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CASK and Protein 4.1 Support F-actin Nucleation on Neurexins*

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Rearrangements of the actin cytoskeleton are involved in a variety of cellular processes from locomotion of cells to morphological alterations of the cell surface. One important question is how local interactions of cells with the extracellular space are translated into alterations of their membrane organization. To address this problem, we studied CASK, a member of the membrane-associated guanylate kinase homologues family of adaptor proteins. CASK has been shown to bind the erythrocyte isoform of protein 4.1, a class of proteins that promote formation of actin/spectrin microfilaments. In neurons, CASK also interacts via its PDZ domain with the cytosolic C termini of neurexins, neuron-specific cell-surface proteins. We now show that CASK binds a brain-enriched isoform of protein 4.1, and nucleates local assembly of actin/spectrin filaments. These interactions can be reconstituted on the cytosolic tail of neurexins. Furthermore, CASK can be recovered with actin filaments prepared from rat brain extracts, and neurexins are recruited together with CASK and protein 4.1 into these actin filaments. Thus, analogous to the PDZ-domain protein p55 and glycophorin C at the erythrocyte membrane, a similar complex comprising CASK and neurexins exists in neurons. Our data suggest that intercellular junctions formed by neurexins, such as junctions initiated by β-neurexins with neuroligins, are at least partially coupled to the actin cytoskeleton via an interaction with CASK and protein 4.1.

Neurexins are neuron-specific cell surface molecules that have been proposed as candidate proteins for synaptic cell adhesion (1). They were initially identified as receptors for α-latrotoxin, a spider venom toxin that triggers massive release of synaptic vesicles from the presynaptic terminal (2). In a splice-site specific manner, a subset of β-neurexins interacts with neuroligins, neuronal cell-surface proteins localized to postsynaptic membranes of excitatory synapses (3, 4). All neurexins have short, highly conserved intracellular C termini, which are recognized by the PDZ domain of CASK, a protein primarily expressed in brain, but also detectable in other tissues (5). CASK is a member of the family of MAGUK[1] proteins that are involved in the organization of membrane-associated protein complexes (6–8). In neurons, CASK is found at synapses and is localized at both the pre- and postsynaptic membranes of mainly excitatory synapses (9). In addition to neurexins, CASK also binds to the syndecan family of neuronal heparan sulfate proteoglycans, members of which are localized at synapses and axons as well as glial cells (10, 11). In epithelial cells, CASK interacts with junctional adhesion molecule at sites of intercellular contacts (12).

The function of CASK is unknown. In neurons, the majority of CASK is associated with membranes and it forms a stable, tripartite complex with the brain-enriched Veli proteins and the brain-specific Mint1 (13, 14). CASK anchors this complex to neurexins (13). One of the complex components, Mint1, provides an alternate mode of anchoring the tripartite complex to neurexins (15) and may be involved in synaptic vesicle traffic (16). This tripartite complex was proposed to serve as nucleation site for the assembly of synaptic plasma membrane proteins. It is evolutionarily conserved, as the *Caenorhabditis elegans* CASK homologue Lin-2 forms the complex by interaction with Lin-10, the Mint1 homologue, and Lin-7, the Velis homologue. In epithelial cells of *C. elegans*, these components are required for the correct localization of an epidermal growth factor-like receptor tyrosine kinase (17, 18).

Interestingly, CASK also binds to protein 4.1 (11), a peripheral membrane protein initially identified in erythrocytes. Protein 4.1 promotes association of F-actin with tetrameric spectrin and thereby stabilizes the cytoskeleton (19). Several genes encode distinct protein 4.1 isoforms that are differentially expressed in various tissues (20). A binding site for protein 4.1 is conserved in the HOOK region of three vertebrate MAGUK proteins, namely erythrocyte p55 (21), CASK (11), and the primarily epithelial/neuronal protein hDlg (human discs-large)/SAP97 (22). Studies in erythrocytes have shown that protein 4.1 interacts not only with p55 but also with the cell-surface protein glycophorin C, which are associated with each other (21, 23–25). This trimeric complex therefore provides two binding sites for protein 4.1, one found on the cytosolic domain of glycophorin C, the other on p55, which binds the extreme C terminus of glycophorin C via its PDZ domain (26). As a result, the majority of protein 4.1 associates with the plasma membrane at sites where glycophorin C is localized (25, 27). Interestingly, erythrocytes lacking protein 4.1 show a secondary loss of p55, hDlg, and glycophorin C (28, 29).

Although CASK has been best defined as a component of the tripartite complex, the fact that CASK was found to interact with several cell adhesion molecules, and is expressed also outside the brain, led us to hypothesize that CASK may have a more general role. We here demonstrate that, in brain, CASK interacts with neuronal protein 4.1N (30, 31). We reconstituted the complex of CASK and protein 4.1N on the cytosolic tail of neurexin I and observed the formation of stable actin/spectrin microfilaments. Importantly, this localized cytoskeletal assembly is temperature-dependent and is abolished in presence of ATP. As predicted by these results, CASK co-fractionates with

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[1] The abbreviations used are: MAGUK, membrane-associated guanylate kinase homologues; GST, glutathione *S*-transferase; GDI, GDP dissociation inhibitor protein.

preparations of the actin cytoskeleton, and neurexins could be recruited into these cytoskeletal preparations. Our data suggest a general function for CASK as an adaptor protein that links sites of cell adhesion to the intracellular actin cytoskeleton. Actin has recently been localized to the active zone of the presynaptic membrane (32), and several components characterized in this study are also synaptically localized, which could indicate a function of CASK in plastic changes of synaptic membranes.

MATERIALS AND METHODS

Vectors and Antibodies—The *Escherichia coli* expression vectors encoding GST-neurexin I and GST-neurexin IΔ10 (originally named GST-neurexin IΔ3) have been described previously (33). The vector encoding full-length GST-CASK was a gift of Dr. Anton Maximov (University of Texas Southwestern, Dallas, TX). Mutations were inserted by PCR using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Monoclonal antibodies directed against CASK were purchased from Transduction Laboratories (Cincinnati, OH). Antibodies directed against actin and spectrin were purchased from Sigma. Antibodies specific for protein 4.1N were a kind gift of Dr. S. Snyder (The Johns Hopkins University, Baltimore, MD). Monoclonal antibodies against GDP dissociation inhibitor protein (GDI) were a kind gift from Dr. R. Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Antibodies directed against Velis were described previously (13).

