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Cysteine String Protein (CSP) Inhibition of N-type Calcium Channels Is Blocked by Mutant Huntingtin*

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Cysteine string protein (CSP), a 34-kDa molecular chaperone, is expressed on synaptic vesicles in neurons and on secretory vesicles in endocrine, neuroendocrine, and exocrine cells. CSP can be found in a complex with two other chaperones, the heat shock cognate protein Hsc70, and small glutamine-rich tetratricopeptide repeat domain protein (SGT). CSP function is vital in synaptic transmission; however, the precise nature of its role remains controversial. We have previously reported interactions of CSP with both heterotrimeric GTP-binding proteins (G proteins) and N-type calcium channels. These associations give rise to a tonic G protein inhibition of the channels. Here we have examined the effects of huntingtin fragments (exon 1) with (huntingtin^exon1/exp) and without (huntingtin^exon1/nonexp) expanded polyglutamine (polyQ) tracts on the CSP chaperone system. *In vitro* huntingtin^exon1/exp sequestered CSP and blocked the association of CSP with G proteins. In contrast, huntingtin^exon1/nonexp did not interact with CSP and did not alter the CSP/G protein association. Similarly, co-expression of huntingtin^exon1/exp with CSP and N-type calcium channels eliminated CSP's tonic G protein inhibition of the channels, while coexpression of huntingtin^exon1/nonexp did not alter the robust inhibition promoted by CSP. These results indicate that CSP's modulation of G protein inhibition of calcium channel activity is blocked in the presence of a huntingtin fragment with expanded polyglutamine tracts.

Molecular chaperones are best known for assisting nascent polypeptides to fold, for protecting mature proteins from stresses (such as heat shock), and for the transferring of mis-folded proteins to the proteasome. They are also important in numerous cellular pathways requiring protein conformation remodeling (*e.g.* recycling of clathrin-coated pits after endocytosis, Ref. 1). At the synapse, chaperones are important regulators of the dynamic complexes underlying neurotransmitter release and allow for the essential speed and high fidelity of the process. Interference with the normal chaperone function due to altered protein levels or activities would be expected to result in pathological consequences. Recently, chaperones have been implicated in diseases involving both the accumulation of unfolded or misfolded proteins and the degeneration of neurons, such as in Huntington's disease (2, 3).

Huntington's disease is an autosomal dominant neurodegenerative disorder caused by a mutation in the gene encoding the 350 kDa cytosolic protein huntingtin (4), which is of unknown but essential function (5). The first exon of the huntingtin gene contains a polymorphic expansion of CAG repeats that encodes a polyglutamine tract. The severity of Huntington's disease depends on the length of the glutamine repeats and is invariably terminal. In unaffected individuals the polyglutamine tract typically contains between 6 and 39 repeats compared with 36–250 repeats in patients with Huntington's disease. Huntington's disease is a member of a class of eight human polyglutamine repeat diseases that includes spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17, dentatorubral pallidoluysian atrophy, and spinobulbar muscular atrophy. Huntington's disease manifests in midlife and causes progressive motor, psychiatric, and cognitive dysfunction. Early symptoms of Huntington's include cognitive defects such as memory and information-processing deficits, mood changes, and aggressive behavior. Initially movement impairments involve shaking/dance like movements and at later stages of the disease the muscles become rigid. At autopsy, late stage brains show extensive striatal, pallidal, and cortical atrophy. The initial target of degeneration in Huntington's disease is the striatal medium spiny GABAergic neuron, and by end stages of the disease up to 95% of these neurons are lost (6). Neuronal loss is also observed in the globus pallidus, cortex, hippocampus, thalamus, and cerebellum. Given the ubiquitous distribution of huntingtin, the underlying mechanisms that elicit atrophy in GABAergic neurons and protect against mutant huntingtin-induced atrophy in other cell types is the subject of intense scrutiny.

Pathological neurodegeneration in Huntington's disease is directly correlated with the expansion of CAG triplets encoding polyglutamine repeats. Expansion of the polyglutamine tract beyond a critical threshold results in the formation of huntingtin inclusion bodies, one of the neuropathological hallmarks of Huntington's disease. While chaperones have been shown to protect against neurodegeneration by inhibiting the early

stages of aggregation (7), the extensive aggregation associated with disease progression is expected to eventually deplete chaperone availability. Exhaustion of molecular chaperones would leave the native targets of chaperones vulnerable to misfolding and result in loss of function.

CSP[1] is a 34-kDa protein present on synaptic vesicles (8) in neurons and on secretory vesicles in exocrine (9), endocrine (10), and neuroendocrine cells (11). It has been proposed to function in association with Hsc70 and SGT (small glutamine-rich tetratricopeptide repeat domain protein) as a trimeric chaperone machine (12, 13). CSP derives its name from a centrally located cysteine string region, which in vertebrates contains 14 cysteine residues, most of which are palmitoylated. CSP contains a J domain, which is a 70-amino acid region of homology shared by DnaJ (a well characterized bacterial chaperone) and many otherwise unrelated eukaryotic proteins (14). The J domain of CSP interacts with and activates the ATPase activity of members of the heat-shock family Hsp70 (9, 15, 16). In 1994, Zinsmaier *et al.* (17) demonstrated that CSP plays a significant role in neurotransmitter release. The deletion of CSP in *Drosophila* was semi-lethal and temperature-dependent. Only 4% of null mutants developed into adulthood at 25 °C, and none survived at 29 °C.

We have recently shown that CSP is capable of binding to both the N-type calcium channel and to $G\beta\gamma$ *in vitro*, and that the interaction between CSP and the N-type calcium channel results in a robust tonic inhibition of channel activity by G protein $\beta\gamma$ subunits (18, 19). Given that proteins with expanded polyglutamine repeats have been proposed to interfere with the chaperone balance of the cell, we have analyzed the effects of huntingtin on CSP modulation of N-type channels. In this study we begin to address the hypothesis that cysteine string protein (CSP) dysfunction might contribute to defects in synaptic transmission or plasticity observed in Huntington's disease. As a first step toward testing this hypothesis, we have examined the chaperone activity of the secretory vesicle chaperone CSP in the presence of huntingtin$^{exon1/exp}$ and huntingtin$^{exon1/nonexp}$. Our findings demonstrate that mutant huntingtin with an expanded polyglutamine region sequesters CSP and blocks CSP inhibition of N-type channels.

EXPERIMENTAL PROCEDURES

Preparation of Rat Hippocampal Homogenate—Rat hippocampi were hand homogenized with a teflon coated homogenizer in 0.32 M sucrose, 10 mM HEPES KOH (pH 7.0), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM PMSF, protease inhibitor mixture (Roche Applied Science), 1 μM microcystin, 1 μM okadaic acid, and 1 mM sodium orthovanadate (2 ml/hippocampus). The homogenate was centrifuged for 10 min at $500 \times g$, and the supernatant collected and subsequently centrifuged for 20 min at $20,000 \times g$ (4 °C). The pellet, containing the synaptic proteins was resuspended in 1% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, and 0.5 mM PMSF, protease inhibitor mixture (Roche Applied Science), 1 μM microcystin, 1 μM okadaic acid, 1 mM sodium orthovanadate, and incubated for 30 min at 37 °C. Next the homogenate was centrifuged at $1000 \times g$ for 5 min, and the pellet was discarded. The resulting supernatant is a crude hippocampal homogenate that contains synaptic proteins. Protein concentrations were determined by Bio-Rad Protein Assay using bovine serum albumin as the standard. All procedures were carried out in strict accordance with a protocol approved by the University of Calgary Animal Care Committee.

