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Palmitoylation and Testis-Enriched Expression of the Cysteine-String Protein β Isoform[†]

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ABSTRACT: Cysteine-string protein α (CSPα) is a DnaJ chaperone that is associated with secretory vesicles in diverse cell types. The cysteine-string region is the signature domain of CSPα and contains 14 closely spaced cysteines, the majority of which are palmitoylated; this post-translational modification mediates stable membrane attachment. CSPα has been proposed to function in regulated exocytosis pathways throughout the body and has an additional neuroprotective function. Two novel CSP isoforms, β and γ, were identified recently, although the expression profile, properties, and functions of these proteins are not clear and in some cases are subject to debate. Here, we report that CSPβ is enriched in rat testis and was not detected in any other tissue that was examined, including brain. Although the cysteine-string domain of CSPβ is distinct from that found in CSPα, the endogenous β isoform expressed in testis is membrane-associated and palmitoylated. However, in agreement with earlier work, we find that the palmitoylation efficiency of CSPβ is reduced compared with that of CSPα. Subsequent analysis of chimeric proteins reveals that regions upstream of the cysteine-string domains of CSPα and CSPβ underlie this difference in palmitoylation efficiency between the two isoforms.

Cysteine-string protein α (CSPα) is a DnaJ chaperone expressed throughout the body (*1*, *2*). The defining feature of CSPα is its cysteine-string domain, which contains 14 cysteines in a span of 24 amino acids; the majority of these cysteines are thought to be palmitoylated (*3*, *4*). CSPα is associated with synaptic vesicles in neurons and secretory vesicles/granules in a range of non-neuronal cell types (*5*−*9*). This localization is consistent with a function in some aspect of vesicle dynamics, such as exocytosis or endocytosis. Indeed, *Drosophila* CSP[1] null mutants are embryonic lethal, and larvae display a defect in evoked presynaptic exocytosis (*10*, *11*). Interestingly, this defect is enhanced at elevated temperatures, suggesting that components of the exocytosis machinery are heat-sensitive in the absence of CSP expression. This observation, coupled with the reported interaction of CSP with the cochaperone HSC70 (*7*, *12*), has generally been proposed to indicate a chaperone function for CSP, regulating the conformational status and folding of one or more exocytotic proteins (*13*). This model is supported by in vitro studies demonstrating that mammalian CSPα together with HSC70 and SGT1 prevents aggregation of model denatured proteins and mediates their refolding (*12*, *14*). However, the in vivo substrates of CSP are not known, but possible candidates include syntaxin (*15*, *16*), VAMP (*17*, *18*), SNAP25 (*19*), synaptotagmin (*20*), and voltage-gated Ca^{2+} channels (*17*, *21*). CSPα has also been implicated in regulated exocytosis pathways in mammalian cells, including PC12 cells (*22*), adrenal medullary chromaffin cells (*23*), and pancreatic β cells (*9*, *24*). In all these cell types, overexpression of CSPα affected the extent of exocytosis. In addition, amperometric analysis suggested that CSPα overexpression also affects the kinetics of secretory vesicle fusion (*23*).

Recently, CSPα null mice were generated (*19*). These mutant mice were apparently normal until ∼2 weeks after birth, at which point they suffered from progressive weakness and ceased gaining weight. The CSPα null mice had a dramatically reduced life span, with none surviving beyond 3 months of age. Intriguingly, neuromuscular junctions and synapses of the CSPα null mice showed marked neurodegenerative changes, suggesting that this protein has an important neuroprotective function. However, no defect in synaptic vesicle exocytosis was apparent in Calyx of Held synapses from young mice (P9−P11), suggesting that CSPα function is not essential for this fusion pathway in mice.

