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Association of adaptor protein TRIP8b with clathrin

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Abstract

TRIP8b is a brain-specific hydrophilic cytosolic protein that contains tetratricopeptide repeats (TPRs). Previous studies revealed interaction of this protein via its TPR-containing domain with Rab8b small GTPase, HCN channels and G protein-coupled receptor CIRL. We identified clathrin as a major component of eluates from the TRIP8b affinity matrix. In the present study, by *in vitro* binding analysis we demonstrate a direct interaction between clathrin and TRIP8b. The clathrin-binding site was localized in the N-terminal (non-TPR containing) part of the TRIP8b molecule that contains two short motifs involved in the clathrin binding. In transfected HEK293 cells, co-expression of HCN1 with TRIP8b resulted in translocation of the channels from the cell surface to large intracellular puncta where both TRIP8b and clathrin were concentrated. These puncta co-localized partially with an early endosome marker and strongly overlapped with lysosome staining reagent. When HCN1 was co-expressed with a clathrin-non-binding mutant of TRIP8b, clathrin did not translocate to HCN1 and TRIP8b-containing puncta, suggesting that TRIP8b interacts with HCN and clathrin independently. We found TRIP8b present in the fraction of clathrin-coated vesicles purified from brain tissues. Stripping the clathrin coat proteins from the vesicles with Tris alkaline buffer resulted in concomitant release of TRIP8b. Our data suggest complex regulatory functions of TRIP8b in neuronal endocytosis through independent interaction with membrane proteins and components of the clathrin coat.

Keywords

TRIP8b; clathrin; CCV; endocytosis; HCN1

Introduction

TRIP8b is a brain-specific, cytosolic, hydrophilic protein that exists both in soluble and membrane-bound forms and contains tetratricopeptide repeats (TPRs). It was originally discovered as a binding partner of small GTPase Rab8b (Chen et al. 2001), a putative regulator of endosomal trafficking (Heidrych et al. 2008). Yeast two-hybrid and affinity purification experiments revealed its binding to the cytoplasmic tails of hyperpolarization-activated cyclic nucleotide-regulated (HCN) channels (Santoro et al. 2004; Zolles et al. 2009) and G protein-coupled calcium-independent receptor of latrotoxin CIRL/latrophilin (Popova et al. 2007). The previously described interactions of TRIP8b are primarily mediated by the TPR domains located in its C-terminal part (Amery et al. 2001; Chen et al.

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The abbreviations used are: AP, adaptor protein complex; CCV, clathrin-coated vesicle; CIRL, calcium-independent receptor of α-latrotoxin; CHC, clathrin heavy chain; CLC, clathrin light chain; CME, clathrin-mediated endocytosis; EEA1, early endosome antigen 1; HCN, hyperpolarization-activated cyclic nucleotide-regulated channel; TPR, tetratricopeptide repeat; TRIP8b, TPR-containing Rab8b interacting protein.

The authors declare that they have no conflicts of interest.

2001; Santoro et al. 2004; Popova et al. 2007) and an internal sequence in the conserved middle core of TRIP8b (Lewis et al. 2009; Han et al. 2011; Santoro et al. 2011).

Several independent functional studies of the TRIP8b interaction with HCN channels provided strong evidence that this protein regulates the channel function and its cell surface expression (Santoro et al. 2004; Lewis et al. 2009; Santoro et al. 2009; Zolles et al. 2009). It was proposed that TRIP8b is an auxiliary subunit of HCN channels that binds to HCN1–4 subunits in a complex manner with at least two distinct domains in TRIP8b and HCN molecules involved (Han et al. 2011; Santoro et al. 2011). HCN channels, members of the superfamily of voltage-gated channels (Santoro et al. 1997; Ludwig et al. 1998; Santoro et al. 1998), are of interest because they were implicated in control of pacemaking activity in the heart and brain, determination of the resting membrane potential, dendritic integration and synaptic transmission (reviewed in (DiFrancesco 1993) and (Robinson and Siegelbaum 2003)). TRIP8b undergoes extensive alternative splicing at its N-terminus, and the effect of splicing was studied on HCN channel traffic and surface expression. It was shown that different splice variants produce diverse effects on HCN channel membrane trafficking, with some causing an increase in surface expression of HCN1 and others a decrease (Lewis et al. 2009; Santoro et al. 2009).

Two types of TRIP8b-knockout mice were obtained to investigate TRIP8b functions. TRIP8b-knockout mouse selectively lacking some TRIP8b isoforms didn't show any overt phenotype (Piskorowski et al. 2011). Mouse, lacking all isoforms ($TRIP8b^{-/-}$), demonstrated impaired motor learning and enhanced resistance to multiple tasks of behavioral despair with high predictive validity for antidepressant efficacy (Lewis et al. 2011).

Recently, we identified clathrin and the subunits of AP-2 complex, proteins that have a key role in endocytosis, as major proteins that bind to immobilized TRIP8b (Popova et al. 2008). Clathrin-mediated endocytosis (CME) is necessary for the transport of lipids and proteins from the plasma membrane and Golgi network and is the best characterized endocytic route for constitutively recycling receptors (Seachrist and Ferguson 2003; Traub 2005). In endocytosed clathrin-coated vesicles (CCVs), clathrin oligomers form a lattice (Kirchhausen and Harrison 1984) that serves as a scaffold but does not bind directly to the membrane (Kirchhausen 2002). Its attachment is mediated by clathrin adaptors, such as AP-2, that can bind directly to both clathrin and the protein components of membranes (reviewed in (Maldonado-Baez and Wendland 2006)).

In the view of previously identified interactions of TRIP8b with plasma membrane proteins, our finding that clathrin coat proteins are major *in vitro* binding partners of TRIP8b was intriguing and thus prompted us to test the specificity of this interaction, and to validate it *in vivo*. In the present study, experiments with truncated and point mutants of TRIP8b reveal the role of the N-terminal (non-TPR containing) domain of the TRIP8b molecule in mediating clathrin binding. In HEK293 cells, cotransfected with HCN1 and TRIP8b, clathrin relocated to the puncta of endocytosed HCN1. These data, together with the shown presence of TRIP8b in the fraction of clathrin-coated vesicles (CCVs), identify TRIP8b as a clathrin-binding protein and suggest its role as an intracellular adaptor.