Preparation of Fusion Proteins—Glutathione *S*-transferase (GST) fusion proteins of CSP and CSP deletion mutants were prepared as described previously (9, 12, 19). The SGT construct (α-SGT) was prepared by subcloning SGT PCR fragments into pGEX-KG (20) and was expressed as a GST fusion protein in AB1899 cells. Myc-tagged hun-

tingtin pGEX-HDQ53 and pGEX-HDQ20 fusion protein constructs were prepared as described previously (7) and resulted in the expression of GST followed by the PreScission protease cleavage site (LEV-LFQGP), nine vector-derived residues (LGSPEFIMC), a Myc epitope (EQKLISEEDL) and exon 1 of the human huntingtin gene containing 53 or 20 glutamines, respectively. The sequences of all constructs were verified. pGEX-HD53Q and pGEX-HDQ20 were transformed into SURE cells and expression was induced with 100 μM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 28 °C. All other proteins were induced with 100 μM IPTG for 4 h at 37 °C. The bacteria were suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$) supplemented with 0.05% Tween 20, 2 mM EDTA, and 0.1% β-mercaptoethanol and lysed by two passages through a French Press (Spectronic Instruments Inc.). The fusion proteins were recovered by binding to glutathione-agarose beads (Sigma). The fusion protein beads were washed extensively and finally resuspended in 0.2% Triton X-100, 20 mM MOPS, pH 7.0, 4.5 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, and 0.5 mM PMSF. Recombinant CSP was purified from the agarose beads by cleavage with 0.2 μM thrombin in 50 mM Tris, pH 8, 150 mM NaCl, 2.5 mM CaCl$_2$ followed by incubation in 0.3 mM PMSF. Myc-HDQ20 and myc-HD53 proteins were cleaved from the GST fusion protein through incubation with PreScission protease (Amersham Biosciences) in 50 mM Tris, pH 7, 150 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol. The concentration of recombinant proteins was estimated by Coomassie Blue or Silver (Bio-Rad) staining of protein bands after SDS-polyacrylamide gel electrophoresis using bovine serum albumin as a standard.

Huntingtin Exon 1 Aggregation in Vitro—GST-huntingtin$^{exon1/exp}$ fusion protein (3 μM) was incubated at 37 °C with Precission protease (Amersham Biosciences) in 50 mM Tris-HCl, pH 7, 150 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 0.5 μM leupeptin, 0.5 μM pepstatin A for up to 7 h. Full-length CSP$_{1-198}$ or mutant CSP$_{1-82}$ were added with the protease cleaved fusion protein. At each time point, aliquots of each protein (500 ng) were diluted into 0.2 ml of 2% SDS, 50 mM dithiothreitol and heated at 95 °C for 5 min. The samples were then filtered through cellulose acetate membranes (0.2-μM pore size) using a Slot Blot Manifold (Amersham Biosciences). Huntingtin aggregates were detected with the MW8 anti-huntingtin antibody (21) and the ECL system (Amersham Biosciences).

Immunoblotting—Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 μm or 0.2 μm) in 20 mM Tris, 150 mM glycine, 12% methanol. Transferred proteins were visualized by staining with Ponceau S. Nitrocellulose membranes were blocked for nonspecific binding using 5% milk, 0.1% Tween 20, PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.4 mM KH$_2$PO$_4$, pH 7.3) and incubated with primary antibody overnight at 4 °C or 2 h at room temperature. The membranes were washed three to four times in the above milk/Tween/PBS solution and incubated for 30 min with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Antigen was detected using chemiluminescent horseradish peroxidase substrate (ECL, Amersham Biosciences). Immunoreactive bands were visualized following exposure of the membranes to Amersham Biosciences Hyperfilm-MP. Bound antisera were quantitated by BioRad Fluor-S MultiImager Max and QuantityOne 4.2.1 software. Differences between mean values from each group were tested using one-way analysis of variance. Differences were considered significant if $p < 0.05$.

Transient Transfection of HEK Cells and Electrophysiological Recordings—N-type calcium channel subunits and CSP (18) were prepared as described previously. Huntingtin-GFP DNA constructs were obtained from (22) and resulted in expression of exon 1 of the huntingtin gene containing either 25, 47, or 72 glutamines followed by GFP. The sequence of all DNA constructs was confirmed. Transfection of tsA-201 cells and electrophysiological recordings were carried out as described in detail previously (Miller *et al.,* Ref. 19).

The external recording solution was comprised of 20 mM BaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 40 mM tetraethylammonium chloride (TEA-Cl), 10 mM glucose, 65 mM CsCl, (pH 7.2 with TEA-OH), the internal pipette solution contained 108 mM CsMS, 4 mM MgCl$_2$, 9 mM EGTA, 9 mM HEPES (pH 7.2). Series resistance was compensated by 85%, and capacitance was partially compensated. Unless stated otherwise, all error bars are S.E., and numbers in parentheses displayed in the figures reflect numbers of experiments. Statistical analysis was carried out using SigmaStat 2.0 (Jandel Scientific). Differences between mean values from each group were tested using analysis of variance followed by a Tukey post-hoc test for multiple comparisons. Differences were considered significant if $p < 0.05$.

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[1] The abbreviations used are: CSP, cysteine string protein; MOPS, 4-morpholinepropanesulfonic acid; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; TEA-Cl, tetraethylammonium chloride.