Two novel isoforms of CSP, CSPβ and CSPγ, are also expressed in mammals (*19*). One explanation for the lack of an effect on synaptic exocytosis in CSPα knockout mice is the functional redundancy between one or both of these novel CSP isoforms and CSPα. This possibility was ruled out as CSPβ and CSPγ mRNA were reportedly detected only in testis (*19*). However, a recent report suggested that CSPβ protein is expressed in brain where it is present as an ∼100 kDa protein complex that is resistant to SDS denaturation (*25*). As CSPβ also interacts with HSC70 (*26*), this report raises the possibility of functional redundancy between CSPα and CSPβ in brain. However, in pancreatic β cells, although overexpressed CSPβ is membrane-associated, it is largely unpalmitoylated and colocalizes with markers of the trans Golgi network (*26*). Thus, even if CSPβ is expressed in brain, it is unclear whether this protein could compensate for a loss of CSPα expression given the reported different localizations and palmitoylation status of these two CSP isoforms.

In light of these uncertainties about CSPβ expression and palmitoylation, we report on the expression profile of CSPβ using a novel CSPβ-specific antibody. In addition, we have also investigated the palmitoylation status of endogenous CSPβ and generated CSPα−CSPβ chimera to identify regions of these

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[1]Abbreviations: CSP, cysteine-string protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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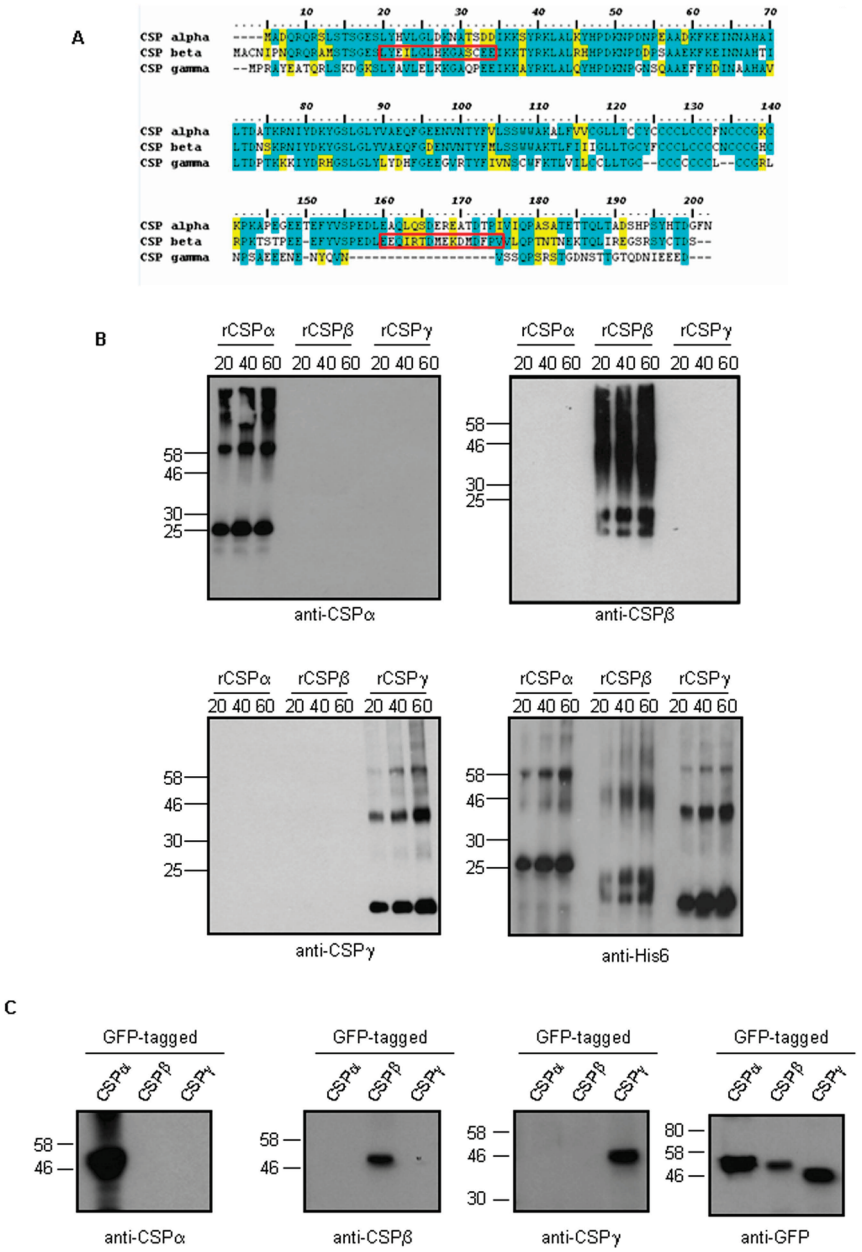