Materials and methods

DNA plasmids and constructions

DNA encoding full-length TRIP8b protein was isolated as a result of yeast two-hybrid followed by rat brain cDNA library screening (Popova et al. 2007) and cloned in pET32a(+) (Novagen) vector via *Nco*I/*Eco*RV sites. This construct encodes TRIP8b fused with Trx-tag,

Histag, and S-tag at the N-terminus (Popova et al. 2008). To obtain GST-tagged protein, TRIP8b was cloned into pGEX-kg vector at the *Nco*I/*Xho*I sites.

In Figures 1b and S1, we used the shortest spliced variant of TRIP8b, containing exon 1a and exons 5–16. It corresponds to TRIP8b IsoA5 isoform, according to Lewis classification (Lewis et al. 2009) or TRIP8b(1a) isoform according to Santoro classification (Santoro et al. 2009). In the case of Figure 6c, Supplemental figure 2 and HEK293 transfection (Figures 2 and 3) we used TRIP8b(1b-2) isoform, containing exons 1b, 2 and 5–16 (IsoB2 or 1b-2, respectively). Plasmids pCI-TRIP8b(1b-2) and pEGFP-HCN1 were kindly provided to us by Dr. Bina Santoro and Prof. Steven A. Siegelbaum.

pGEX-TRIP8b(1a) 1–236 and pGEX-TRIP8b(1a) 237–566 were generated by partitioning of the sequence at *EcoR*V site. pGEX-TRIP8b(1a) 42–567 and 145–567 were generated by removing first 41 or 145 amino acids at *Aat*II or *BamH*I site, respectively.

The pGEX-TRIP8b(1a) DCM (double clathrin mutant), containing two mutated sites ^{51}DLLDL/^{51}AAAAA and ^{107}LDLD/^{107}AAAA (TRIP8b numbering based on isoform TRIP8b(1a)), and pGEX-TRIP8b(1a) SCM1 and SCM2 (single clathrin mutant) were generated using megaprimer PCR approach. The following primers were used: For-TTAGATATCCAAACACAACTGGAAAAATG and Rev-CCCAAGCTTGAGATCTATGTTCTGAGGACCAC; For-GTTGACCACTGGCTTGGCTGCCGCAGCAGCTAGCGAACCCGTCTCTC and Rev-GGCAGCAGCTGCGGATGATGTCTCTGGCCCTCG; For-GGGCCATGGGGATGTACCAGGGACACATGCAGC.

To obtain pCI-TRIP8b(1b-2) DCM, containing two mutated sites DLLDL/AAAAA and LDLD/AAAA the same megaprimer PCR approach was used with the following primers: For-GAGGGCCAGAGACATCAAGCGCTGCCGCTGCCATTCAAACACAACTGGAAAAAT Rev-TTCTCGGATATCCAGTTCCTCC For-GTTGACCACTGGCTTGGCTGCCGCAGCAGCTAGCGAACCCGTCTCTC For-GGGGAATTCATGTCTGACAGTGAAATGGATGG

The correct sequence of all constructs obtained by PCR was additionally verified by DNA sequencing.

Protein expression

To obtain GST-fusion proteins, *Escherichia coli* BL21(DE3) strain cells (Invitrogen) were transformed with constructs of TRIP8b in pGEX-kg vector, described in the previous section. pGEX-kg encoding glutathione S-transferase (GST) was used as control. Cells were grown at 37°C, 200 rpm, in LB medium containing 100 μg/ml ampicillin to OD_{600} = 0.6. Protein synthesis was induced by the addition of IPTG to final concentration 0.5 mM and cells were grown at RT for 4 h. Then cells were harvested for 15 min at 3,000 × g at 4°C, pellets were suspended in PBS containing 4 mM DTT, 1 mM PMSF, 0.1 mg/ml lysozyme, and incubated for an hour at 4°C. The digested cells were disrupted by sonication, and the insoluble material was removed by centrifugation at 15,000 × g for 30 min.

Antibodies and immunoblotting

Antibodies specific to TRIP8b were described previously (Popova et al. 2008). The following primary antibodies were also used: rabbit polyclonal antibody to clathrin heavy chain (P1663, Cell Signaling), mouse monoclonal antibody to clathrin heavy chain (CBL188, Chemicon), or mouse monoclonal antibody to N-terminus of clathrin heavy chain TD.1 (sc-12734, Santa Cruz Biotechnology), mouse monoclonal antibody to adaptin β

(610381, BD Transduction Laboratories), mouse monoclonal antibody to adaptin α (610501, BD Transduction Laboratories), mouse monoclonal antibody to adaptin γ (610385, BD Transduction Laboratories), mouse monoclonal antibody to EEA1 (610456, BD Transduction Laboratories), mouse antibody to synaptophysin (S5768, Sigma), rabbit antibody to synaptotagmin (V761, a kind gift of Dr. T.C. Südhof). For lysosome labeling, LysoTracker dye (L7528 Red DND-99, Invitrogen) was used.

SDS-polyacrylamide gel electrophoresis and Western blotting were performed by standard procedures. Blots were blocked overnight in 5% non-fat milk in 10 mM Tris-HCl, pH 7.8, 150 mM NaCl and 0.1% Tween 20 and then incubated with primary antibodies, as indicated in the individual figure legends. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were visualized by enhanced chemiluminescence as described (Deyev and Petrenko 2010).

Subcellular Fractionation

CCVs were purified from rat brain according to standard procedures (Girard et al. 2005). Briefly, adult rat brains were homogenized in buffer A (100 mM MES-NaOH, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM PMSF) using glass-Teflon homogenizer. The homogenate was sedimented for 30 min at 15,000 × g at 4°C, and a low-speed pellet (P1) and supernatant (S1) were obtained. The S1 was subjected to centrifugation at 56,000 × g for 60 min at 4°C, and P2 and S2 were obtained. Pellet 2 was resuspended in buffer A and an equal volume of ice-cold Ficoll-sucrose solution was added. The solution was subjected to centrifugation for 20 min at 43,000 × g at 4°C, generating sucrose gradient pellet (SGp) and sucrose gradient supernatant (SGs). SGs was diluted 5 times in buffer A and centrifuged 60 min at 91,000 × g at 4°C to obtain partially purified CCVs. Equal protein amounts were subjected to SDS-PAGE and then detected with appropriate antibodies.