FIG. 1. **HDQ53 blocks CSP interactions with G proteins.** *A*, immunoblot analysis showing the effect of cleaved HDQ53 on the interaction between CSP and $G\alpha$ or $G\beta$. SGT (1.3 μM), HDQ20 (0.1 μM), HDQ53 (0.05 μM), and aggregated HDQ53 were preincubated with immobilized CSP (0.3 μM) for 10 min at 37 °C prior to the addition of rat hippocampal homogenate (200 μg). Beads were washed with 200 μl of buffer (0.2% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCl, and 0.5 mM PMSF), and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-$G\beta$ monoclonal from Transduction Labs (*top*) and anti-$G\alpha$ polyclonal from Calbiochem (*bottom*). The *panels* on the *right* show an experiment with a higher concentration of HDQ53 (0.2 μM). The *last lane* in each *panel* represents 30 μg of rat hippocampal homogenate loaded directly onto the gel. The pixel values are: *top left panel*: 563, 482, 481, 254, 104, 584; *top right panel*: 14, 37, 430; *bottom left panel*: 320, 310, 385, 294, 40, 306; *bottom right panel*: 77, 62, 322. *B*, bar graph summarizing the effect of HDQ20 and HDQ53 on the association of G proteins with CSP. The numbers in parenthesis indicate the number of experiments. The G protein:CSP association was reduced significantly in the presence of HDQ53 and HDQ53*. *C*, HDQ53 (0.04 μM) was preincubated with immobilized GST (0.1 μM) and CSP (0.2 μM) for 10 min at 37 °C prior to the addition of rat hippocampal homogenate (150 μg). Beads were washed, and bound proteins eluted in sample buffer, fractionated by SDS-PAGE and subjected to Western blot analysis. *Lane 5* shows 30 μg of rat hippocampal homogenate loaded directly onto the gel. The pixel values are: *top panel*: 85, 79, 194, 74, 216; *bottom panel*: 89, 71, 201, 76, 136.
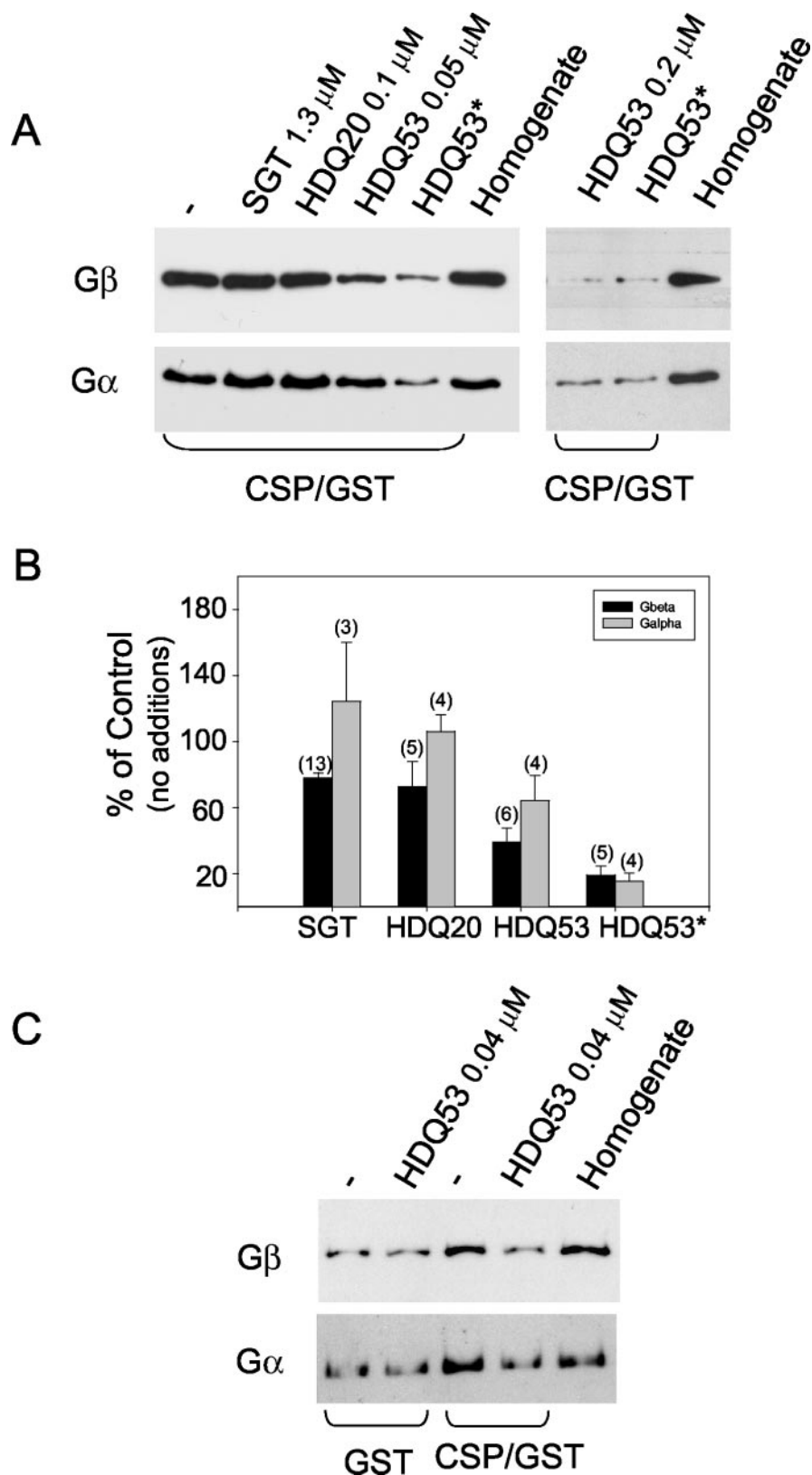
RESULTS

Exon 1 of Huntingtin with an Expanded Polyglutamine Tract (huntingtin^(exon1/exp)) Blocks the CSP Interaction with G Proteins—In order to investigate the possibility that mutant forms of huntingtin with expanded glutamine repeats alter the association of G protein with CSP, a GST fusion protein consisting of full-length CSP was coupled to glutathione-agarose beads

and used in an *in vitro* binding assay. In each binding assay an equal amount of fusion protein was immobilized on agarose beads and confirmed by Ponceau S staining. Fusion proteins composed of GST and exon 1 of huntingtin with normal (HDQ20) and expanded (HDQ53) polyglutamine repeats were expressed in *Escherichia coli* and purified as soluble proteins. Proteolytic cleavage of HDQ20 by PreScission protease yields
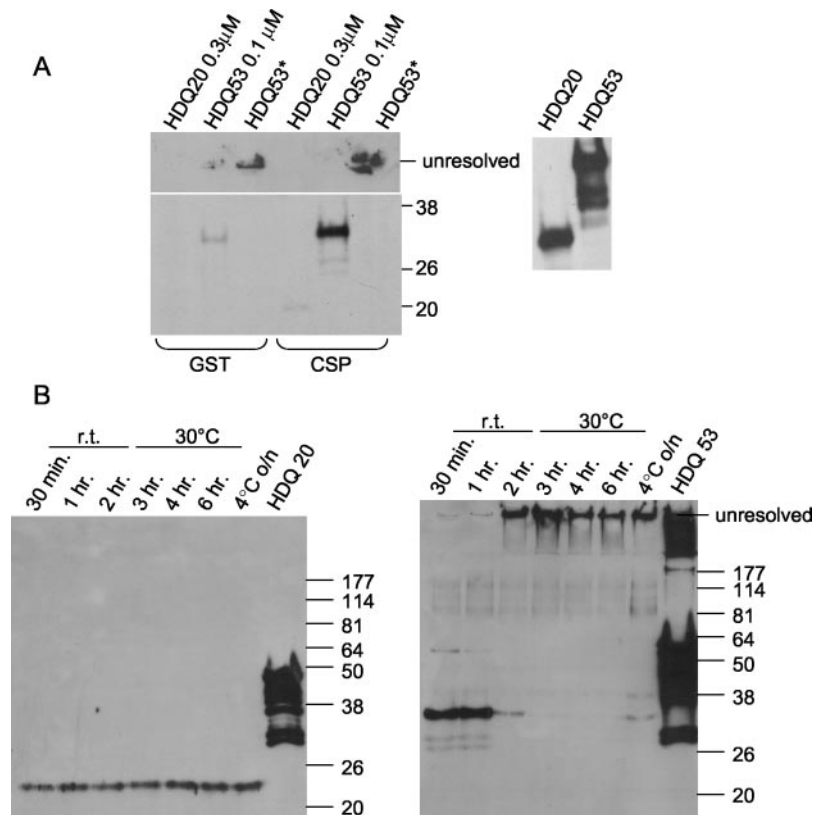
FIG. 2. **HDQ53 interacts with immobilized CSP.** *A*, immunoblot analysis showing binding of PreScission protease cleaved HDQ20 and HDQ53 to CSP-GST immobilized on agarose. HDQ20 and HDQ53 were incubated for 30 min at 37 °C with immobilized CSP (0.3 μM) or GST (0.6 μM) in 0.2% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM Mg(CH$_3$COO)$_2$, 150 mM KCl, and 0.5 mM PMSF in a total volume of 300 μl. Beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-c-Myc monoclonal. The *left top panel* shows aggregated HDQ53. The blot on the *right* shows cleaved HDQ20 and HDQ53 loaded onto the gel. These results are representative of four experiments. *B*, time course of PreScission protease cleavage of HDQ20 (*left panel*) and HDQ53 (*right panel*). Sample 1 (*lanes 1–6*) 10 μl of the supernatant was removed at the indicated time points, added to 5 μl of 3× sample buffer, boiled, and fractionated by SDS-PAGE. Sample 2 (*lane 7*) 10 μl of the supernatant was removed after 22 h at 4 °C, added to 5 μl of 3× sample buffer, boiled, and fractionated by SDS-PAGE. Sample 3 (*lane 8*) purified polyglutamine-GST fusion proteins; HDQ20 (0.3 μM), HDQ53 (0.2 μM). Immobilized proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-c-Myc monoclonal. Migration of molecular mass standards (Invitrogen) at 177, 114, 81, 64, 50, 38, 26, and 20 kDa is shown on the *left hand side* of each panel. The pixel values of the *bottom panel* of *A* are 5, 52, 0, 16, 566, 0.