FIGURE 1: Analysis of CSP isoform-specific antibodies. (A) Alignment of the sequences of rat CSPα, -β, and -γ. Identical residues are highlighted in blue and similar residues in yellow. The peptides used to generate a CSPβ antibody are outlined with red boxes. (B) Aliquots of His_6-tagged recombinant CSP (rCSP) proteins (20, 40, and 60 ng) were resolved by SDS−PAGE and probed with antibodies against CSPα, CSPβ, CSPγ, and the hexahistidine tags. (C) HEK293T cells were transfected with 1 μg of GFP-tagged CSPs and probed with antibodies against the CSP isoforms or against GFP. The GFP−CSPα fusion protein migrates between the 46 and 58 kDa markers as previously reported (*4*). Positions of molecular mass standards are shown on all panels.

proteins that contribute to the reported difference in palmitoylation efficiency.

MATERIALS AND METHODS

Commercial Antibodies. A rabbit antibody against CSPα was purchased from Stressgen (Victoria, BC) and was used at a 1:1000 dilution for immunoblotting. The SNAP23 (rabbit) antibody was from Synaptic Systems (Goettingen, Germany) and was used at a 1:1000 dilution for immunoblotting. A monoclonal anti-GFP antibody (JL8) was supplied by Clontech and used at a 1:5000 dilution for immunoblotting. A monoclonal HSP70 antibody and a syntaxin 1 antibody (HPC1) were provided by Sigma (Poole, U.K.) and used at 1:5000 and 1:1000 dilutions, respectively, for immunoblotting.

Generation of CSPβ and CSPγ Antibodies. A rabbit affinity-purified antibody recognizing CSPβ was generated by Eurogentec (Seraing, Belgium) using a double peptide strategy. The peptide sequences used for immunization corresponded to the following sequences from rat CSPβ: LYEILGLHKGASCEE and EQIRTDMEKDMDFPV (highlighted in red boxes in Figure 1A). The preparation used throughout this work was affinity purified against the EQIRTDMEKDMDFPV peptide. A rabbit CSPγ antiserum was produced by immunizing rabbits with full-length His_6-tagged rat CSPγ. The CSPβ and CSPγ antibodies were used at a 1:1000 dilution for immunoblotting.

DNA Cloning. Rat testis total RNA was purified using an RNeasy kit (Qiagen) and used as the template to amplify CSPβ and CSPγ. For cloning into pQE30, the primers included flanking *Bam*HI (5′) and *Hin*dIII (3′) restriction sites to facilitate

directional cloning and lacked the initiating methionine ATG coding sequence of CSPβ and -γ. pQE30-CSPα has been previously described (*1*).

For expression in mammalian cells, CSPβ was amplified from testis cDNA with flanking *Hin*dIII (5′) and *Bam*HI (3′) sites incorporated for directional cloning into pEGFPC2 (Clontech). CSPα in pEGFPC2 has been previously described (*4*).

To replace the N-terminal domain of CSPβ with that from CSPα, an initial round of site-directed mutagenesis was used to insert *Eco*RI sites immediately upstream from the first cysteines of the cysteine-string domains of both proteins. The N-terminal domains of CSPα and CSPβ were then excised using *Hin*dIII and *Eco*RI and ligated into the corresponding DNA backbone. A final round of site-directed mutagenesis was used to remove the *Eco*RI restriction sites.

