CASK: A Novel *dlg*/PSD95 Homolog with an N-Terminal Calmodulin-Dependent Protein Kinase Domain Identified by Interaction with Neurexins

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Neurexins are neuronal cell surface proteins with hundreds of isoforms. In yeast two-hybrid screens for intracellular molecules interacting with different neurexins, we identified a single interacting protein called CASK. CASK is composed of an N-terminal Ca^{2+} , calmodulin-dependent protein kinase sequence and a C-terminal region that is similar to the intercellular junction proteins *dlg*-A, PSD95/SAP90, SAP97, ZO1, and ZO2 and that contains DHR-, SH3-, and guanylate kinase domains. CASK is enriched in brain in synaptic plasma membranes but is also detectable at low levels in all tissues tested. The cytoplas-

mic domains of all three neurexins bind CASK in a salt-labile interaction. In neurexin I, this interaction is dependent on the C-terminal three residues. Thus, CASK is a membrane-associated protein that combines domains found in Ca^{2+} -activated protein kinases and in proteins specific for intercellular junctions, suggesting that it may be a signaling molecule operating at the plasma membrane, possibly in conjunction with neurexins.

Key words: *CaM* kinase; synapse; neurexins; yeast-two hybrid; intercellular junctions; disks-large

Neurexins are highly polymorphic cell surface proteins expressed in central and peripheral neurons. There are at least three neurexin genes in mammals, each of which contains two independent promoters that initiate transcripts for α - and β -neurexins (Ushkaryov et al., 1992, 1994; Ushkaryov and Südhof, 1993). The primary transcripts of neurexins are extensively alternatively spliced, resulting in hundreds to thousands of isoforms (Ullrich et al., 1995). The six principal neurexins (neurexins 1 α –3 α and 1 β –3 β) and their alternatively spliced forms are expressed in a differential manner in brain, with each type of neuron coexpressing different subsets of neurexins.

The structure of the neurexins resembles that of a cell surface receptor. Three putative extracellular ligands for neurexins have been identified. (1) Neurexin 1 α binds with high affinity to α -latrotoxin, a potent excitatory neurotoxin from black widow spider venom (Ushkaryov et al., 1992; Davletov et al., 1995). (2) A subset of β -neurexins lacking an insert in splice site #4 binds to neuroligins, a recently discovered family of neuronal cell surface receptors (Ichtchenko et al., 1995). Similar to neurexins, neuroligins are encoded by at least three genes and are subject to alternative splicing (Ichtchenko et al., 1996). (3) A 29 kDa protein called neurexophilin copurifies with neurexin 1 α and structurally resembles a neuropeptide, suggesting that it may represent a soluble ligand for α -neurexins (A. Petrenko and T. Südhof, unpublished observations).

The structures of neurexins, their highly polymorphic nature, and the splice site-specific interaction of β -neurexins with neuroligins suggest a function as cell adhesion/signaling molecules and a possible role in mediating intercellular recognition events. Because α -latrotoxin acts at synapses and at least some neurexins localize close to synapses, it has been speculated that neurexins may be involved in the formation of synaptic junctions. Such a role is supported by the interaction of the intracellular domains of neurexins with synaptotagmin I, a synaptic vesicle Ca^{2+} -sensor (Petrenko et al., 1991; Hata et al., 1994). However, although this interaction is stoichiometric and sequence-specific, it could not be detected by a yeast two-hybrid assay (Hata et al., 1995), and synaptotagmin I is not required for α -latrotoxin action (Geppert et al., 1994). Therefore, the functional importance of the interaction of neurexins with synaptotagmin I is unclear.

The molecular components of several types of intercellular junctions have been characterized in detail in recent years. Pioneered by the description of the *dlg-A* gene in *Drosophila* (Woods and Bryant, 1991), a class of molecules was discovered with members at different types of intercellular junctions. The *dlg-A* gene encodes a component of septate junctions that are disrupted by mutations in *dlg-A* (Woods and Bryant, 1991). The gene is evolutionarily conserved, with at least two homologs in humans (Lue et al., 1994; Mazoyer et al., 1995). In addition, *dlg-A* is homologous to the tight junction proteins ZO-1 and ZO-2 (Itoh et al., 1993; Tsukita et al., 1993; Willott et al., 1993; Jesaitis and Goodenough, 1994) and the synaptic proteins PSD95/SAP90 and SAP97 (Cho et al., 1992; Kistner et al., 1993; Müller et al., 1995). All of these proteins exhibit similar domain structures: a set of N-terminal repeats called DHR domains (for “disks-large homology region”), a middle SH3 domain, and a C-terminal region comprised of a guanylate kinase domain that is probably not catalytically active. An additional member of this protein family is p55, a plasma membrane protein of erythrocytes that is involved in attaching the cytoskeleton to the membrane and may be im-

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portant for determining erythrocyte shape (Ruff et al., 1991; Alloisio et al., 1993; Marfati et al., 1994). The function of p55 suggests that members of this protein family may be involved in establishing specialized membrane structures via interactions with the membrane cytoskeleton and integral membrane proteins, resulting either in intercellular junctions between cells or a particular membrane shape in a cell.