soluble Myc-tagged HDQ20. Cleavage of purified GST-HDQ53 led to the formation of soluble Myc-tagged HDQ53, which after a time lag entered an aggregation phase (Fig. 2*B*). The aggregated HDQ53 (HDQ53*) was SDS-insoluble and did not migrate into the gel (Fig. 2*B*) as previously described (7). Soluble HDQ20, soluble HDQ53 and HDQ53* were incubated with the immobilized CSP prior to the addition of rat hippocampal homogenate. The beads were washed, and the bound proteins eluted. The presence of Gα and Gβ were determined through Western blotting with anti-Gα polyclonal and anti-Gβ monoclonal, respectively. Fig. 1 shows that the association of Gβ and Gα with CSP was decreased in the presence of HDQ53. Further reduction in the G protein:CSP association was observed in the presence of HDQ53*. Neither HDQ20 nor SGT reduced the interaction between CSP and G proteins to the same extent as HDQ53. No reduction in the association of GST (background) with either Gβ or Gα was observed in the presence of HDQ53 (Fig. 1*B*), demonstrating the specificity of the HDQ53-induced reduction in the G protein:CSP association. These results show that huntingtin[exon1/exp] specifically prevents the association of CSP with Gβ and Gα.

To further evaluate the interference of HDQ53 in CSP protein-protein interactions we tested the possibility that CSP directly interacts with HDQ53. Immobilized full-length CSP was incubated with soluble Myc-tagged HDQ20, HDQ53, and HDQ53*. The beads were washed to remove unbound protein

and bound proteins were eluted with sample buffer. The presence of the huntingtin proteins was determined through Western blot analysis using anti-c-Myc monoclonal. Fig. 2*A* shows that HDQ53 directly bound to immobilized CSP. In contrast HDQ20 was not observed to associate with either CSP or GST. Aggregated HDQ53* was detected in pull-down assays with both GST and CSP as shown in the unresolved portion of the gel (Fig. 2*A*) and likely represents the insolubility of the aggregated protein rather than a specific protein interaction. Fig. 2*B* demonstrates that huntingtin[exon1/exp] aggregated *in vitro* after proteolytic cleavage by Prescission protease and that these aggregates did not resolve by SDS-PAGE. In contrast, Prescission protease cleavage of native huntingtin[exon1/nonexp] resulted in a soluble protein that is clearly resolved by SDS-PAGE. These results suggest that huntingtin[exon1/exp] directly and specifically associates with CSP and that this association, in turn, blocks the CSP:G protein interaction.

To further evaluate the interactions between CSP, G proteins, and polyglutamine proteins, GST, GST-SGT, GST-HDQ20, and GST-HDQ53 fusion proteins were immobilized on beads and incubated with purified G$\beta\gamma$ proteins (Calbiochem) or rat hippocampal homogenate. In each assay, equal amounts of fusion proteins were immobilized on beads and confirmed by Coomassie (Fig. 3*B*) and Ponceau S staining. Fig. 3*B* shows the Coomassie-staining profile of purified immobilized GST-CSP, GST-HDQ20, GST-HDQ53, and GST-SGT. The beads were

CSP Associates with Mutant Huntingtin
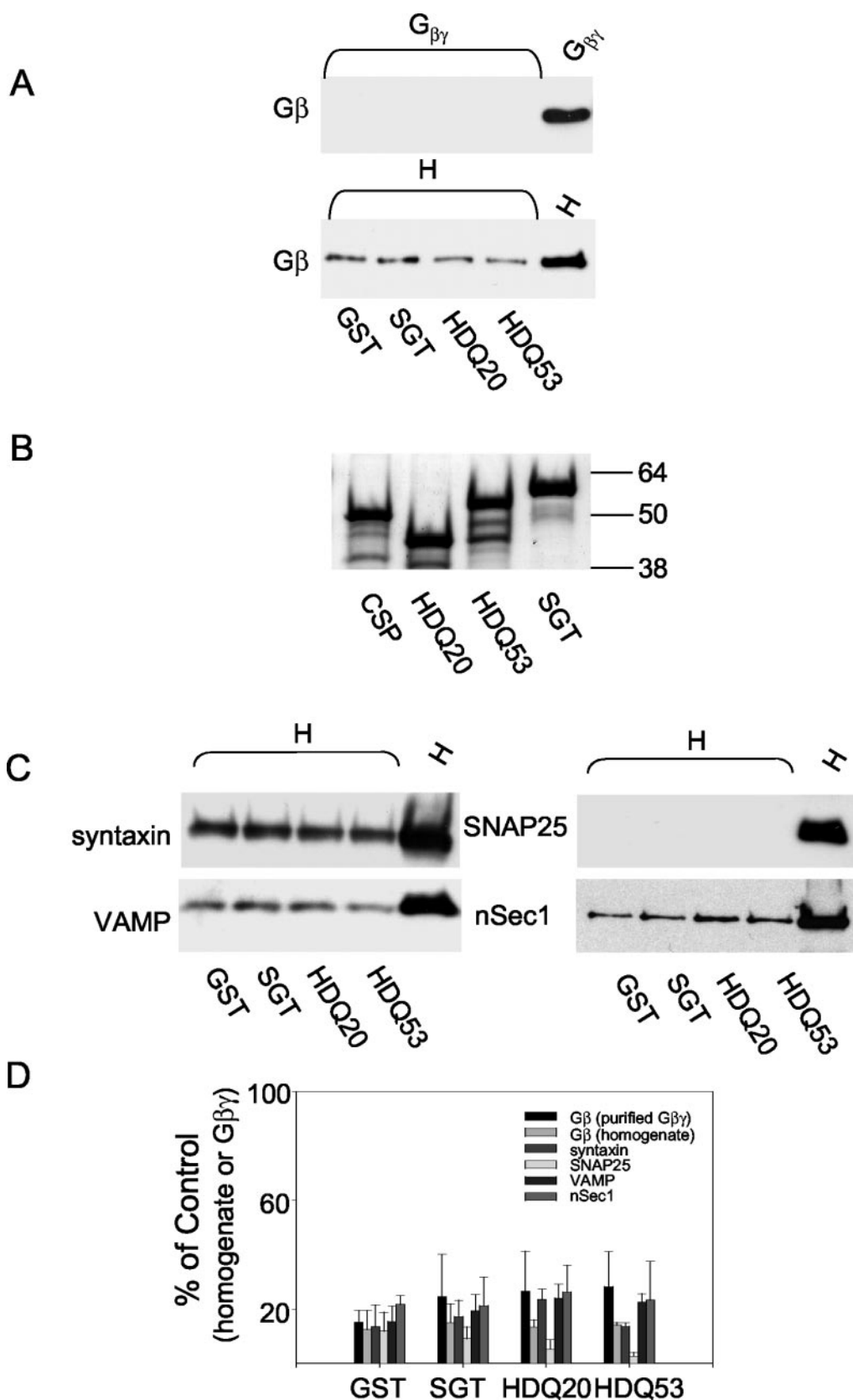
FIG. 3. **Gβ does not directly interact with huntingtin[exon1].** *A*, immunoblot analysis showing that Gβ does not associate with SGT, HDQ20, or HDQ53. 250 ng of purified Gβγ (Calbiochem) or 200 μg of crude hippocampal homogenate was incubated with GST (0.6 μM), GST-SGT (0.3 μM), GST-HDQ20 (0.3 μM), and GST-HDQ53 (0.2 μM) in a final volume of 300 μl. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-Gβ monoclonal (Transduction Labs). *Lane 5* shows 50 ng of Gβγ (*top panel*) and 30 μg of rat hippocampal homogenate (*bottom panel*) loaded directly onto the gel. *B*, Coomassie stain of GST fusion proteins separated by SDS-PAGE. *C*, association of synaptic proteins with HDQ20 and HDQ53. Immunoblot analysis showing association of synaptic complexes with HDQ20 and HDQ53. Crude hippocampal homogenate (200 μg) was incubated with GST (0.6 μM), GST-SGT (0.3 μM), GST-HDQ20 (0.3 μM), and GST-HDQ53 (0.2 μM) in a final volume of 300 μl. The beads were washed and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to Western blot analysis. *Lane 5* shows 30 μg of rat hippocampal