The sequences of all constructs were verified by sequencing (University of Dundee DNA Sequencing Service, Dundee, U.K.).

PC12 Cell Culture, Transfection, and Fractionation. PC12 cells were grown in RPMI1640 medium containing 10% horse serum and 5% fetal calf serum. Cells were plated on poly-D-lysine-coated 24-well plates and transfected the following day with 1 μg of the appropriate plasmid using Lipofectamine 2000 (Invitrogen). Two days later, the cells were fractionated into cytosol and membrane fractions using an SPEK kit (Merck) as previously described (*27*, *28*).

Tissue Lysates and Fractionation. Tissues were extracted from adult Sprague-Dawley rats. For the preparation of lysates, the tissues were homogenized in ice-cold lysis buffer [20 mM Hepes, 1 mM $MgCl_2$, 250 mM sucrose, 2 mM EDTA, 1% NP40, and protease inhibitor cocktail (Roche) (pH 7.4)]. The lysate was centrifuged at 14000*g* for 3 min to remove insoluble material, which was then subjected to a repeat round of homogenization in fresh lysis buffer and centrifuged again. The solubilized supernatant fractions obtained were pooled, and the protein content was determined. Twenty micrograms of each tissue lysate was resolved by SDS−PAGE for immunoblotting analysis.

Brain Region Lysates. Lysates from various brain regions taken from 10-week-old Sprague-Dawley rats were supplied by Zyagen (San Diego, CA).

Testis Fractionation. Testis was homogenized in a Dounce homogenizer in ice-cold buffer containing 20 mM Tris, 100 mM KCl, 250 mM sucrose, 2 mM $MgCl_2$, 1 mM DTT (pH 8), and protease inhibitors. The sample was separated into cytosol (supernatant) and membrane (pellet) fractions by centrifugation at 196000*g* for 30 min. Equal volumes of the recovered cytosol and membrane fractions were analyzed by immunoblotting. For chemical depalmitoylation, 1 M hydroxylamine (pH 7) or 1 M Tris (pH 7, control) was added to the membrane fractions and incubated at room temperature overnight in the presence of protease inhibitors.

RESULTS

A rabbit antiserum was generated following immunization with the peptide sequences from CSPβ highlighted in the boxed regions of Figure 1A. The downstream peptide (EQIRTDMEKDMDFPV) shown in Figure 1A was then used for affinity purification. As shown in Figure 1B, this antibody specifically recognized bacterially produced recombinant His_6-tagged CSPβ and was not reactive against either His_6-CSPα or His_6-CSPγ. In addition, an antiserum was generated against full-length His_6-CSPγ, which was specific for this CSP isoform, and the commercial CSPα antibody used in this study was also highly specific (Figure 1B). An antibody against the hexahistidine tag was used to show the similar loading of the His_6-tagged CSP isoforms. The multiple bands that were detected by all antibodies used to probe bacterially expressed proteins (Figure 1B) represent dimers and higher-molecular mass oligomers of CSPs that have been extensively reported and characterized (see, for example, ref *29*).

The antibody specificity observed against bacterially expressed proteins was also reproduced when the antibodies were used to probe lysates of HEK293T cells that had been transfected with EGFP-tagged CSP proteins (Figure 1C). In this case, the GFP antibody was used to confirm similar loadings.

The CSPγ antibody recognized multiple bands when used to probe tissue lysates (not shown), and we were unable to ascertain the expression profile of CSPγ; this antibody was not used further.