Solubilization of Rat Forebrains—Rat forebrains (PelFreez, Rogers, AR) were homogenized in 3 ml of buffer A (20 mM Hepes (pH 7.4), 125 mM potassium acetate, 5 mM magnesium chloride, 320 mM sucrose, 1.0% Triton X-100)/brain in the presence of protease inhibitors. The homogenate was incubated 60 min on ice and homogenized 30 times in a pestle tissue grinder using a slow speed stirrer. The samples were then centrifuged using a Sorvall S80-AT3 rotor with 80,000 rpm (280,000 × *g*) for 15 min at 2 °C, which pellets particles larger than 150 S. This first supernatant was kept on ice. The resulting pellet was suspended again in 2 ml of buffer A/brain in presence of protease inhibitors, incubated 60 min on ice, homogenized 20 times, and spun again at 150 S. The supernatants of both centrifugations were combined and passed through a 0.2-μm SFCA syringe filter (Nalgene).

GST Fusion Protein and Peptide Bead Affinity Chromatography—GST fusion proteins were expressed in *E. coli* and immobilized on glutathione-agarose beads (Sigma). Peptides of 12 amino acids length were synthesized on an ABI synthesizer. The peptide sequence CNKKNKDKEYYV corresponds to the 11 C-terminal amino acids of neurexin I, with an N-terminal cysteine for coupling to SulfoLink beads (Pierce). Proteins solubilized from rat forebrain were incubated with immobilized GST fusion proteins or peptides for 14 h at 4 °C. Where indicated, the samples were warmed to 30 °C for 60 min at the end of this incubation. For ATP regeneration, samples were adjusted to 1 mM ATP, 10 mM creatine phosphate, 1 mg/ml creatine kinase (Sigma). At the end of the binding, the beads were layered on 30 bead volumes of a sucrose cushion consisting of buffer A adjusted to 600 mM sucrose on ice, allowed to sink through the cushion, and then washed repeatedly. Samples were eluted first with one column volume of buffer A containing 800 mM potassium acetate for 10 min on ice. Where indicated, the second elution was performed using one column volume of 200 mM sodium carbonate (pH 11.5), 1.0% Triton X-100 for 20 min on ice. Finally, beads were eluted with one column volume of 2.0% SDS, 200 mM Tris (pH 6.8). For quantification, samples were analyzed by immunoblotting using ^{125}I-labeled secondary antibodies and the signals were quantified using a Fuji PhosphorImager and the ImageQuant program.

Formation of Cytoskeletal Assemblies in Vitro—Rat forebrains were homogenized as described above with the exception that the solubilization times on ice were 2 h each. Aliquots of the detergent solubilizate were incubated under mild agitation for 12–16 h at 4 °C. The samples were then diluted 1:4 with buffer A, and particles larger than 1000 S were pelleted using a Sorvall S80-AT3 rotor with 30,000 rpm (45,000 × *g*) for 11 min at 2 °C. The supernatants were discarded, and the pellets (P_1) suspended in buffer A. To wash these P_1 preparations, they were diluted in buffer A and incubated for 30 min on ice. Particles larger than 1000 S were then pelleted a second time in an Hitachi S45-A rotor with 34,000 rpm (52,000 × *g*) for 11 min at 2 °C to prepare P_2 and S_2 fractions. For stringent treatment of P_1 preparations, this washing step was performed in buffer A containing 400 mM potassium iodide or 400 mM potassium acetate, leading to salt-extracted P_2 and S_2 fractions. To analyze the cytoskeletal recruitment of GST-neurexin I and GST-neurexin IΔ10, these proteins were immobilized on glutathione-agarose beads and eluted using reduced glutathione. Rat brain detergent solubilizate was prepared as described above. The glutathione eluates were added at a final concentration of 20 μg of GST fusion protein/ml, bovine serum albumin was added at 1 mg/ml, and the samples were processed as described.

RESULTS

Protein 4.1N Binds to a Positively Charged Stretch of CASK and Recruits Actin and Spectrin—CASK and the two other MAGUK family members p55 and hDLG/SAP97 have been reported previously to interact with protein 4.1 from erythrocytes (11, 22, 23). In case of the erythrocyte MAGUK p55, the protein 4.1 binding site is located in the HOOK region, *i.e.* between the Src homology 3 and guanylate kinase domains (21). This sequence contains a stretch of four lysines, which is conserved in the three MAGUKs known to bind protein 4.1, but not in other members of this protein family (Fig. 1*A*). To address whether this tetralysine motif helps to constitute the protein 4.1 binding site on CASK, we mutated the amino acids $(KKKK)_{691-694}$ of CASK to TTTT and expressed this mutant CASK*4.1 as GST fusion protein. Identical amounts of wild-type GST-CASK and mutated GST-CASK*4.1 were immobilized on beads and were incubated with proteins solubilized from rat forebrain homogenates. We analyzed binding of a neuronal isoform of protein 4.1, protein 4.1N, that has been identified recently (30, 31). In initial experiments, the binding of protein 4.1N to GST-CASK was observed, but only at comparatively low yields (Fig. 1*A*, *lane 3*; data not shown). After varying the binding conditions, we noted that an elevation of the temperature at the end of the incubation led to markedly increased retention of protein 4.1 on CASK (Fig. 1*B*, *lane 5*). In contrast, binding of protein 4.1N to the GST-CASK*4.1 mutant was strongly reduced at elevated temperature (Fig. 1*B*, *lane 7 versus lane 5*) and in the cold (Fig. 1*C*). These results demonstrate that CASK interacts with this brain-specific protein 4.1 isoform, and that the conserved tetralysine motif contributes strongly. The salt resistance of this binding indicates that this motif provides not only electrostatic, but also hydrophobic interactions.