washed, bound proteins were eluted, and the presence of Gβ was determined through Western blotting with anti-Gβ monoclonal. No association of Gβ with GST-SGT, GST-HDQ20, and GST-HDQ53 was observed above control (GST) (Fig. 3, *A* and *D*). Also, no association above control of syntaxin, VAMP, SNAP25, or nSec1 with huntingtin[exon1] fusion proteins was observed (Fig. 3, *C* and *D*). Overall, these results suggest that huntingtin[exon1/exp] associates with CSP but not G proteins and that the huntingtin[exon1/exp]:CSP interaction precludes the association of CSP with G proteins.

Next we examined the effects of purified CSP on huntingtin[exon1] aggregation *in vitro*. We have previously shown that *E. coli* Hsp70 (DnaK) and Hsp40 (DnaJ) efficiently suppressed the formation of SDS-insoluble aggregates of HDQ53 (7). In contrast, while the human DnaJ homologue, Hdj-1, alone was unable to suppress HDQ53 aggregation, the Hdj-1: Hsc70 complex efficiently suppressed aggregation in an ATP-dependent manner (7). Although CSP and Hsp40 both contain a J domain, outside of the J domain these proteins are unrelated. The effect of CSP on polyglutamine aggregation has not previously been reported. Fig. 4 shows that CSP does not suppress formation of SDS insoluble aggregates of HDQ53 in the filter assays at equimolar or subequimolar ratios.

Huntingtin[exon1/exp] Blocks CSP Regulation of N-type Calcium Channels—Previous work in our laboratory has shown that CSP modulates G protein-mediated inhibition of N-type calcium channels (18, 19). Thus, the N-type calcium channel can be used as a functional readout of CSP-G protein interactions in live cells. To test if the presence of huntingtin fragments interferes with CSP modulation of channel function, we transfected HEK 293 cells with N-type Ca^{2+} channels (α_{1B} + $\alpha_2 - \delta + \beta_{1b}$), CSP, and GFP-tagged exon 1 of the huntingtin gene containing either 25, 47, or 72 glutamines. Subsequently, the CSP-mediated effects on channel function were assessed via whole-cell patch-clamp recordings. As shown in Fig. 5 the channels exhibited a slow current waveform typically observed with N-type calcium channels that are tonically inhibited by G$\beta\gamma$. Upon application of a strong depolarizing prepulse, peak current amplitude was increased. This is consistent with removal of a G protein-mediated inhibitory effect, which we have characterized in detail (18, 19). When N-type channels were co-transfected with HDQ25, HDQ47, or HDQ72 no effect on channel function (*i.e.* activation and inactivation) was evident, and these constructs did not induce a G protein-mediated inhibition of the channels. However co-expression of HDQ47 or HDQ72 with CSP and N-type calcium channels eliminated the CSP tonic G protein inhibition of the channels, while co-expression of HDQ25 did not alter the robust inhibition promoted by CSP. These results indicate that CSP modulation of G protein inhibition of calcium channel activity is blocked in the presence of huntingtin fragments with expanded polyglutamine tracts, suggesting that CSP becomes functionally inactivated in the presence of huntingtin[exon1/exp].

To evaluate the structural requirements for CSP association with HDQ53, a series of CSP deletion mutants were constructed, expressed, and purified. The regions of CSP required for binding HDQ20 and HDQ53 were determined through binding experiments to the CSP deletion mutants. The CSP fusion proteins were coupled to glutathione-agarose beads and incubated with soluble HDQ53 or HDQ20. An interesting pattern of binding was revealed through this analysis. All the CSP dele-
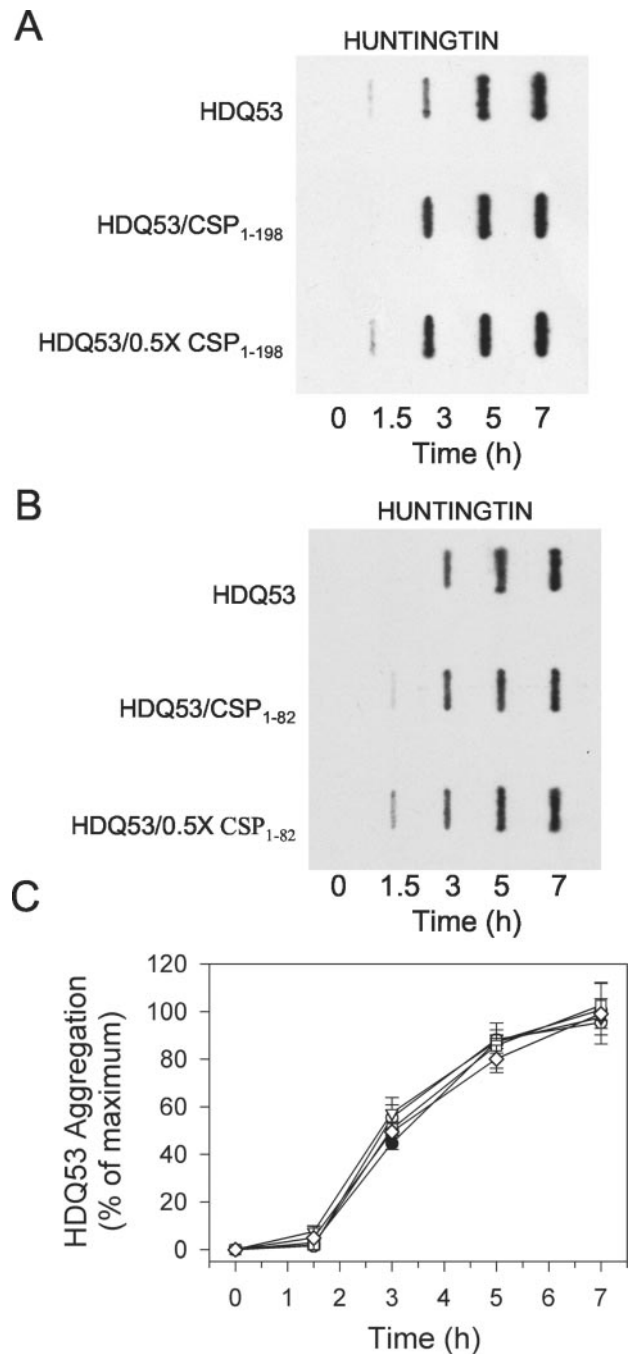
FIG. 4. **Effect of Csp$_{1-198}$ and CSP$_{1-82}$ on HDQ53 aggregation *in vitro*.** HDQ53 (3 μM) forms SDS-insoluble aggregates in a time-dependent manner as detected by a filter-trap assay. *A*, addition of an equimolar amount of CSP$_{1-198}$ (3 μM) or subequimolar (1.5 μM) does not alter HDQ53 exon 1 aggregation. The pixel values for HDQ53 were 0, 10,490, 78,373, 201,778, 246,778, for HDQ53/CSP$_{1-198}$ were 0, 32, 139,950, 201,072, 200,394 and for HDQ53/0.5XCSP$_{1-198}$ were 0, 27,614, 154,681, 179,342, 212,211. *B*, addition of equimolar (3 μM) or subequimolar (1.5 μM) amounts of CSP$_{1-82}$ does not have a significant effect on HDQ53 exon1 aggregation. The pixel values for HDQ53 were: 0, 0, 704, 612, 1, 547,125, 1,645,825; for HDQ53/CSP1–82 were 0, 38,985, 786, 121, 1,152,888, 1,253,035; for HDQ53 0.5XCSP1–82 were 0, 210,790, 687, 127, 1,027,377, 1,492,929. *C*, summary of HDQ53 aggregation. The symbols are as follows: ● control; ○ 1× CSP$_{1-198}$; ▽ 0.5× CSP$_{1-198}$; □ 1× CSP$_{1-82}$, 224 0.5× CSP$_{1-82}$.