To examine the tissue distribution of CSPβ protein, lysates were prepared from a panel of rat tissues and probed with CSPα and CSPβ antibodies. Figure 2A confirms previous work showing that CSPα is enriched in brain but also expressed at varying levels in other non-neuronal tissues (*1*, *2*). In contrast, immunoreactivity against CSPβ was only detected in testis (Figure 2A). CSPβ in testis migrated as two bands on SDS gels, one which was the same size as recombinant CSPβ (arrow) and another more prominent higher-molecular mass band (arrowhead). SNAP23 is ubiquitously expressed, and an antibody against this protein confirmed protein loading (Figure 2A). An antibody against the neuron-specific SNARE protein syntaxin 1 (HPC-1) was used to confirm the integrity of the brain lysate (Figure 2A). We also screened a range of common cell lines for CSPβ expression but did not detect immunoreactivity (Figure 2B).

We next set out to determine the properties of CSPβ in testis. Figure 2C shows that the upper band detected by the CSPβ antibody (arrowhead in Figure 2A) cofractionates with membranes whereas the lower band (arrow in Figure 2A) is cytosolic. Antibodies against GAPDH and HSP70, proteins that are largely cytosolic, were used as controls to assess the efficiency of the fractionation procedure (Figure 2C). Given that CSPα is extensively palmitoylated (*3*), an obvious possibility for explaining the increased apparent molecular mass of membrane-associated CSPβ is that this protein is also highly palmitoylated. To test this possibility, membranes were isolated from testis and incubated overnight in either 1 M hydroxylamime (pH 7) to cleave thioester linkages between fatty acids and cysteines or 1 M Tris (pH 7) as a control. Figure 2D shows that hydroxylamine treatment decreased the apparent molecular mass of CSPα as previously reported (*3*). In addition, hydroxylamine caused a marked downward shift in the migration of CSPβ but had no effect on the migration of a nonpalmitoylated protein, GAPDH (Figure 2D). Thus, panels C and D of Figure 2 reveal that CSPβ is membrane-associated in testis and palmitoylated.

A higher-molecular mass band was recognized by the CSPβ antibody (above the 25 kDa marker) in all tissues and is visible in the lung, adrenal, and spleen samples shown in Figure 2A. It was possible that this band represented a differentially palmitoylated form of CSPβ. If this were the case, then this band would be predicted to be membrane-associated. However, fractionation of spleen revealed that this band was entirely cytosolic (Figure 2E), strongly suggesting that it is not a palmitoylated form of CSPβ.

It is possible that CSPβ is expressed at low levels in specific brain region(s) (*30*) and that a signal might be detectable following enrichment of these brain areas. Thus, a panel of samples containing different brain regions isolated from Sprague-Dawley rats