Protein 4.1 promotes interaction of spectrin and actin, and indeed both proteins were retained on immobilized CASK in presence of higher amounts of protein 4.1 (Fig. 1*B*, *lane 5*). Veli proteins, which interact with CASK on a domain distant to the protein 4.1-interaction domain, bound both wild-type and mutated CASK identically and this interaction was independent of the temperature (Fig. 1*A*, *lanes 3*, *5*, and *7*). Rab GDI, monitored as a negative control, was not retained (Fig. 1*A*).

Assembly of Actin Microfilaments on the CASK/Neurexin I Complex—CASK binds to the intracellular C termini of neurexins and other cell-surface proteins (5). To further characterize the interaction of CASK with neurexins and protein 4.1, we performed affinity chromatography experiments with the complete cytosolic sequence of neurexin I. The 56 C-terminal amino acids of neurexin I were expressed as a GST fusion protein (GST-neurexin I), and immobilized on beads. As a control, we used a truncated GST-neurexin fusion protein that lacked the 10 C-terminal amino acids (GST-neurexin IΔ10), and does not bind CASK (5). The experiment was performed in two affinity chromatography steps, using proteins solubilized from rat forebrain as starting material (Fig. 2*A*). First, proteins that bind to neurexin I independent of its C-terminal residues were removed by passing detergent-solubilized forebrain proteins over a column containing GST-neurexin IΔ10. One half of the flow-through from this column was loaded onto a first GST-neurexin I column, and the other half was adjusted to contain an ATP-regenerating system and was then loaded, in parallel, onto a second GST-neurexin I column. All three columns (the initial

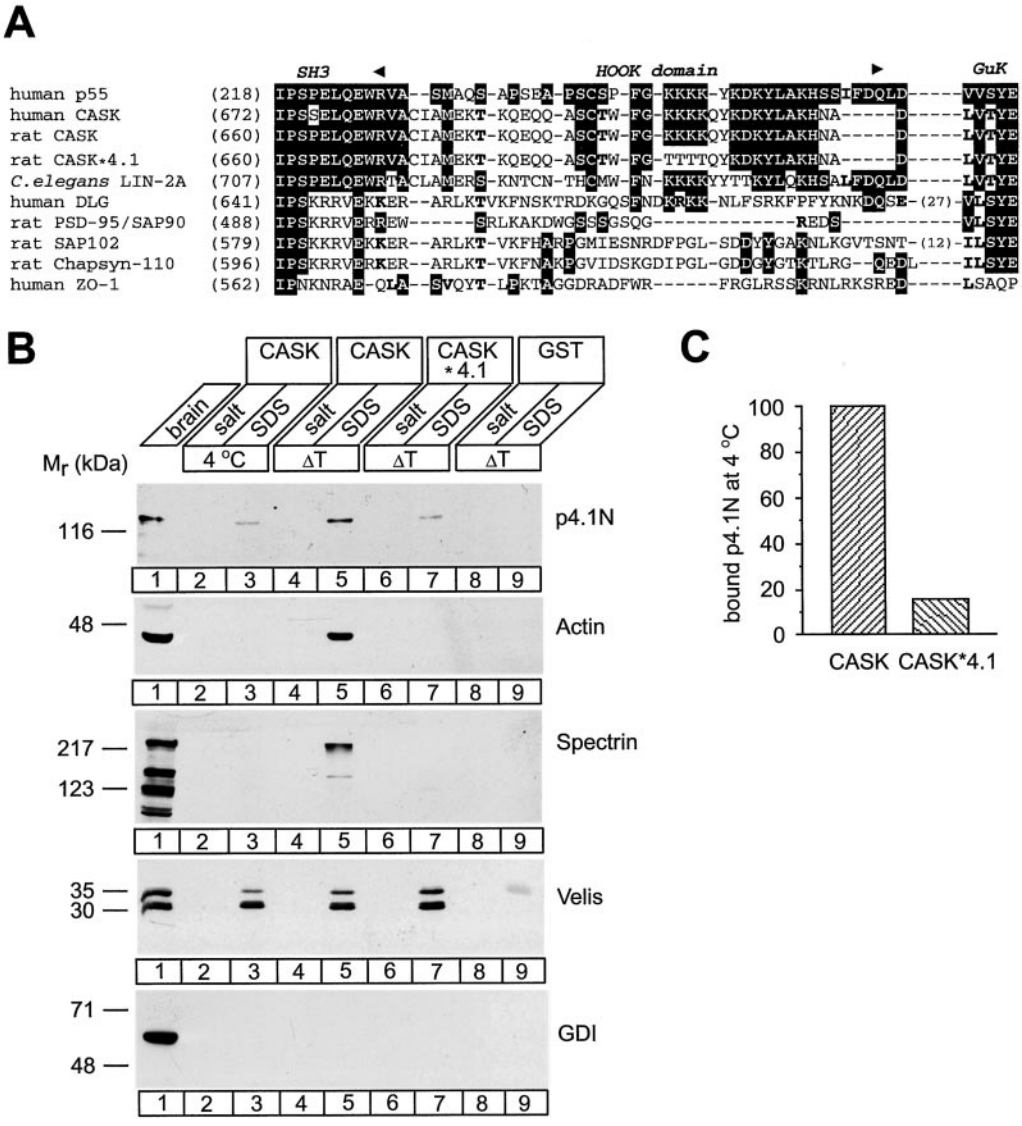

FIG. 1. **Specific binding of protein 4.1N to a positively charged domain in CASK is accompanied by immobilization of actin and spectrin.** *A*, sequence alignment of the HOOK region of different MAGUK proteins. CASK*4.1 is a point-mutated protein used in this study. Sequences are shown in *single-letter* amino acid code, with gaps indicated by *hyphens*. In *brackets*, the number of amino acids omitted in the alignment is indicated. Residues shared by the sequences with p55 are *highlighted* with a *black background*; similar residues are shown in *bold typeface*. *B*, Identical amounts of GST-CASK and a GST-CASK $KKKK_{691-694}$ to TTTT mutant (GST-CASK*4.1) were immobilized and incubated with solubilized rat forebrain proteins at 4 °C. Where indicated (ΔT), the samples were warmed to 30 °C for 60 min at the end of the incubation. Beads were first eluted at high ionic strength with 800 mM potassium acetate, then with SDS, and the eluted material was analyzed by immunoblotting. *C*, binding was performed at 4 °C, bound proteins were eluted with SDS, and retention of protein 4.1N was analyzed by quantitative immunoblotting.