For the extraction of coat proteins, aliquots of CCVs were centrifuged for 40 min at 91,000 × g at 4°C. The pellets were resuspended in buffer A or Tris buffer (0.5 M Tris-HCl, pH 7.8, 1 mM EDTA, 0.2 mM DTT). The samples were centrifuged for 40 min at 91,000 × g at 4°C, and the pellets were resuspended in buffer A and analyzed in parallel with the supernatant fraction. In other cases, CCVs in buffer A and CCVs extracted with Tris buffer were loaded on the top of linear 20–50% sucrose gradients prepared in buffer A and Tris buffer, respectively, and were centrifuged in SW 50.1 swing rotor for 90 min at 145,000 × g at 4°C. The gradients were fractionated from the bottom, and equal volumes of gradient fractions were analyzed by Western blotting.

Clathrin isolation

Clathrin was isolated from CCVs according to the published protocols (Jackson 1993; Girard et al. 2005). CCVs, obtained as described in the previous section, were centrifuged for 10 min at 15,000 × g at 4°C, the supernatant was removed and underlain with D_2O-sucrose solution (8% sucrose on buffer A, prepared on D_2O). Samples were centrifuged for 2 h at 116,000 × g at 4°C in SW 50.1 swing rotor. The pellets were resuspended in Dissociation buffer (0.5 M Tris-HCl, pH 7.8, 1 mM EDTA, 0.2 mM DTT, and 1 mM PMSF), homogenized and incubated on ice for 1 h. The suspension was clarified by ultracentrifugation for 40 min at 91,000 × g at 4°C and the supernatant was loaded onto Superose 12 HR 10/30 column (Amersham Pharmacia Biotech), pre-equilibrated with Dissociation buffer. Proteins were eluted from the column in Dissociation buffer at 0.25 ml/min and analyzed by SDS electrophoresis followed by Coomassie staining, silver staining or Western blotting for clathrin. The clathrin-containing peak was pooled, dialyzed against Assay buffer (20 mM Hepes-KOH, pH 7.3, 100 mM NaCl, 0.5 mM $MgCl_2$, 2 mM EGTA, and 1 mM PMSF) and further used in the GST-pull down experiment as described below.

Protein mass spectrometry

For protein identification by mass-spectrometry after SDS-PAGE, the gel was stained with Coomassie R-250 and protein band was excised and cut into 1 × 1-mm pieces. Then sample was prepared and analyzed as described in (Popova et al. 2008).

Binding Assays

The association of brain proteins with GST-TRIP8b fusion proteins was assayed in 20 mM Hepes-KOH, pH 7.3, 100 mM NaCl, 0.5 mM $MgCl_2$, 2 mM EGTA, and 1 mM PMSF (assay buffer) in a final volume of 900 μl. To obtain matrices with similar amounts of GST and GST-fusion proteins on glutathione-Sepharose, excess amounts of these proteins (1.5 ml solution with protein concentration about 0.2 μg/μl) were incubated with 40 μl of resin at 4°C overnight. The matrices were then extensively washed with the assay buffer. Rat brain cytosol was obtained from adult rat brains homogenized in the assay buffer (10 ml per 1 g) using glass-Teflon homogenizer. The homogenate was centrifuged for 1 h at 15,000 × g at 4°C, and 900 μl of supernatant (cytosol) were added to 40 μl of glutathione-Sepharose with preadsorbed GST fusion proteins, and the tubes were further incubated at 4°C for 3 h with continuous gentle mixing. The beads were pelleted and, after washing with assay buffer with 0.1% Triton X-100, the bound proteins were eluted first with 20 mM Tris-HCl, pH 8.0, 1 M NaCl, and 2 mM EDTA buffer and then with SDS-containing Laemmli buffer. About 8 μg total protein of rat brain cytosole and 1/8 part of the high-salt eluates were subjected to SDS-PAGE followed by Western blotting. To estimate the amount of preadsorbed fusion proteins, the Laemmli buffer eluates were analyzed by SDS-PAGE followed by Coomassie staining and visual comparison to purified GST or GST-fusion protein standards run side-by-side as in (Krasnoperov et al. 2009). Typically, we detected about 50 μg of GST or GST-fusion proteins bound to 40 μl of matrices. In experiments with purified clathrin, the bound proteins were eluted by Laemmli buffer and 1/10 part of the load, flow-through and eluates were used in Western blot analysis.

Cells and Immunocytochemistry

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. The cells were transfected with pEGFP-HCN1 together with pCI-TRIP8b(1b-2) or pCI-TRIP8b(1b-2) DCM using Unifectin-56 (UnifectGroup). In 28–40 hours after transfection, cells were washed twice with PBS and further fixed in PBS containing 3% formaldehyde for 20 min at room temperature. The cells were blocked and permeabilized with a blocking solution (10% goat serum, 0.2% Triton X-100 in PBS) followed by antibody labeling in 0.2% Triton X-100/PBS for 1 h at room temperature. Primary antibodies used were rabbit anti-TRIP8b (1:3000), rabbit anti-CHC (1:600) or mouse anti-EEA1 (1:1000). Labeling with secondary goat anti-rabbit or anti-mouse Cy3-cojugated IgG (Jackson ImmunoResearch; 1:5000) was performed for 50 min at RT and was detected using fluorescent microscope Olympus IX51.

For lysosomal labeling, transfected cells adhered to coverslips were incubated with 75 nM LysoTracker Dye at 37°C for 1 h. After the incubation, cells were washed with warm PBS, fixed as indicated above and visualized by fluorescent microscopy.

Results

We previously used affinity chromatography to isolate soluble brain proteins that are potential binding partners of TRIP8b (Popova et al. 2008). The brain extracts were passed over immobilized TRIP8b(1a) N-terminally fused to pET32 vector tags (Trx, His6, and S-tags). We originally identified clathrin as a major TRIP8b-binding protein by mass spectrometry and anti-clathrin light chain antibody staining (Popova et al. 2008), and further

confirmed its identity by Western-blot analysis with anti-clathrin heavy chain antibody (Fig. S1). In a control, the matrix with immobilized tags from pET32 vector was used that did not show any substantial clathrin enrichment in this chromatography.