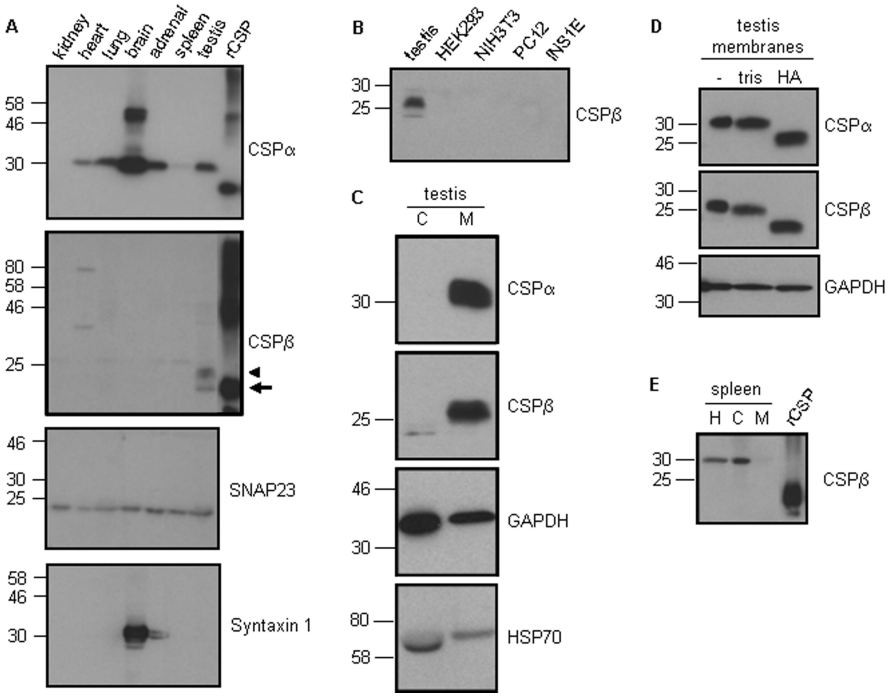

FIGURE 2: Tissue distribution and membrane binding of CSPβ. (A) Rat lysates (10 μg) were resolved by SDS−PAGE and probed with the indicated antibodies; 20 ng of recombinant His_6-CSPα or His_6-CSPβ (rCSP) were used as positive controls. (B) Lysates were prepared from the indicated cell lines, resolved by SDS−PAGE beside a testis lysate, and probed using a CSPβ antibody. (C) Rat testis was fractionated into cytosol (C) and membrane (M) fractions which were resolved by SDS−PAGE and probed with the indicated antibodies. (D) Membranes isolated from testis were untreated (−) or treated with Tris or hydroxylamine (HA), and the samples were probed with the antibodies shown. (E) Rat spleen was fractionated into cytosol (C) and membrane (M) fractions and resolved by SDS−PAGE beside a spleen homogenate (H) fraction and 20 ng of recombinant His_6-CSPβ (rCSPβ) and probed with a CSPβ antibody. The positions of molecular mass markers are shown on the left side of each panel.

was obtained from Zyagen. These samples were resolved by SDS−PAGE and probed with CSPα and CSPβ antibodies, compared with an aliquot of the appropriate His_6-tagged recombinant CSP isoform. Figure 3 shows that CSPα was expressed throughout the brain, with enrichment in the hippocampus and cerebrum. In contrast, we did not detect any signal with the CSPβ antibody, suggesting that this isoform is not expressed at levels comparable to that of CSPα in any of the brain regions tested. Note that the CSPβ antibody gave a strong signal against recombinant CSPβ, providing a positive control for the immunoblotting procedure using this antibody.

Previous work reported that when a plasmid encoding CSPβ was transfected into a pancreatic β cell line the protein was membrane-associated but only weakly palmitoylated (*26*). In agreement with the work by the Lang group, we also found that the EGFP−CSPβ fusion protein was inefficiently palmitoylated when expressed in PC12 cells and that a pool of the protein associated with membranes in an apparently unpalmitoylated form (arrowhead, Figure 4B, second panel). This is in contrast to the EGFP−CSPα fusion protein, which is almost entirely palmitoylated when expressed in PC12 cells (Figure 4B, top panel). Quantified data for membrane binding and the extent of palmitoylation of the membrane-bound pools of CSPα and CSPβ are shown in Figure 4C. Thus, although the expression levels of endogenously expressed CSPβ allow for efficient palmitoylation in testis, it is clear that CSPβ is palmitoylated less efficiently than CSPα when the proteins are overexpressed.

As palmitoylation is central to the function of CSP (*31*), we created chimeric constructs in an effort to understand the basis for the different efficiencies of CSPα and CSPβ palmitoylation in PC12 cells. Work from Lang and co-workers has shown that the different configurations of cysteines in the cysteine-string domains