homogenate loaded directly onto the gel. The nitrocellulose membrane was probed with anti-syntaxin monoclonal (Sigma), anti-VAMP polyclonal (Stressgen), anti-SNAP25 monoclonal (Sternberger) and anti-nSec1 polyclonal (Stressgen). The pixel values are: *A*, *top panel*: 0, 0, 4, 0, 240; *A*, *bottom panel*: 98, 106, 70, 57, 375; *C*, *top left panel*: 373, 339, 290, 181, 1147; *C*, *top right panel*: 0, 1, 0, 0, 478; *C*, *bottom left panel*: 226, 253, 234, 156, 795; *C*, *bottom right panel*: 75, 80, 148, 81, 532. *D*, bar graph summarizing the lack of association of specific proteins with HDQ20 and HDQ53.
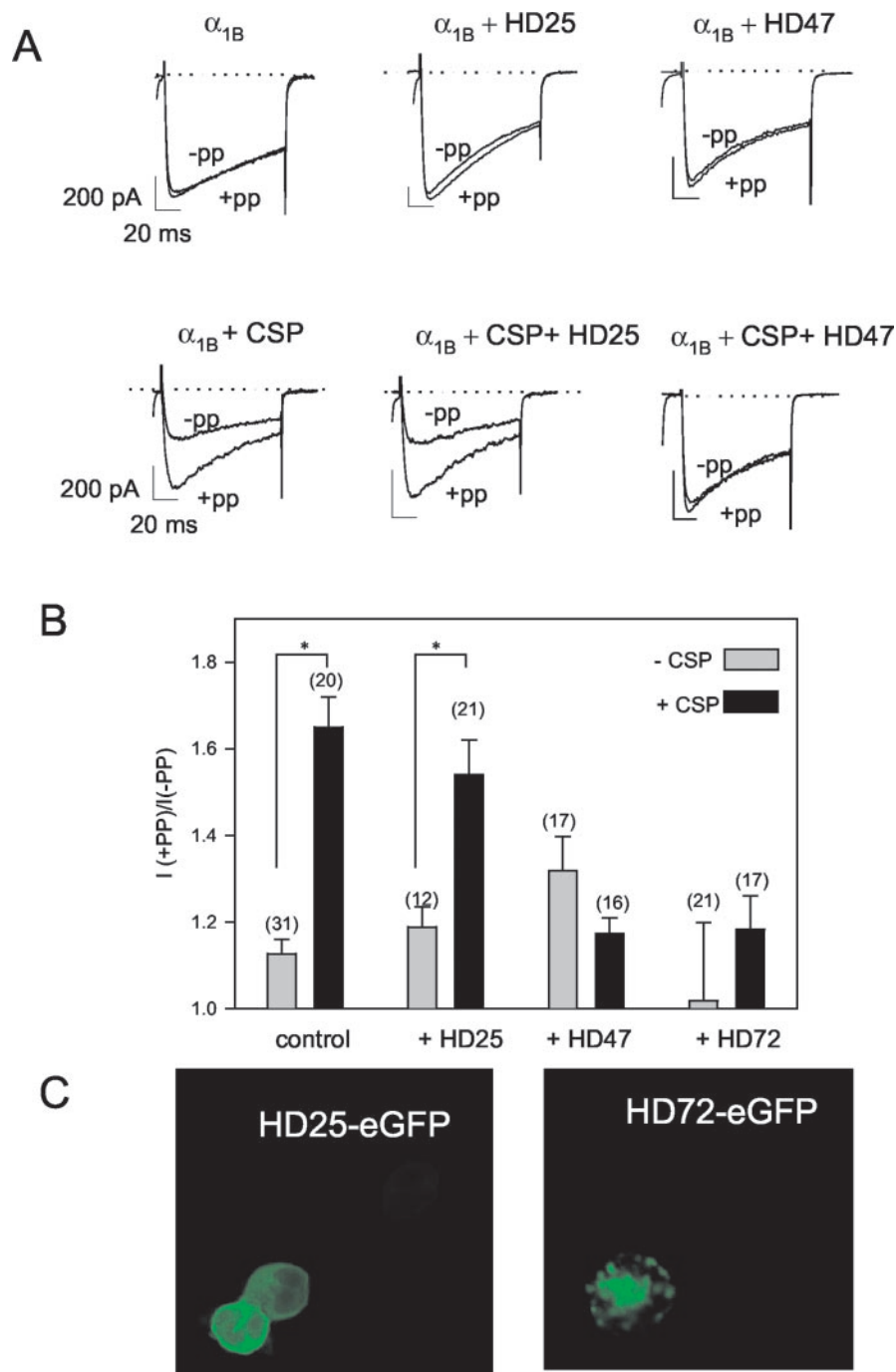
FIG. 5. **Effects of huntingtin[exon1] on CSP-induced tonic G protein inhibition of $Ca_v2.2$ ($\alpha_{1B} + \alpha_2 - \delta + \beta_{1b}$) N-type calcium channels coexpressed with an EGFP marker in HEK (tsA-201) cells.** *A*, current records obtained from transiently expressed N-type ($\alpha_{1B} + \alpha_2 - \delta + \beta_{1b}$) calcium channels in the presence of various combinations of CSP and huntingtin proteins, before and after application of a 50 ms prepulse (pp) to $+150$ mV. Currents were elicited by stepping from a holding potential of -100 mV to a test potential of $+20$ mV. In the absence of CSP (*top traces*), the prepulses do not affect peak current amplitude. Note that HDQ25 and HDQ47 do not mediate a G protein effect in the absence of CSP and that HDQ47, but not HDQ25 blocks the CSP-mediated G protein inhibition of the channel. Following coexpression of CSP, the channels undergo a tonic G protein inhibition that is reversed by a prepulse. In all traces, the *vertical* and *horizontal bars* indicate, respectively, a current amplitude of 200 pA and a 20 ms duration. *B*, summary of the effects of huntingtin proteins on CSP-mediated G proteins inhibition of N-type channels. *Error bars* are S.E. The *numbers in parentheses* reflect the number of experiments, the *asterisks* indicate statistical significance relative to control conditions at $p < 0.05$ level. *C*, fluorescence micrographs (Olympus V300 confocal) showing expression and distribution of normal length (HDQ25-GFP) and extended polyglutamine huntingtin constructs (HDQ72-GFP) in HEK cells. HDQ25 has a diffuse cytosolic expression. HDQ72 forms multiple aggregates.