GST-neurexin IΔ10 column, and the two subsequently loaded GST-neurexin I columns) were sequentially eluted with high salt solution, alkaline sodium carbonate buffer, and SDS.

Analysis of the eluted material by immunoblotting confirmed that CASK specifically bound to full-length GST-neurexin I independent of ATP (Fig. 2*B*, *lanes 6* and *7* and *lanes 9* and *10*) but not to GST-neurexin IΔ10 (Fig. 2*B*, *lanes 2–4*). This interaction was very strong, as CASK was quantitatively depleted from the flow-through of the column (data not shown). The interaction of CASK with neurexin I was resistant to elution with high salt, and CASK could only be recovered by stringent elutions with alkaline pH and SDS (Fig. 2*B*, *lanes 5–7*). If CASK serves as an adaptor molecule for protein 4.1 in neurons, we should be able to observe CASK-mediated recruitment of protein 4.1N on neurexins. Indeed, salt-resistant binding of protein 4.1N occurred and was dependent on neurexin's intact C terminus, suggesting that CASK was an intermediary (Fig. 2*B*, *lanes 6* and *7* and *lanes 9* and *10*). The negative control protein GDI was not retained (Fig. 2*B*).

Coomassie staining of these fractions revealed that CASK or protein 4.1N were not the most abundant proteins to be affinity-purified (Fig. 2*C*). Two proteins of ~230 and 43 kDa were the major protein components captured on GST-neurexin I but not on GST-neurexin IΔ10 (Fig. 2*C*, *lanes 6* and *7*). These proteins were identified as spectrin and actin by mass spectrometry and microsequencing. This suggests that, under our conditions, the complex of protein 4.1 and CASK immobilized on neurexins promotes salt-resistant assembly of actin microfilaments and spectrin. Interestingly, the recovery of spectrin and actin filaments was abolished in presence of ATP (Fig. 2*C*, compare *lanes 6* and *7* and *lanes 9* and *10*). In addition, a specifically bound protein of ~55 kDa was identified by mass spectrometry as fascin, an actin-bundling protein that is brain-specific in vertebrates (34).

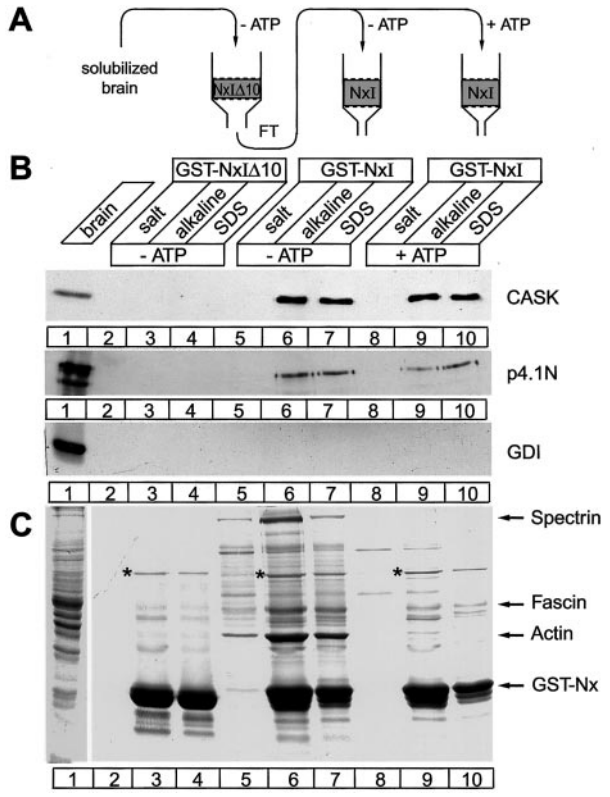

FIG. 2. **Binding of CASK to the cytosolic domain of neurexin mediates binding of protein 4.1N and is paralleled by assembly of actin microfilaments.** *A*, schematic description of the experiment. Rat forebrain proteins were solubilized in detergent passed over an affinity column containing a truncated form of the cytosolic domain of neurexin IΔ10 (*GST-NxIΔ10*). The flow-through (*FT*) was then loaded onto an affinity column containing the full-length cytoplasmic domain of neurexin I (*GST-NxI*) either in absence or presence of ATP. After binding overnight at 4 °C, the samples were warmed to 30 °C for 60 min. After washing, proteins bound to the three columns were sequentially eluted at high ionic strength with 800 mM potassium acetate, followed by carbonate buffer at pH 11.5 and by SDS. After SDS-PAGE, the resulting fractions were analyzed by immunoblotting in *B*, and by Coomassie blue staining in *C*. The amount of brain input shown corresponds to 10% of the eluted material. In *C*, the *asterisk* marks a protein band that is a contaminant of the expressed GST fusion proteins.

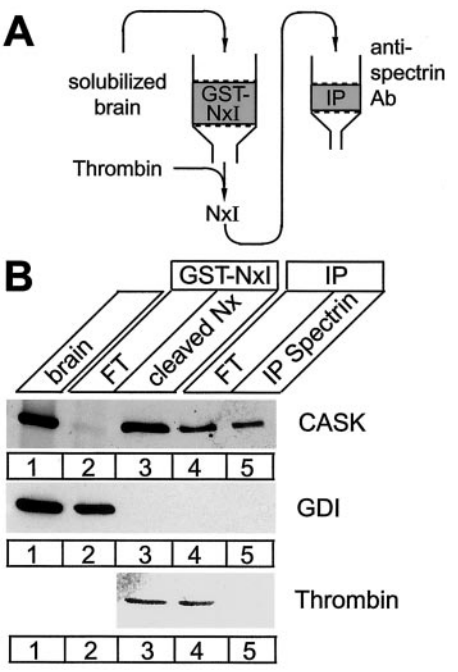

FIG. 3. **CASK and spectrin are components of a single protein complex bound to the C-terminal domain of neurexin I.** *A*, schematic description of the experiment. Rat brain proteins were solubilized and incubated with immobilized GST-neurexin I to allow for binding of CASK and actin cytoskeleton assembly as described in Fig. 2. The bound proteins were then eluted from the column by cleavage of the GST-neurexin I with thrombin, which cuts the linker between GST and the cytoplasmic neurexin domain. The thrombin eluate was then subjected to immunoprecipitation (*IP*) with antibodies (*Ab*) directed against spectrin. *B*, the fractions of the experiment described in *A* were subjected to SDS-PAGE and analyzed by immunoblotting for CASK and for GDI (as a negative control of the GST affinity chromatography), and by Amido Black staining for thrombin. The amounts of inputs shown in *lanes 1* and *3* correspond to 20% of the eluates in *lanes 3* and *5*, respectively. Identical amounts of input and flow-through fractions are loaded for the analysis of both chromatographic steps.