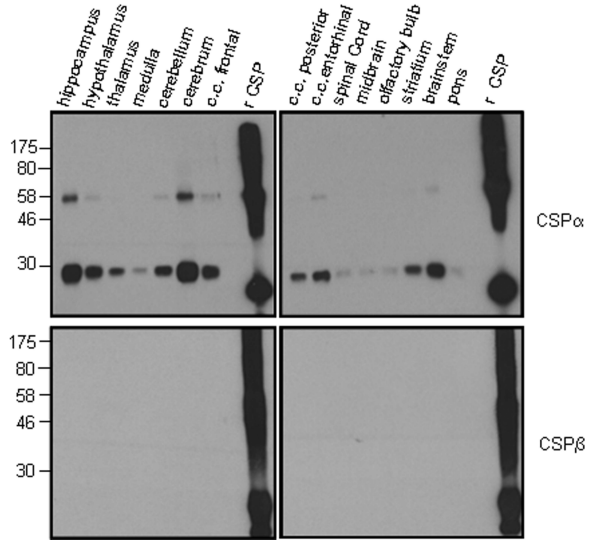

FIGURE 3: CSP expression in brain regions. Lysates from the various brain regions shown (10 μg) were resolved by SDS−PAGE beside a 20 ng aliquot of His_6-tagged CSPα or CSPβ (rCSP) and probed with antibodies against CSPα (top) or CSPβ (bottom). c.c denotes the cerebral cortex. Positions of molecular mass markers are shown at the left.

of CSPα and CSPβ are not responsible for the observed differences in palmitoylation (*26*). Thus, we generated a chimeric construct in which the N-terminal domain of CSPβ was replaced with that from CSPα (Figure 4A). This chimeric construct was transfected into PC12 cells, which were then fractionated into cytosol and membrane fractions. The representative immunoblots (Figure 4B) and quantified data from several experiments (Figure 4C) show that this chimera had an increased level of membrane binding and

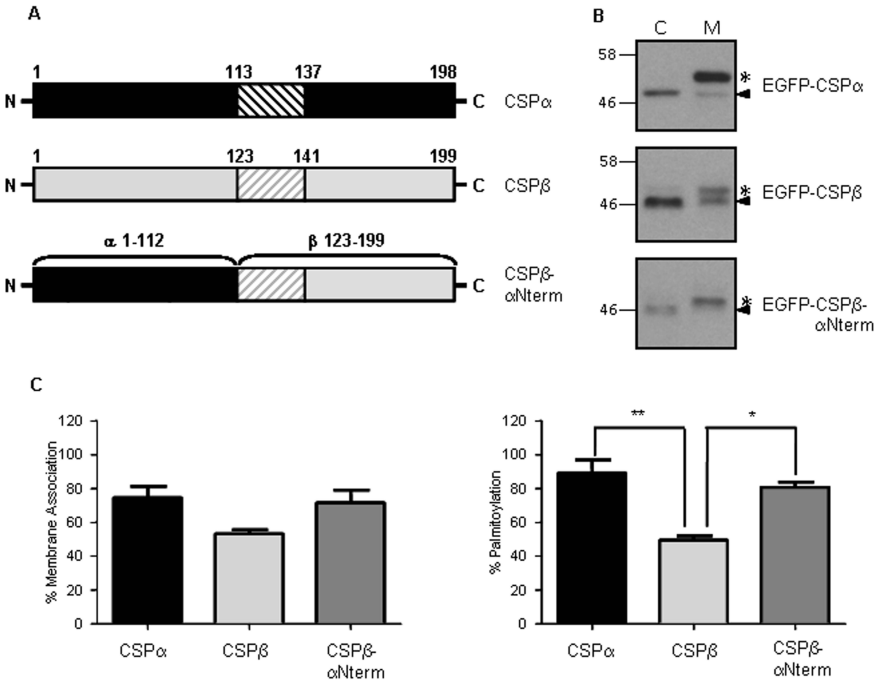

FIGURE 4: Membrane binding and palmitoylation of GFP-tagged CSPα and CSPβ and a chimeric construct. (A) Domain structure of CSPs and representation of the chimeric construct that was synthesized. The numbers indicate the position of amino acids at the boundaries of the cysteine-string domains. (B) CSP constructs were transfected into PC12 cells, which were then fractionated into cytosol (C) and membrane (M) fractions. The distribution of the proteins between cytosol and membrane fractions was determined by immunoblotting with a GFP antibody. The arrowhead denotes unpalmitoylated CSP and the asterisk palmitoylated CSP. Positions of molecular mass standards are shown at the left. (C) Quantified data ($n = 3$) for the percent membrane association (left) and the percent palmitoylation of the membrane-associated fraction (right). There were no significant differences in the extent of membrane binding for the three proteins, but palmitoylation was significantly different for CSPα and CSPβ (**$p < 0.005$) and CSPβ and CSPβ-αNterm (*$p < 0.05$) as judged by one-way ANOVA.