To confirm specificity of TRIP8b interactions with clathrin and to probe its structural characteristics, we designed a set of N-terminal GST-fused TRIP8b expression constructs based on the TRIP8b(1a) isoform (Fig. 1a). GST, GST-TRIP8b full-length and truncated proteins were immobilized on glutathione-Sepharose. After washes, the matrices were incubated with rat brain aqueous extracts. The bound proteins were eluted and analyzed by Western blotting with anti-clathrin heavy chain and anti-adaptin β subunit antibodies which react with β subunits of heterotetrameric adaptor protein complexes (AP). We found clear binding of clathrin from soluble rat brain extracts to TRIP8b(1a) full-length protein, 1–237 and 42–567 constructs, whereas the 145–567 and 238–567 constructs did not show any affinity to clathrin (Fig. 1b, upper panels), indicating that the clathrin binding site is localized between the 42nd and 145th residues of TRIP8b(1a) isoform. Unlike clathrin heavy chain, AP β subunits were detected in all precipitates with TRIP8b(1a) mutants but this binding was TRIP8b-dependent, as indicated by control GST-Sepharose precipitation. To distinguish between AP-1 and AP-2 complexes, we also blotted membranes with antibodies specific to γ-subunit of AP-1 and α-subunit of AP-2. Unexpectedly, differential binding of AP-1 and AP-2 to various TRIP8b-truncated proteins was detected (Fig. 1b, upper panels). These data suggest that the 1–237 fragment of TRIP8b(1a) mediates AP-1 binding whereas 42–567 fragment of TRIP8b(1a) is only responsible for AP-2 interaction.

The computer-assisted analysis of TRIP8b sequence revealed two boxes potentially important for clathrin-binding, ^{51}DLLDL and ^{107}LDLD, in the N-terminal part of its molecule (Fig. 1a). These short regions of alternating hydrophobic and acidic residues resemble the type 1 clathrin motif that was shown earlier to mediate the binding to the clathrin heavy chain in several clathrin-associated proteins (Dell'Angelica et al. 1998; ter Haar et al. 2000; McPherson and Ritter 2005).

To determine whether the short peptide motifs ^{51}DLLDL and ^{107}LDLD are indeed involved in clathrin binding, we mutated them to ^{51}AAAAA and ^{107}AAAA. Precipitation experiments with GST-tagged full length TRIP8b(1a) mutants and rat brain cytosole revealed that both single and double mutations dramatically reduced the binding of the clathrin heavy chain to TRIP8b(1a) mutants (Fig. 1b, lower panels). Association of TRIP8b with adaptin subunits was not strongly affected by these mutations.

Since TRIP8b was shown to produce a robust effect on HCN channels cell distribution in transfected HEK293 cells (Santoro et al. 2004), possibly due to endocytosis, we also explored an impact of clathrin-non-binding mutant of TRIP8b, TRIP8b DCM, on HCN1 cell surface expression. In this experiment, we used the TRIP8b(1b-2) isoform, which was shown to have the strongest effect on HCN1 downregulation through internalization (Lewis et al. 2009; Santoro et al. 2009), together with the corresponding TRIP8b(1b-2) DCM mutant as a negative control. The 1b-2 isoform and its clathrin non-binding mutant precipitated clathrin in the same way as the 1a isoform as assessed by GST-pull down experiments (Fig. S2).

Co-expression of HCN1 with TRIP8b produced translocation of channels to large intracellular puncta, sometimes only one or two per cell, where both TRIP8b and clathrin were concentrated (Fig. 2a,b,c). When HCN1 was co-expressed with a clathrin-non-binding mutant of TRIP8b, the mutant still co-localized with the channels but clathrin no longer translocated to the areas of endocytosed HCN1 (Fig. 2d,e).

To probe the nature of the observed HCN-containing intracellular entities we stained them with endosomal and lysosomal markers. Staining of cells with anti-EEA1 antibody showed only partial co-localization of the puncta with early endosome marker (Fig. 3a), whereas LysoTracker dye revealed strong co-localization of HCN1 channels with lysosomes, with a majority of the puncta being positive for LysoTracker dye staining (Fig. 3c). The same general pattern was also observed when cells were co-transfected with the clathrin non-binding mutant of TRIP8b, suggesting that the mutation did not cause a substantial redistribution in the identity of the intracellular puncta (Fig. 3b,d).

To examine, whether TRIP8b and clathrin co-distributed at the subcellular level in the native environment, we partially purified clathrin-coated vesicles (CCV) according to the standard protocol (Girard et al. 2005) involving differential and gradient centrifugation (Fig. 4a), and analyzed the preparations at different purification stages by Western blotting with antibodies against TRIP8b, clathrin coat, and synaptic vesicle proteins. A major portion of TRIP8b was detected in the dense microsomal pellet SGp, similarly to synaptotagmin and synaptophysin. The SGs and CCV preparations, enriched with clathrin and representing CCVs of a significant purity degree (Fig. 4b), still contained a significant amount of TRIP8b (Fig. 4c).

The clathrin coat and some adaptor proteins can be stripped from CCVs by *in vitro* treatment with alkaline Tris buffer (Keen et al. 1979). TRIP8b contains no hydrophobic domains, it is present in the soluble protein fraction and can be completely released from membranes by treatment with Na_2CO_3, pH 11 buffer (Chen et al. 2001), thus its binding to membranes most likely result from protein-protein interactions. To determine the nature of TRIP8b association with CCVs, the pSGs fraction was incubated in CCV purification buffer A, pH 6.5 (as in Experimental Procedures) or in Tris-buffer pH 7.8, and further centrifuged to separate soluble proteins. Western blotting with specific antibodies revealed efficient (though not complete) release of clathrin and adaptor protein complex to the solution whereas a minor portion of TRIP8b became soluble (Fig. 5a).

In a complementary approach, we analyzed intact and stripped CCVs by sedimentation in a sucrose gradient. When CCV preparation was sedimented under “native” conditions (buffer A), clathrin and adaptin β subunit, the markers of CCVs, co-migrated in the gradient (Fig. 5b). Synaptophysin staining revealed the presence of lighter vesicles, in addition to CCVs. TRIP8b distribution was essentially similar to that of synaptophysin, indicating its association with both coated and non-coated vesicles. In the stripped CCV sample (Tris-buffer, pH 7.8), clathrin was detected at the top of the gradient (maximum at fraction 10, Fig. 5b). TRIP8b also shifted to the top, but still, a significant portion of it co-migrated with synaptophysin, a vesicular marker. These data correlate with the results of differential centrifugation analysis and can be interpreted as association of TRIP8 with both clathrin and integral membrane proteins of the vesicles.