tion constructs were observed to bind soluble HDQ53; however, binding to the J domain (CSP_{1-82}) was more robust. In contrast no interaction was observed between HDQ20 and any of the CSP deletion constructs examined (Fig. 6*B*).

We have previously shown that two distinct domains of CSP trigger G protein inhibition of N-type calcium channels (19) albeit through different mechanisms. While the cysteine string domain appeared to colocalize G protein $\beta\gamma$ subunits and the N-type calcium channel α_1 subunit, the J domain of CSP appeared to induce G protein inhibition of the channel independent of CSP association with the channel (*i.e.* perhaps by triggering the dissociation of the heterotrimeric G protein complex, Ref. 19). We therefore assessed whether CSP_{1-82} and CSP_{83-198}, shown to promote G protein inhibition of N-type calcium channels (19), were able to elicit G protein inhibition in the presence of HDQ47. Cells were transfected with N-type

calcium channels, HDQ47 and either CSP_{1-82} or CSP_{83-198}. In these experiments, HDQ47 eliminated the modulation of N-type calcium channels by both CSP_{1-82} and CSP_{83-198} (Fig. 6*C*). Taken together, our results show that huntingtin[exon1/exp] binds CSP at multiple regions and sequesters the CSP-specific binding site, thereby mediating a general dysfunction of CSP.

DISCUSSION

We have found that the vesicle protein CSP interacts with and is sequestered by huntingtin[exon1/exp]. The association of CSP with the mutant huntingtin fragments blocks CSP regulation of N-type calcium channels. In contrast, CSP does not associate with huntingtin with a non-expanded polyglutamine repeat nor was the CSP modulation of calcium channels altered. Therefore the CSP: huntingtin[exon1/exp] interaction is directly mediated through the expanded polyglutamine domain.
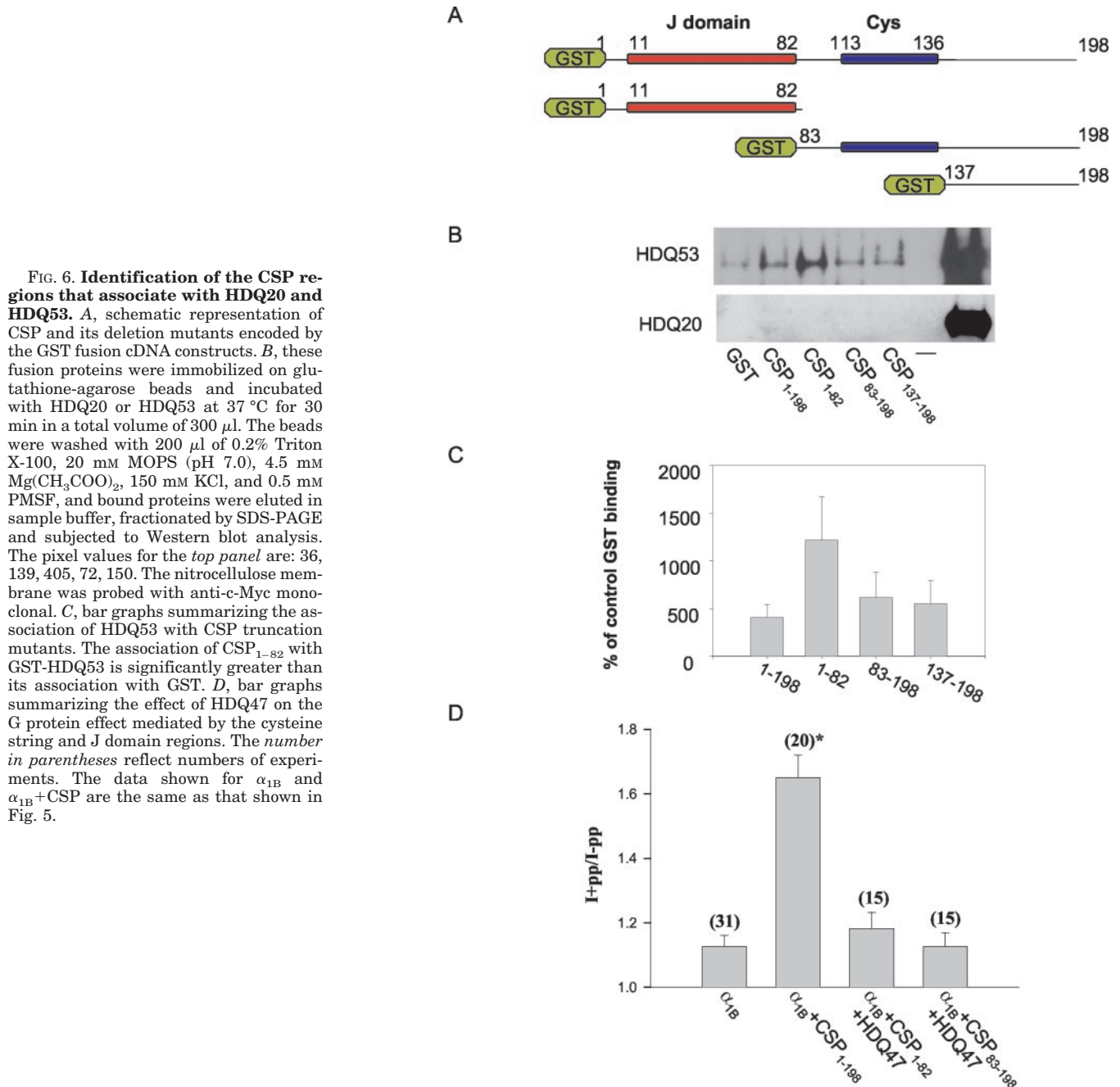
FIG. 6. **Identification of the CSP regions that associate with HDQ20 and HDQ53.** *A*, schematic representation of CSP and its deletion mutants encoded by the GST fusion cDNA constructs. *B*, these fusion proteins were immobilized on glutathione-agarose beads and incubated with HDQ20 or HDQ53 at 37 °C for 30 min in a total volume of 300 μl. The beads were washed with 200 μl of 0.2% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCl, and 0.5 mM PMSF, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE and subjected to Western blot analysis. The pixel values for the *top panel* are: 36, 139, 405, 72, 150. The nitrocellulose membrane was probed with anti-c-Myc monoclonal. *C*, bar graphs summarizing the association of HDQ53 with CSP truncation mutants. The association of CSP_{1-82} with GST-HDQ53 is significantly greater than its association with GST. *D*, bar graphs summarizing the effect of HDQ47 on the G protein effect mediated by the cysteine string and J domain regions. The *number in parentheses* reflect numbers of experiments. The data shown for α_{1B} and α_{1B}+CSP are the same as that shown in Fig. 5.