CASK, Protein 4.1, and Actin/Spectrin Filaments Are in the Same Protein Complex—The above results do not exclude that two different neurexin populations exist, one attached to protein 4.1 and CASK, the other to filamentous actin immobilized independently of protein 4.1. To address the composition of these complexes more directly, we took advantage of a thrombin cleavage site between the GST moiety and neurexin I. This makes it possible to use mild thrombin treatment to elute neurexin's cytoplasmic domain together with proteins bound to neurexin (Fig. 3*A*). Detergent-solubilized forebrain proteins were loaded onto immobilized GST-neurexin I as described above, and proteins bound to neurexin were cleaved off the beads with thrombin together with the cytoplasmic domain of neurexin. The cytoskeletal components of this complex were immunoprecipitated with antibodies directed against spectrin, and the distribution of CASK was determined. CASK was quantitatively bound to GST-neurexin I and, as expected, could be eluted by thrombin, whereas GDI was completely recovered in the initial flow-through (Fig. 3*B*, *lanes 1–3*). Coomassie staining and immunoblotting confirmed that the spectrin-containing actin microfilaments were formed on the beads and released after thrombin cleavage of the neurexin domain (data not shown). The material eluted with thrombin was subsequently subjected to immunoprecipitation with antibodies directed against spectrin and was shown to contain CASK (Fig. 3*B*, *lanes 4* and *5*). Thrombin served as negative control for the immunoprecipitation (Fig. 3*B*). This allows to conclude that CASK is an integral part of the spectrin/actin assemblies formed around the cytosolic domain of neurexin I *in vitro*.

CASK Partitions into in Vitro Assembled Actin Microfilaments—The results presented above suggest that CASK together with members of the protein 4.1 family may function to link the actin cytoskeleton to cell-surface molecules like neurexins or syndecans. This would predict that CASK is incorporated into actin cytoskeleton preparations assembled *in vitro* from brain extracts. To test this, we incubated detergent extract from total rat brain at a high protein concentration, a condition under which actin microfilaments rapidly assemble *in vitro* once filament nucleation is provided (35). The brain extract was incubated overnight in the cold and subsequently centrifuged. Immunoblotting (Fig. 4*A*) and Coomassie Blue staining of SDS gels (Fig. 4*B*) revealed that CASK was partially recovered in the P_1 pellet together with the cytoskeletal assemblies containing actin, spectrin, protein 4.1N, and fascin (Fig. 4, *A and B*, *lanes 1* and *2*; data not shown). Veli proteins, binding partners of CASK, were detected in the cytoskeletal preparation at a lower yield than CASK itself, consistent with an indirect recruitment via CASK (Fig. 4*A*). GDI, used as a negative control, was absent (Fig. 4*A*). After resuspension of the P_1 pellet and repeated centrifugation, all of CASK was recovered in P_2 together with actin, spectrin, and fascin (Fig. 4*A*, *lanes 3* and *4*). However, when the P_1 pellet was resuspended in buffer

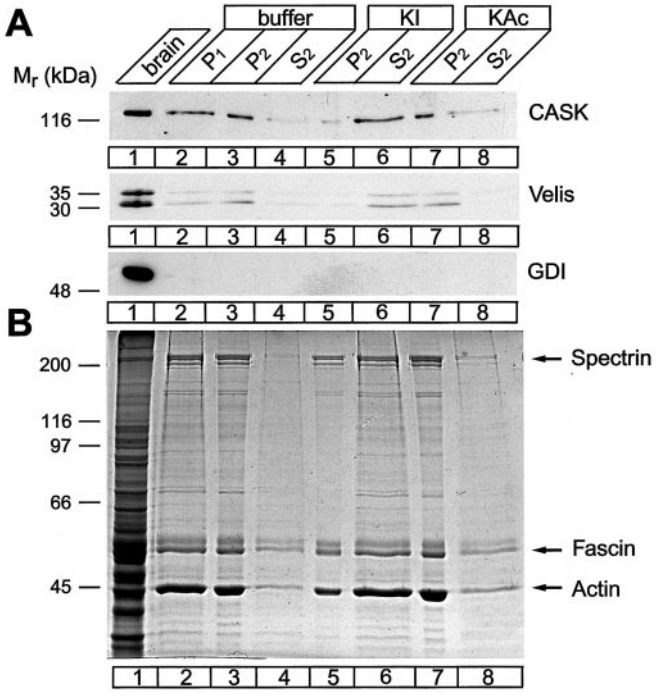

FIG. 4. **CASK and Mint1 are incorporated into actin microfilaments assembled *in vitro*.** Detergent-solubilized rat brain proteins (*lane 1*) were incubated at 4 °C overnight and centrifuged to obtain pellet P_1 (*lane 2*). The P_1 pellet was then resuspended either in the original buffer, or in buffer containing 400 mM of either potassium iodide (*KI*) or potassium acetate (*KAc*). These suspensions were centrifuged again to prepare the second pellet P_2 and the supernatant S_2. *A*, analysis of the samples by immunoblotting using antibodies directed against the indicated proteins. *B*, samples were subjected to SDS-PAGE followed by Coomassie Blue staining. The amount of solubilized brain input shown in *lane 1* corresponds to 1% of the fractions in *panel A*, and to 5% of the fractions in *panel B*.

