) 35–47.
[39] P. Manzerra, S.J. Rush, I.R. Brown, Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit, J. Cell Physiol. 170 (1997) 130–137.
[40] A. Mastrogiacomo, S.M. Parsons, G.A. Zampighi, D.J. Jenden, J.A. Umbach, C.B. Gundersen, Cysteine string proteins: a potential link between synaptic vesicles and presynaptic Ca^{2+}channels, Science 263 (1994) 981–982.
[41] L.C. Miller, L.A. Swayne, L. Chen, Z.P. Feng, J.L. Wacker, P.J. Muchowski, G.W. Zamponi, J.E.A. Braun, Cysteine string protein (CSP) inhibition of N-type calcium channels is blocked by mutant huntingtin, J. Biol. Chem. 278 (2003) 53072–53081.
[42] L.C. Miller, L.A. Swayne, J.G. Kay, Z.P. Feng, S.E. Jarvis, G.W. Zamponi, J.E.A. Braun, Molecular determinants of cysteine string protien modulation of n-type calcium channels, J. Cell Sci. 116 (2003) 2967–2974.
[43] M. Morales, A. Ferrus, M. Martinez-Padron, Presynaptic calcium-channel currents in normal and csp mutant *Drosophila* peptidergic terminals, Eur. J. Neurosci. 11 (1999) 1818–1826.
[44] J.R. Morgan, K. Prasad, S. Jin, G.J. Augustine, E.M. Lafer, Uncoating of clathrin-coated vesicles in presynaptic terminals: roles for Hsc70 and auxilin, Neuron 32 (2001) 289–300.
[45] M. Natochin, T.N. Campbell, B. Barren, L.C. Miller, S. Hameed, N.O. Artemyev, J.E. Braun, Characterization of the G alpha(s) regulator cysteine string protein, J. Biol. Chem. 280 (2005) 30236–30241.
[46] R. Ranjan, P. Bronk, K.E. Zinsmaier, Cysteine string protein is required for calcium secretion coupling of evoked neurotransmission in *Drosophila* but not for vesicle recycling, J. Neurosci. 18 (1998) 956–964.
[47] F. Schmitz, L. Tabares, D. Khimich, N. Strenzke, P. de la Villa-Polo, M. Castellano-Munoz, A. Bulankina, T. Moser, R. Fernandez-Chacon, T.C. Sudhof, CSPalpha-deficiency causes massive and rapid photoreceptor degeneration, Proc. Natl. Acad. Sci. USA 103 (2006) 2926–2931.
[48] B. Stahl, S. Tobaben, T.C. Sudhof, Two distinct domains in hsc70 are essential for the interaction with the synaptic vesicle cysteine string protein, Eur. J. Cell. Biol. 78 (1999) 375–381.
[49] S. Tobaben, P. Thakur, R. Fernandez-Chacon, T.C. Sudhof, J. Rettig, B. Stahl, A trimeric protein complex functions as a synaptic chaperone machine, Neuron 31 (2001) 987–999.
[50] J.A. Umbach, M. Saitoe, Y. Kidokoro, C.B. Gundersen, Attenuated influx of calcium ions at nerve endings of csp and shibire mutant *Drosophila*, J. Neurosci. 18 (1998) 3233–3240.
[51] J.A. Umbach, K.E. Zinsmaier, K.K. Eberle, E. Buchner, S. Benzer, C.B. Gundersen, Presynaptic dysfunction in *Drosophila* csp mutants, Neuron 13 (1994) 899–907.
[52] E. Ungewickell, H. Ungewickell, S.E.H. Holstein, R. Linder, K. Prasad, W. Barouch, B. Martin, L.E. Greene, E. Eisenberg, Role of auxilin in uncoating clathrin-coated vesicles, Nature 378 (1995) 632–635.
[53] G.J. van de, R.B. Kelly, Association of *Drosophila* cysteine string proteins with membranes, FEBS Lett. 380 (1996) 251–256.
[54] N. Vitale, M. Gensse, S. Chasserot-Golaz, D. Aunis, M.F. Bader, Trimeric G proteins control regulated exocytosis in bovine chromaffin cells: Sequential involvement of Go associated with secretory granules and Gi(3) bound to the plasma membrane, Eur. J. Neurosci. 8 (1996) 1275–1285.
[55] P. Walsh, D. Bursac, Y.C. Law, D. Cyr, T. Lithgow, The J-protein family: modulating protein assembly, disassembly and translocation, EMBO Rep. 5 (2004) 567–571.
[56] M. Yamada, S. Yamazaki, K. Takakashi, K. Nara, H. ozawa, s. Yamada, Y. kiuchi, K. Oguchi, K. Kamijima, T. Higuchi, K. Momose, Induction of cysteine string protein after chronic antidepressant treatment in rat frontal cortex, Neurosci. Lett. 301 (2001) 183–186.
[57] H. Zhang, W.L. Kelley, L.H. Chamberlain, R.D. Burgoyne, J. Lang, Mutational analysis of cysteine-string protein function in insulin exocytosis, J. Cell Sci. 112 (Pt 9) (1999) 1345–1351.
[58] H. Zhang, W.L. Kelley, L.H. Chamberlain, R.D. Burgoyne, C.B. Wollheim, J. Lang, Cysteine-string proteins regulate exocytosis of insulin independent from transmembrane ion fluxes, FEBS Lett. 437 (1998) 267–272.
[59] W. Zhang, A. Khan, C.G. Ostenson, P.O. Berggren, S. Efendic, B. Meister, Down-regulated expression of exocytotic proteins in pancreatic islets of diabetic GK rats, Biochem. Biophys. Res. Commun. 291 (2002) 1038–1044.
[60] C.M. Zhao, G. Jacobsson, D. Chen, R. Hakanson, B. Meister, Exocytotic proteins in enterochromaffin-like (ECL) cells of the rat stomach, Cell Tissue Res. 290 (1997) 539–551.
[61] K.E. Zinsmaier, P. Bronk, Molecular chaperones and the regulation of neurotransmitter exocytosis, Biochem. Pharmacol. 62 (2001) 1–11.
[62] K.E. Zinsmaier, K.K. Eberle, E. Buchner, N. Walter, S. Benzer, Paralysis and early death in cysteine string protein mutants of *Drosophila*, Science 263 (1994) 977–980.