The preparation of CCVs was also used to obtain purified clathrin to test whether its interaction with TRIP8b is direct or requires adaptor or auxiliary proteins. For this experiment, CCVs were additionally purified using D_2O-sucrose solution centrifugation, then proteins of the clathrin coat were stripped from CCVs in Dissociation buffer and clathrin triskelions were separated from adaptins using size-exclusion chromatography on Superose 12. Clathrin purity was determined by Coomassie, silver and anti-adaptins antibody staining (Fig. 6a,b,c and Fig. S3). Neither AP-1 nor AP-2 subunits were detected by appropriate antibodies (Fig. 6c and Fig. S3) or silver staining (Fig. 6b). However, on a silver stained gel, an additional minor band of the size ~ 70 kDa became visible (Fig. 6b, arrowhead). This protein was identified by mass-spectrometry as chaperone Hsc70 that binds near the C-terminus of the clathrin heavy chain and stimulates dissociation of clathrin lattice (Eisenberg and Greene 2007; Xing et al. 2010). The intensity of this band was about

1% of the intensity of the clathrin heavy chain band, as was calculated with ImageJ software.

The preparation of purified clathrin was incubated with GST-TRIP8b(1b-2) full or GST-TRIP8b(1b-2) DCM immobilized on glutathione-Sepharose, as described above. The bound proteins were eluted and equal parts of input, flow-through and eluates were analyzed by Western blotting with anti-clathrin heavy chain antibody. Fig. 6d shows that at least 2/3 of loaded clathrin were detected in the eluate from TRIP8b-resin but not in the eluates from mutant TRIP8b-containing matrices. Since clathrin heavy chain/Hsc70 ratio is about 100/1 in the clathrin preparation, it is highly unlikely that Hsc70 may serve as a mediator of TRIP8b/clathrin interaction.

Discussion

To investigate a potential function of TRIP8b as a signaling adaptor, we searched for its binding partners by affinity chromatography and, unexpectedly, identified clathrin and AP subunits as major components of the column eluates (Popova et al. 2008). In this study, we show that, in contrast with known TRIP8b partners, clathrin binds to the N-terminal part of the protein. We provide the evidence that TRIP8b and clathrin can interact in live cells, and that TRIP8b is present in clathrin-coated vesicles of the brain tissue.

TRIP8b was originally discovered as a prey in several independent yeast two-hybrid screens with cytoplasmic domains of membrane proteins as baits. In particular, we found (Popova et al. 2007) its interaction with the C-terminal tail of CIRL (Calcium-independent Receptor of Latrotoxin) which is a large neuronal adhesion G protein-coupled receptor implicated in regulation of secretion (Krasnoperov et al. 1997; Lelianova et al. 1997; Bittner et al. 1998; Krasnoperov et al. 2002). The amino acid sequence of TRIP8b suggests its hydrophilicity, although both soluble and membrane-bound forms of this protein were detected in biochemical tests (Chen et al. 2001). The analysis of TRIP8b structure reveals the presence of two clusters of TPR repeats in its C-terminal part with strongest homology to Pex5p, a protein which is involved in peroxisomal protein import (Amery et al. 2001; Fransen et al. 2008) and no significant homologies in its N-terminal part. TPR modules are indicative of protein to protein binding, however, they mediate a variety of intracellular interactions (Blatch and Lassle 1999; D'Andrea and Regan 2003) and thus cannot provide any valuable information about the protein function.

The specificity and structural characteristics of TRIP8b/clathrin interaction were studied with a set of TRIP8b mutants (Fig. 1a). In our *in vitro* binding assays, N-terminal truncations located the clathrin binding site in the N-terminal part of TRIP8b. Mutations in either ^{51}DLLDL or ^{107}LDLD clathrin-binding motif abolished binding thus indicating that there is a multi-point interaction between these two proteins. Using purified clathrin and TRIP8b, we also confirmed that the TRIP8b/clathrin interaction is direct and does not critically require the presence of other proteins, in particular, components of AP complexes, although we cannot completely exclude that a low molecular weight compound(s) may work as a cofactor of this interaction.

Besides two found clathrin-binding sites, TRIP8b contains tyrosine-based trafficking motif (YXXØ) in exon 2 (**Y**GK**L**) and four dileucine-based [DE]XXXL[LI] motifs in exons 4, 5, 6, and 16 (**E**EKP**LL**, **E**SRP**LL**, **D**GSD**LI** and **D**LDV**LL** respectively). These signals mediate interaction with μ and β subunits of AP complexes (Bonifacino and Traub 2003). Mutations in two of these signals (**Y**GK**L** and **E**SRP**LL**) appeared to be critical in order for TRIP8b to downregulate I_h (Santoro et al. 2009). Our data also suggest importance of other

TRIP8b regions, different for different adaptors, for the interaction with AP complexes (Fig. 1b).

To demonstrate the interaction of TRIP8b with clathrin in live cells, we chose a previously designed test system based on TRIP8b translocation in the course of HCN endocytosis in HEK293 cells, co-transfected with HCN and TRIP8b (Santoro et al. 2004). In this test, all of the cell surface HCN channels move to large intracellular membrane compartments that we found to strongly co-localize with a lysosome marker, and TRIP8b strongly concentrates in the region of these puncta. When we stained these cells for endogenous clathrin, we observed its translocation to the areas enriched with TRIP8b. The TRIP8b mutant, that did not bind clathrin, did not cause any change in the clathrin pattern, although it still co-localized with HCN1 channels. These data indicate that TRIP8b can bind to clathrin in *ex vivo* conditions independently from HCN interaction, and indirectly confirm our *in vitro* finding that clathrin and HCN interact with two different domains within the TRIP8b sequence.