palmitoylation, similar to that observed for wild-type CSPα. Thus, the different N-terminal domains of CSPα and CSPβ are important in determining palmitoylation efficiency.

DISCUSSION

Our results demonstrate that CSPβ is highly enriched in rat testis compared with any other rat tissue that was examined. This finding is consistent with previous analysis of the expression profile of CSPβ mRNA (*19*). While we did not detect expression of CSPβ in any brain region tested, we cannot rule out the possibility that levels of this isoform are below the sensitivity of our antibody. However, by comparing antibody signals from known concentrations of recombinant CSPs with lysates from selected brain regions (Figure 3), we can be confident that CSPα is substantially more abundant in brain than CSPβ. Our antibody did not recognize the high-molecular mass band reported in a recent study (*25*).

In testis, CSPβ is largely membrane-associated. Furthermore, when these membranes were treated with hydroxylamine, CSPβ underwent a marked band shift, consistent with this isoform being palmitoylated in testis. This result is in apparent contrast to work suggesting that CSPβ is a nonpalmitoylated CSP isoform, a conclusion that was based on analyses of CSPβ overexpressed in pancreatic β cells (*26*). Indeed, our own work is in accordance with this study as we showed that the GFP−CSPβ fusion protein expressed in PC12 cells also exhibits a reduced efficiency of palmitoylation compared with that of CSPα. There are two main possibilities that might explain the apparent lack of CSPβ palmitoylation in β cells and PC12 cells. (i) Palmitoylation of CSPβ is less efficient than palmitoylation of CSPα, and a difference in relative palmitoylation becomes apparent when the proteins are expressed at high levels. (ii) The palmitoyl transferase that palmitoylates CSPβ is expressed at higher levels in testis than in pancreatic β cells or PC12 cells. We favor the first possibility, and indeed, palmitoylation of CSPα is also inefficient when overexpressed in certain non-neuronal cells (*27*). It is interesting to note that Lang and colleagues showed that the inefficient palmitoylation of CSPβ in β cells was most likely not caused by differences in the cysteine-string domains of CSPα and CSPβ (*26*). In support of these findings, our own study revealed that the N-terminal domains of CSPα and CSPβ were apparently responsible for the different palmitoylation efficiencies: replacement of the N-terminal domain immediately preceding the cysteine-string domain of CSPβ with the N-terminal domain of CSPα led to efficient palmitoylation in PC12 cells. This finding is in agreement with recent studies that have shown that palmitoylation of substrate proteins can be influenced by residues some way displaced from the modified cysteines (*28*).

In summary, our work is consistent with the notion that CSPβ is enriched in testis and suggests that if this isoform is expressed in brain then it is likely to be at levels substantially lower than that of CSPα. We never detected the higher-molecular mass CSPβ band reported by Gundersen and colleagues (*25*). However, as our antibody was raised against a peptide different from that for the antibody used by Gundersen et al., it is possible that antigen masking prevents us from detecting this complex. It will be interesting in future studies to examine by immunohistochemistry whether CSPβ is expressed in some minor neuronal subpopulations; our efforts using this type of approach have not been successful to date. Finally, while we have been unable to generate a useful CSPγ antibody, it will be intriguing in future studies to uncover the expression profile of the CSPγ isoform and in the longer term to uncover the function(s) of these novel CSP isoforms.

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