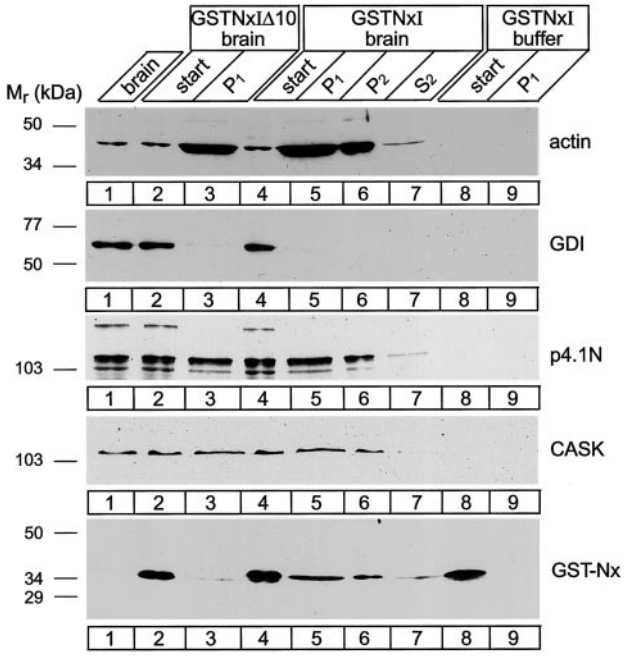

FIG. 5. **The cytoplasmic domain of neurexin I is recruited into actin microfilaments.** Soluble GST-neurexin I and GST-neurexin IΔ10 corresponding to the full-length or truncated cytoplasmic domain of neurexin I, respectively, were prepared. They were incubated with detergent-solubilized rat brain proteins at 4 °C overnight. A P_1 fraction was prepared by centrifugation of the samples, then resuspended and centrifuged again to prepare the second pellet P_2 and the supernatant S_2. Samples were analyzed by immunoblotting using antibodies directed against the indicated proteins. Neurexin was detected using antibodies that had been raised against GST-neurexin I. The amount of solubilized brain input in *lane 1* is identical to the start amounts shown in *lanes 2*, *4*, and *8* and corresponds to 1% of the prepared fractions.

containing potassium iodide, a chaotropic salt, the majority of CASK as well as the cytoskeletal proteins were released into the supernatant S_2 (Fig. 4, *A* and *B*, compare *lanes 4* and *6*). A high salt wash with potassium acetate neither extracted CASK nor the cytoskeletal assemblies, further demonstrating their firm association (Fig. 4, *A* and *B*, *lanes 7* and *8*). Notably, actin microfilament assembly from brain was not affected under our conditions by a final increase in the incubation temperature to 30 °C, and only weakly reduced in presence of ATP (data not shown). This provides evidence that the effects of temperature and ATP on localized actin assembly described above are specific.

The Cytoplasmic Domain of Neurexin I Associates with Actin Microfilaments—To further characterize the interaction of neurexin I with the actin cytoskeleton, we established an assay to analyze cytoskeletal recruitment of recombinant proteins. Soluble GST-neurexin I and GST-neurexin IΔ10 were prepared, and added to detergent-solubilized rat brain proteins (Fig. 5, *lanes 1*, *2*, and *4*). Actin microfilaments were formed as described and recovered in a high speed P_1 fraction (Fig. 5, *lanes 3* and *5*), which excluded the negative control protein GDI. CASK, as well as protein 4.1N, could be recovered in these cytoskeletal assemblies (Fig. 5, *lanes 3* and *5*). After resuspension of P_1 and a second centrifugation, CASK and protein 4.1N were found almost completely in the P_2 fraction (Fig. 5, *lanes 6* and *7*). The truncated GST-neurexin IΔ10 was absent from the pelleted cytoskeletal fraction (Fig. 5, *lanes 2* and *3*). However, GST-neurexin I was recruited into the P_1 fraction together with actin microfilaments (Fig. 5, *lanes 4* and *5*).

After resuspension of P_1 and repeated centrifugation, this fraction of GST-neurexin I was almost quantitatively recovered in the P_2 preparation (Fig. 5, *lanes 6* and *7*). In absence of brain proteins, GST-neurexin I remained completely soluble (Fig. 5, *lane 9*). These observations confirm that the cytosolic domain of neurexin I interacts with cytoskeletal assemblies in a stable manner dependent on its C terminus, presumably via CASK and protein 4.1.

Protein 4.1 Binding to CASK and Neurexins Analogous to p55 and Glycophorin C—The cytoplasmic domain of neurexins contains a motif similar to a protein 4.1 binding site described in glycophorin C, a membrane protein in erythrocytes (20). Similarities were only found in the protein 4.1- and CASK-interacting sequences and are also conserved in the syndecan family of proteins (Fig. 6*A*). Therefore, two sites in neurexins could cooperate in the recruitment of protein 4.1, one provided by neurexin-bound CASK, the second on the cytosolic domain of neurexin itself. To test for such a cooperation, CASK was solubilized from rat brain and captured either on the complete cytoplasmic domain of neurexin I, or on a peptide corresponding to the extreme C terminus of neurexin. The latter condition would provide only a single binding site for protein 4.1, namely on CASK that was retained via its PDZ domain interaction with the neurexin peptide. Identical amounts of CASK were captured under both conditions (Fig. 6*B*, *lanes 5* and *7*). Protein 4.1N was recovered at a higher yield on the cytoplasmic domain of neurexin containing CASK than on peptide-bound CASK (Fig. 6*B*, *lanes 5* and *7*). In independent experiments, identical amounts of CASK were immobilized either indirectly on the cytosolic domain of neurexin or as recombinantly expressed GST-CASK. Protein 4.1N recovery was markedly higher in the first condition (data not shown). Notably, the juxtamembraneous binding motif of neurexin for protein 4.1 alone is not sufficient, as the C-terminally truncated neurexin does not retain