In support of the postulated role of TRIP8b in endocytosis, we detected TRIP8b presence in the preparation of clathrin-coated vesicles partially purified from brain. When the clathrin coat was stripped from purified vesicles by treatment with alkaline Tris buffer, TRIP8b was also released into the solution, albeit partially, indicating the binding of TRIP8b to membrane proteins and/or lipids that may be explained by the presence of non-CCV membranes in the partially purified preparation. Sucrose density sedimentation experiments with intact and stripped CCVs showed association of TRIP8b with both clathrin coat and vesicular proteins. To some extent, these properties of TRIP8b resemble those of connecdenn, a new component of the machinery involved in synaptic vesicles (SV) endocytosis. In particular, connecdenn was shown to resist extraction with alkaline Tris buffer, and most of it co-migrated with SV fraction on a sucrose gradient in the Tris buffer (Allaire et al. 2006). Altogether, our data suggest that TRIP8b is an adaptor-like protein, capable of simultaneous multiple interactions with both membrane proteins and intracellular components of the endocytotic machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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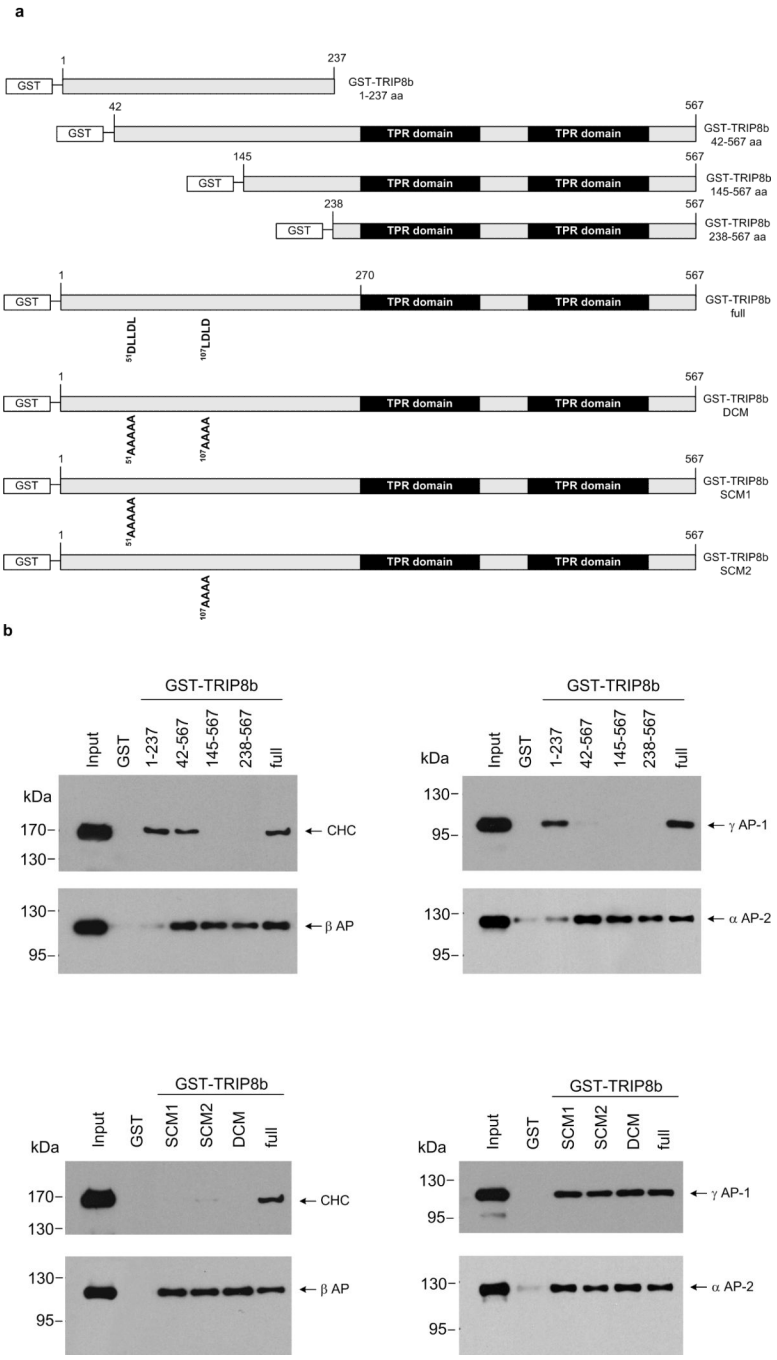

Fig. 1.

Clathrin-binding sites of TRIP8b. (a) Schematic representation of the GST-fusion mutant proteins used in the binding assays. (b) Soluble proteins from brain extracts were precipitated with equal amounts of GST or GST-TRIP8b(1a)-truncated proteins (upper panels) or GST-TRIP8b(1a) point mutants (lower panels) bound to glutathione-Sepharose beads. About 1/400 part of the precipitated rat brain extract (8 μg total protein) and 1/8 part of the eluates were analyzed by Western blotting with antibodies against CHC, β-adaptin subunit of AP complexes, γ-subunit of AP-1 or α-subunit of AP-2.

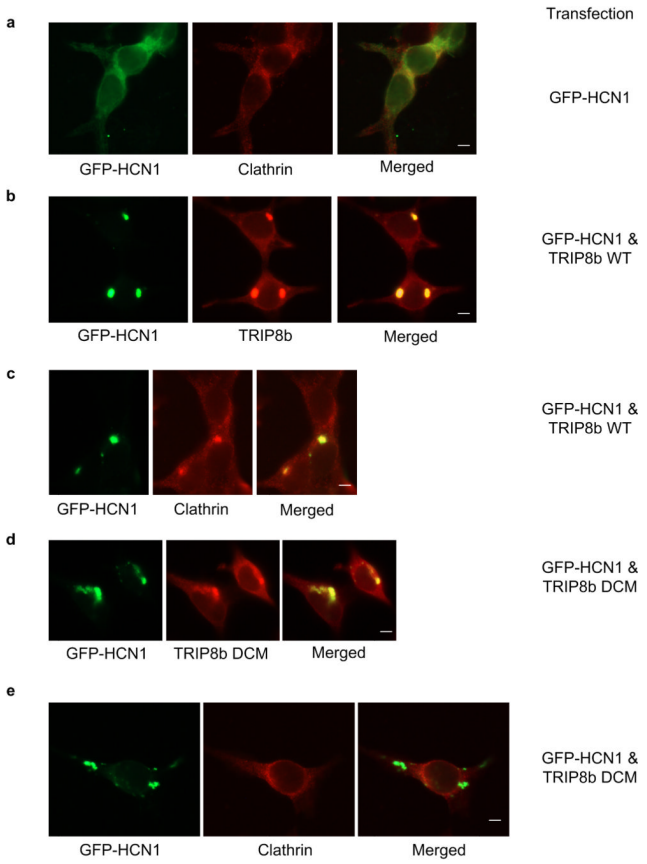

Fig. 2.

Analysis of the TRIP8b interaction with clathrin in transfected HEK293 cells. The cells were transfected with GFP-HCN1 only (a), GFP-HCN1 and TRIP8b(1b-2) (b, c), GFP-HCN1 and TRIP8b(1b-2) DCM (d, e) and stained with anti-TPIR8b (b, d) or rabbit anti-CHC (a, c, e) antibodies (red). Scale bar: 20 μm.