Although our primary objective was to use the N-type calcium channel as a functional readout of CSP function, these data underline the complexity of N-type calcium channel regulation and its sensitivity to the sequestration of regulators such as CSP. Several lines of evidence prompted us to explore the association between CSP and mutant huntingtin. First, since the glutamine-rich protein, SGT, has been shown to be a component of the active CSP complex, it seemed likely that proteins with expanded polyglutamine tracts like huntingtin would interfere with CSP chaperone activity (13). Secondly, while other J domain-containing proteins have been implicated in the suppression of Huntington disease progression in different cell models including HSP40, HDJ2, HSDJ, and MRJ (7, 23–25), no one has yet examined CSP role in polyglutamine aggregation. Finally, the *Drosophila* CSP-null mutant phenotype is characterized by paralytic uncoordinated sluggish movements, spasmic jumping, intense shaking, temperature sensitive paralysis, and reduced lifespan (17), which are phe-

notypes that in some ways mirror what is observed in Huntington's patients and animal models.

Regulation of N-type calcium channels is complex (26, 27). Investigations concerning the role of CSP as a calcium channel regulator have used several experimental approaches. We have observed that CSP promotes G protein inhibition of N-type calcium channels in transiently transfected human embryonic kidney cells (18, 19). Consistent with our results, calcium signals in boutons from *Drosophila* CSP-null mutants were larger than controls indicating CSP had an inhibitory effect in depolarization-dependent calcium entry (28). In contrast, injection of CSP antisense RNA into *Xenopus* oocytes was reported to inhibit the activity of ω-conotoxin-sensitive calcium channels (29). Influx of calcium into the nerve terminal was reported to be reduced in *Drosophila* CSP mutants (30). Introduction of recombinant CSP into the calyx nerve terminal results in an increase in presynaptic calcium currents suggesting a role for CSP in the recruitment of

calcium channels (31). Finally, several studies conclude that CSP is important in exocytosis rather than the regulation of calcium transmembrane fluxes (10, 32–38). Perhaps the explanation of these seemingly paradoxical results lies in the disruption of distinct signaling pathways and chaperone systems in the different models employed.

Here we have presented evidence that the chaperone activity of the synaptic vesicle protein CSP is compromised by huntingtin$^{exon1/exp}$. Our experiments are suggestive of CSP dysfunction in Huntington's disease. The exhaustion of CSP by huntingtin$^{exon1/exp}$ disrupts the signaling pathway by which G proteins modulate calcium channels. Interestingly, several other signaling pathways have been proposed to be compromised during Huntington's disease progression. For example, impairment in synaptic plasticity has been observed in presymptomatic *Hdh* knock-in Huntington's disease mice, which indicate that the synapse is less able to sustain transmitter output (39). Severe deficiencies in dopamine signaling have been reported in presymptomatic R6/2 Huntington's disease mice (40). Abnormal phosphorylation of synapsin I in the striatum and cerebral cortex has also been reported in R6/2 Huntington's disease mice (41). Aberrant neuronal calcium signaling has been reported in Huntington disease models (42, 43), and recently huntingtin with expanded polyglutamine repeats has been shown to increase the sensitivity of the inositol 1,4,5-trisphosphate receptor to inositol trisphosphate (44). Activation of the NR2B-subtype NMDA receptor has been proposed to be central in the selective neuronal degeneration of striatal cells in FVB/N Huntington's disease mice (45, 46). Finally, activation of caspase signaling cascades and induction of transcriptional abnormalities by mutant huntingtin have been observed (5). Thus, changes in several signaling pathways may underlie Huntington's disease and further studies are required to address the sequence of disease progression.

The identification of neural chaperones and the proteins they regulate *in vivo* remains an important biological question. Several neural J domain-containing proteins have been identified (47). The overall amino acid identity between the J domain of rat CSP (NP_077075) and other neural CSP homologues ranges from 32–59% (rat Hsp40: 52% (NP_114468); mouse HSJ1: 55% (NP_064662); mouse HDJ2: 59% (XP_227379); rat MRJ: 59% (AAC16759); bovine auxilin: 32% (S68983)). Outside of the J domain these proteins are unrelated. The presence of distinct chaperones in neurons supports the idea that several folding events in synaptic transmission are managed by specific chaperone complexes (48, 49). The target for auxilin/Hsc70 is clathrin, while the targets for the other J domain proteins remains to be established. The physiological targets of these chaperones are expected to be specific and are likely to be determined by their expression levels and tissue localization. In addition to G proteins (18) and calcium channels (18, 31, 50, 51), several other targets of CSP chaperone activity have been proposed including syntaxin (51–53;53), VAMP (also called synaptobrevin) (50), synaptotagmin I (54), αGDI (55), and CFTR (56). Future experimentation is required to reveal the role of CSP chaperone activity in the function of these proteins.

In this study we provide evidence that the introduction of huntingtin$^{exon1/exp}$ results in a loss of CSP's modulation of N-type calcium channels. Our previous studies have shown that in the presence of CSP, calcium channels become subject to substantial prepulse facilitation, one of the hallmarks of G$\beta\gamma$ modulation of voltage-dependent calcium channels (18, 19). We proposed that CSP associates with G$\beta\gamma$ and presynaptic calcium channels and results in tonic channel inhibition. G proteins bind two separate sites on CSP, such that the N terminus binds the Gα subunit while the C terminus of CSP associates

with either free G$\beta\gamma$ subunits or G$\beta\gamma$ in complex with Gα (19). In neurons, CSP is associated with synaptic vesicles, and is thought to function as a trimeric chaperone machine along with Hsc70 and SGT (13). The regulation of G protein and calcium channel activity by the CSP chaperone complex would most likely occur when synaptic vesicles are in close proximity to the active zone. We speculate that the N terminus of CSP stimulates dissociation of Gα and G$\beta\gamma$, while the C terminus of CSP targets G$\beta\gamma$ to its site of action on the N-type calcium channel (19). Huntingtin$^{exon1/exp}$ was observed to bind CSP and eliminate the CSP modulation of the G protein inhibition of N-type calcium channels. Huntingtin$^{exon1/exp}$ may also eliminate other putative CSP-related G protein-mediated events. Our work identifies CSP/huntingtin as a potential target for therapeutic intervention of the progression of Huntington's disease in that reversal of CSP depletion may relieve some symptoms associated with Huntington's disease.

In conclusion, in Huntington's disease the polyglutamine tract of huntingtin is expanded beyond threshold, inducing a conformational change that triggers a cascade of pathogenic events that remains to be characterized. Changes to the chaperone balance of the cell, disruption of various signaling pathways, as well as polyglutamine aggregation have been implicated in Huntington's disease progression but the precise sequence of events remains to be identified. Our findings demonstrate that huntingtin$^{exon1/exp}$ sequesters CSP, and blocks CSP inhibition of N-type channels. Thus, chaperone activity and G protein signal transduction pathways are compromised in the presence of huntingtin$^{exon1/exp}$. Dysregulation of cellular calcium involving both (1) elimination of G protein inhibition of N-type calcium channels and (2) hypersensitivity of inositol trisphosphate regulation of inositol 1,4,5-trisphosphate receptors (44) emphasizes the importance of perturbation of calcium signaling in Huntington's disease pathology.

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Cysteine String Protein (CSP) Inhibition of N-type Calcium Channels Is Blocked by Mutant Huntingtin

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