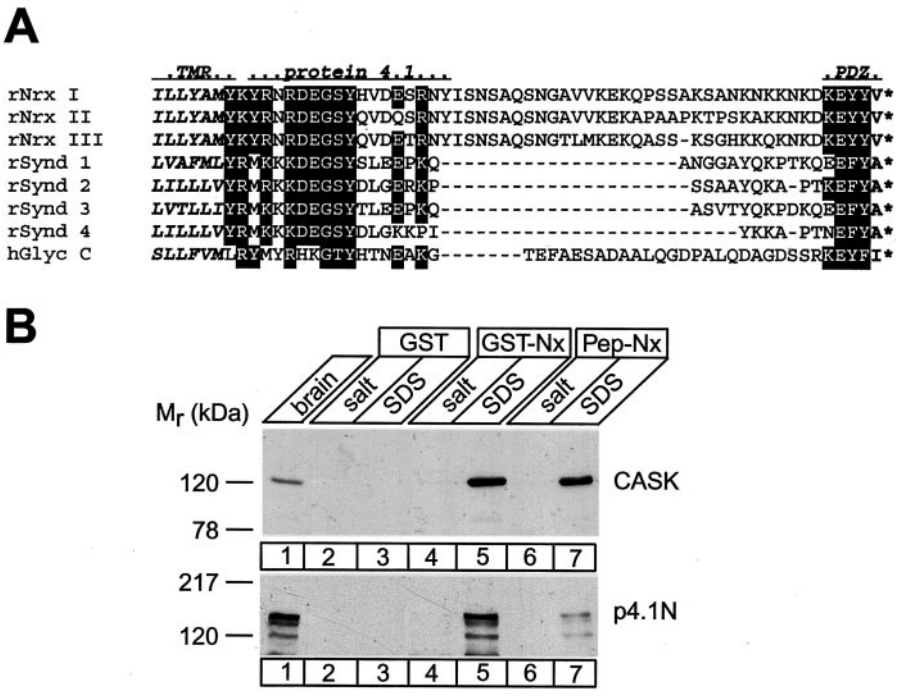

FIG. 6. **Analogy of protein 4.1 recruitment to the plasma membrane of neurons and erythrocytes.** *A*, sequence alignment of the three rat neurexins (*rNrx I*, *II*, and *III*), four rat syndecans (*rSynd 1*, *2*, *3*, and *4*), and human glycophorin C (*hGlyc C*). Sequences are shown in *single-letter* amino acid code, with gaps indicated by *hyphens*. The locations of the transmembrane region (*TMR*), the binding site for protein 4.1 as deduced in glycophorin C, and the binding site for the PDZ domains of CASK or p55 are identified on top of the alignment. Amino acids in the transmembrane region are shown in *bold italic typeface*, and residues in the cytoplasmic tails that are shared by the sequences are *highlighted* with a *black background*. *B*, improved binding of protein 4.1N to neurexin-bound CASK as compared with CASK alone. Rat brain proteins were solubilized and incubated with immobilized GST, GST-neurexin I, or a peptide corresponding to the extreme neurexin C terminus at 4 °C overnight. Beads were first eluted at high ionic strength with 800 mM potassium acetate, then with SDS, and the eluted material was analyzed by immunoblotting. The amount of brain input shown corresponds to 5% of the eluted material.

protein 4.1 under these conditions (compare Fig. 2*B*). Our results therefore indicate that, in the presence of the additional site provided by CASK, protein 4.1 can be efficiently captured by neurexins.

DISCUSSION

CASK is a ubiquitously expressed, but brain-enriched, member of the MAGUK family of adaptor proteins (5). It has been functionally implicated in two different contexts. In neurons, it assembles into a tripartite complex with Velis and Mint1, and links this complex to the cytosolic tail of neurexins (13). This complex has been proposed to couple neurexin-mediated cell adhesion to the exocytotic machinery of synapses. CASK may exert a second, more general function as adaptor for the actin cytoskeleton, as it binds protein 4.1R from erythrocytes (11). Members of the protein 4.1 family promote the interaction of actin and spectrin and lower the critical concentration needed for actin assembly (36, 37). We here describe and characterize the binding of CASK and protein 4.1N, which is enriched in central and peripheral neurons (30, 38), and propose that this interaction may be involved in the localized assembly of actin filaments.

We first characterized the interaction of neuronal protein 4.1N to CASK and observed it to be salt-resistant and temperature-dependent. The reason for the effect of elevated temperature is unclear, but it may promote greater flexibility in the binding domains involved. Mutation of a tetralysine motif in the HOOK region of CASK strongly reduced binding of protein 4.1, indicating that this motif provides electrostatic and hydrophobic interactions in the context of the protein 4.1 binding domain. The interaction with protein 4.1N was not abolished in the CASK mutant, indicating that other residues in the HOOK region are participating. Notably, this tetralysine motif is present in several MAGUK proteins but alone is not sufficient to confer protein 4.1 binding (39). Importantly, actin and spectrin were immobilized concomitantly to protein 4.1 on CASK. We reconstituted the CASK-mediated recruitment of protein 4.1N on the cytosolic domain of neurexins and observed temperature-dependent actin/spectrin microfilament assembly in the same complex. As protein 4.1N binding to CASK is enhanced at elevated temperatures, a threshold concentration of protein 4.1 may have to be exceeded before actin assembly is initiated. Additionally, the rate of reaction between protein 4.1 and actin depends on elevated temperatures consistent with a high activation energy (37). Interestingly, actin microfilament assembly on the cytosolic domain of neurexin was blocked in the presence of ATP. A likely explanation is the inhibition of protein 4.1 function by phosphorylation, which reduces its ability to promote binding of F-actin to spectrin (40, 41). These microfilaments also contained the actin-bundling protein fascin, which induces the formation of membrane protrusions, and in vertebrates is mostly expressed in brain (34, 42, 43). To approach these assemblies from the cytoskeletal side, we prepared actin/spectrin microfilaments *in vitro*. Their protein composition was similar to the microfilaments formed on neurexin, and they contained CASK along with protein 4.1N. Notably, actin assembly in this preparation is not affected by an increase in temperature and only weakly reduced in presence of ATP. Therefore, the blockade of actin assembly on the cytosolic domains of neurexin is likely to be specific for actin/spectrin filament formation as nucleated by protein 4.1. Importantly, recruitment of neurexin into these actin microfilaments was observed together with CASK and protein 4.1N, and these proteins were stably associated. This retention of neurexin was dependent on the extreme C-terminal amino acids, in agreement with an involvement of CASK, as this truncation of neurexin disrupts their interaction.