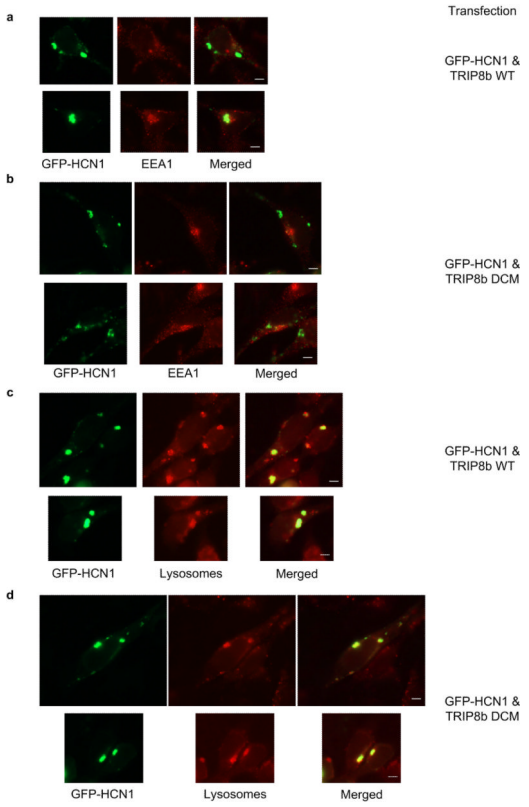

Fig. 3.

Analysis of the GFP-HCN1 localization in transfected HEK293 cells. The cells were transfected with GFP-HCN1 and TRIP8b(1b-2) (a, c) or GFP-HCN1 and TRIP8b DCM(1b-2) (b, d) and stained with anti-EEA1 antibody for early endosomes (a, b) or with LysoTracker dye for lysosomes (c, d) (red). Scale bar: 20 μm.

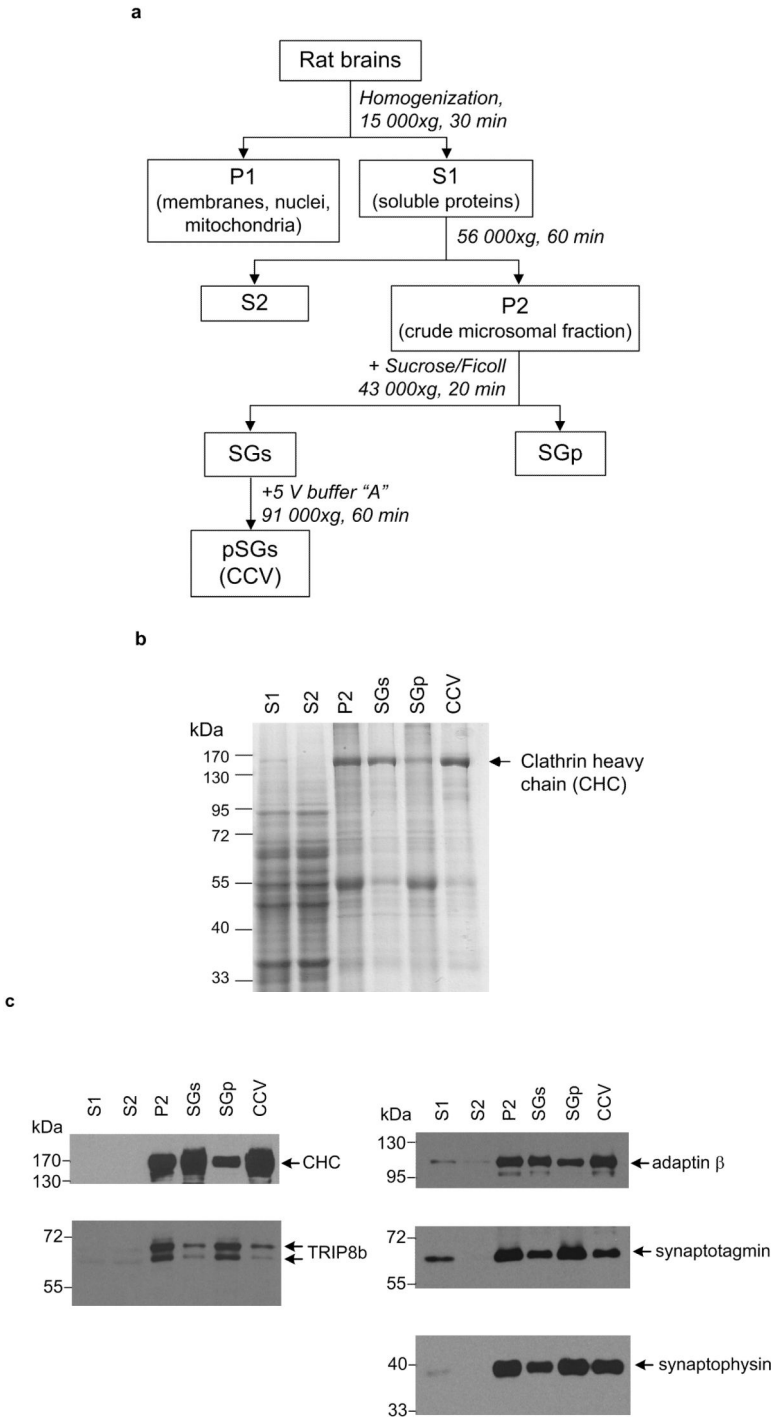

Fig. 4.

Purification of CCVs. (a) Scheme of CCV purification (adapted from (Girard et al. 2005)). S1, supernatant after low-speed centrifugation; P, pellet, SGs, supernatant from Ficollsucrose density gradient centrifugation; SGp, suspension of pellet from Ficoll-sucrose density gradient centrifugation. Equal protein aliquots of the successive fractions of CCVs isolation were analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (b) or

Western blotting with anti-CHC mouse antibody, anti-TRIP8b, adaptin β, synaptotagmin and synaptophysin antibodies (c).