We present a model that suggests that the actin cytoskeleton is assembled by neurexins at neuronal plasma membranes and by glycophorin C at the erythrocyte membrane via analogous mechanisms (Fig. 7). Both of these membrane proteins contain one conserved binding site for protein 4.1 close to their transmembrane domain. A member of the MAGUK family of adaptor proteins provides the second site. In erythrocytes, this is p55, which attaches to glycophorin C via its PDZ domain and contains a binding site for protein 4.1 (21, 25). At neuronal plasma membranes, CASK interacts with neurexins and members of the protein 4.1 family, engaging the same domains. Interestingly, CASK also binds strongly to the cytosolic C terminus of glycophorin C (data not shown). As glycophorin C is expressed in a variety of tissues, this interaction may be relevant outside

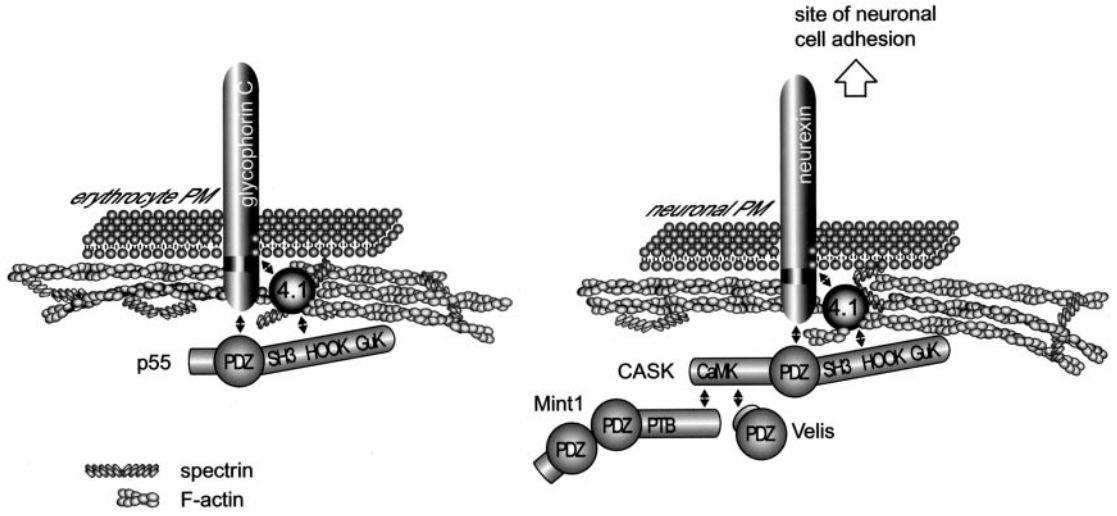

FIG. 7. **Model of CASK function as an adaptor for protein 4.1 that links cell-surface proteins to the actin cytoskeleton.** Protein 4.1 binds simultaneously to a juxtamembraneous sequence in the cytoplasmic domains of the cell-surface proteins glycophorin C and neurexins and to a short sequence in p55 and CASK, respectively. Thereby a stable complex is formed, which serves as an anchor for actin microfilament assembly. In neurons, CASK binds to both Velis and Mint1 in addition to protein 4.1.

the brain to provide CASK and members of the protein 4.1 family with a membrane-bound partner.

The actin/spectrin cytoskeleton is involved in many aspects of membrane dynamics from locomotion to cell polarity and the formation of local specializations of the plasma membrane like cell junctions (44–46). Generally, linker proteins couple the positional extracellular information provided by adhesion molecules to intracellular changes of the actin cytoskeleton. In the case of tight junctions, the connection of occludin to the submembraneous actin cytoskeleton is mediated by the MAGUK proteins ZO-1 and ZO-2 (47) Adherens junctions, formed by cadherins, are linked to the actin cytoskeleton by α-catenin (48). Integrins mediate cell contact with the extracellular matrix, and the β-integrins interact with several actin-binding proteins (49). For neurexins, we propose that CASK serves as an adaptor to the actin cytoskeleton and links extracellular binding to the cytoskeleton by recruiting protein 4.1. This hypothesis is supported by the following results. First, the C terminus of neurexin I is a high affinity binding partner for CASK (5).[2] Second, affinity purification of neurexins on immobilized α-latrotoxin leads to the efficient copurification of CASK (50). Third, affinity chromatography on immobilized CASK purifies neurexins (13) and protein 4.1N (this study). Fourth, the actin cytoskeleton assembles efficiently on the cytosolic domain of neurexins *in vitro*, but only when the CASK binding site is present (this study). Fifth, neurexins and CASK are targeted into cytoskeletal assemblies (this study). Sixth, the proteins described here to participate in localized actin filament assembly are colocalized at synapses. CASK and protein 4.1N are primarily expressed in neurons and synaptically localized (10, 30, 31), and neurexins are exclusively expressed in neurons where they are either synaptic or close to a synapse (2, 51). In the case of CASK as adaptor to the cytoskeleton, the extracellular event in question could be cell-cell interaction as mediated by β-neurexins. This may take place at synapses as neuroligins, the splice site-specific adhesion partners of β-neurexins, are at the postsynaptic membrane (4). Like glycophorin C (20), neurexins could be recruited with CASK and protein 4.1N to the submembraneous actin/spectrin cytoskeleton, and formation of this complex could affect the protein composition of the neuronal plasma membrane. Interestingly, protein 4.1N participates in the targeting of a glutamate receptor subunit to the neuronal cell surface (52). Furthermore, the protein 4.1 binding domain in the MAGUK protein Dlg in *Drosophila* participates in synaptic targeting and is necessary for Dlg recruitment into the subcortical network of neuromuscular junctions (53). Additionally, actin/spectrin microfilament assemblies induced on neurexins in presence of CASK and protein 4.1N could potentially alter the membrane morphology. Finally, both CASK and protein 4.1N can enter the nucleus and interact with regulatory nuclear proteins, which potentially transduces signals from sites of cell adhesion to gene expression (54–57).

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