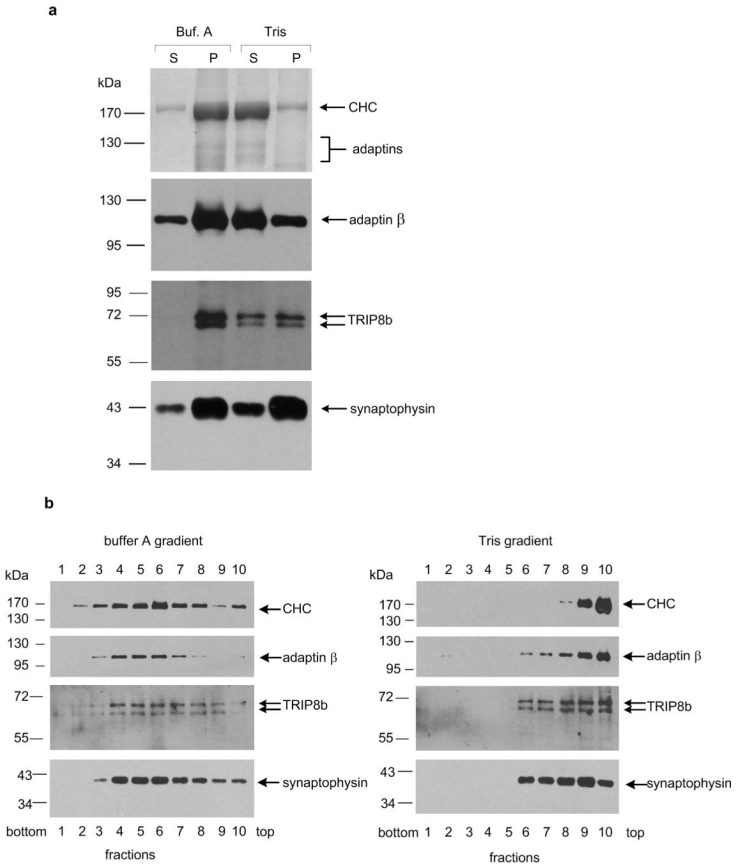

Fig. 5.

TRIP8b association with CCVs. (a) Partially purified rat brain CCVs were resuspended in buffer A or 0.5 M Tris buffer. The samples were centrifuged at 91,000 × g, and equal parts of the supernatant (S) and pellet (P) were analyzed by Coomassie brilliant blue staining (upper panel) or Western blotting with indicated antibodies (lower panels). (b) CCVs in buffer A and CCVs extracted with Tris buffer were loaded on the top of linear 20–50% sucrose gradients prepared in buffer A and Tris buffer, respectively, and were centrifuged at 145,000 × g for 1.5 h. The gradients were fractionated from the bottom, and equal volume aliquots of each fraction were analyzed by Western blotting with the indicated antibodies.

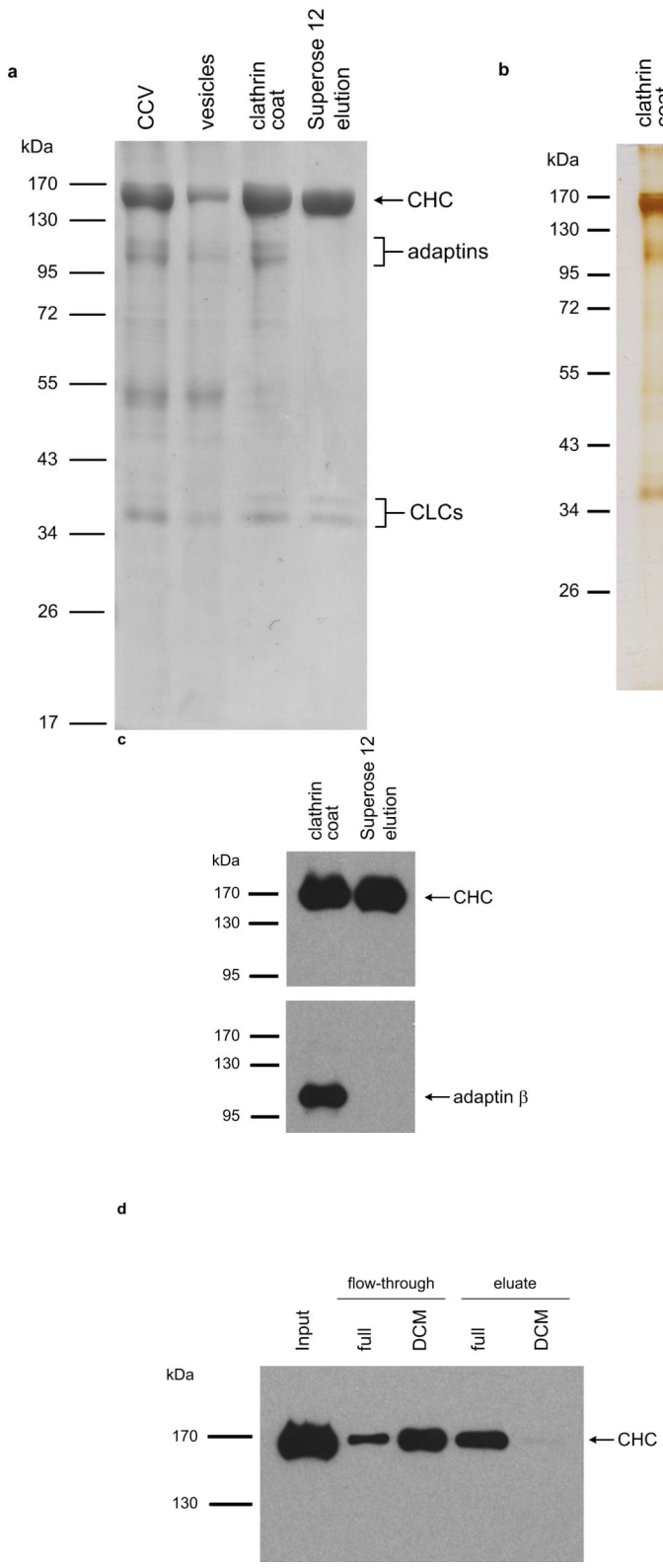

Fig. 6.

Direct binding of TRIP8b to clathrin. CCVs, purified as described in Materials and Methods, were incubated with alkaline Tris buffer and further centrifuged at 91,000 × g, resulting in a pellet (vesicles) and supernatant (clathrin coat). The supernatant was chromatographed on Superose 12 column and the elution fraction was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining (a), silver staining (b) or Western blotting with ant-CHC or adaptin β mouse antibodies (c). In GST-pull down experiments, 100 μl of purified clathrin solution (appr. 1 μg) were incubated with 40 μl of glutathione-Sepharose beads with prebound ~50 μg of GST-TRIP8b(1b-2) or GST-TRIP8b(1b-2) DCM. The bound proteins were eluted by Laemmli buffer, and about 1/10 part of the Superose 12 elution (input), the flow-through and the eluates from glutathione-Sepharose beads were analyzed by Western blotting with antibodies against CHC (d).