We have now used yeast two-hybrid (Y2 H) screens to identify molecules that bind to the intracellular C terminus of neuexins. A single interacting protein was discovered that is composed of an unusual combination of domains. Its N-terminal region is highly homologous to Ca^{2+} , calmodulin-dependent protein kinases (CaM kinases) that are ubiquitous Ca^{2+} -dependent signaling proteins. Its C terminus is very similar to the dlG-homologs DLG2, p55, PSD95/SAP90, and ZO-1/ZO-2. This is the first time that a fusion between a CaM kinase and a junction-like protein has been observed, suggesting a signaling molecule that may be involved in cell–cell communication events.

MATERIALS AND METHODS

Y2 H screens and interaction assays. The bait vectors pBTM116Ne1A, 1AΔ1, 1AΔ2, 1AΔ3, 1AΔ4, 2A, and 3A were obtained by subcloning the *SmaI*-*PstI* insert fragments from the corresponding pGEX-vectors (Hata et al., 1993) into the same sites of pBTM116 (Vojtek et al., 1993). A cDNA library was constructed in the *NotI*-site of Y2 H prey vector pVP16 (Vojtek et al., 1993) from poly(A⁺)-enriched rat brain RNA using the GIBCO Choice system (Grand Island, NY). Yeast strain L40 (Vojtek et al., 1993) was sequentially transfected with the bait vector and the cDNA library using lithium acetate (Schieffl and Gietz, 1989). Transformants were selected on plates lacking histidine, uracil, tryptophan, and leucine. Positive clones were picked after 4–6 d at 30°C, and the β-galactosidase activity of the clones was assayed by the filter method. Extrachromosomal DNA was isolated from clones that grew in the absence of histidine and that were β-galactosidase positive using the glass bead method (Ward et al., 1990). Prey plasmids were rescued in *Escherichia coli* HB101 cells by electroporation and selection on M9 plates containing 50 mg/l proline and 0.1 gm/l ampicillin. For quantitation of β-galactosidase activity, yeast strains transfected with bait and prey vectors were grown in supplemented minimal medium lacking uracil, tryptophan, and leucine in a shaking incubator at 30°C for 48 hr. β-Galactosidase assays were performed on yeast extracts with protein concentrations of 20–40 mg/l per assay as described previously (Rose et al., 1990).

cDNA cloning, DNA sequencing, construction of expression vectors, and COS cell expression. To determine the complete structure of CASK, the insert of pPeyCASK600 was used to screen a rat brain cDNA library in λZAPII (Stratagene, La Jolla, CA) by standard cDNA cloning methods (Sambrook et al., 1989). Thirty positive clones were isolated from ~3 million plaques. The six largest clones were selected for sequence analysis (pCASH2A, 3A, 3–33b, 3–27a, 3–22a, 3–14a), of which 3–33b, 3–27a, 3–22a, and 3–14a included the initiator methionine, and 2A had a rearranged 5' end. No sequence differences were noted between the different cDNA clones except for one short region where one cDNA clone (pCASH2A) had a distinct in-frame sequence that probably arose by alternative splicing (see Results). The insert of pCASH-27a was cloned as a 4.5 kb *KpnI*-*XbaI* fragment into pCMV5 to generate a eukaryotic expression vector (pCMVCASK581). Vectors encoding GST-CASK fusion proteins were constructed in pGEX-KG (Guan and Dixon, 1991) by PCR and preexisting restriction enzyme sites. The following vectors encoded the following residues of CASK: pGexCash3–9, 1–275; pGex-Cash3–13, 1–337; pGEXCash3–3, 508–909; pGEX-Cash3–18, 600–675; pGEXCash3–15, 600–909; pGpGEXCash3–16, 710–909. pMalSNAP25A encoding the maltose binding fusion protein with SNAP-25 was constructed similarly in pMalC2 (New England Biolabs, Beverly, MA). The vectors encoding the full-length and deleted version of the cytoplasmic tail of neuexin I (pMalNe2A, 2AΔ1, 2AΔ2, 2AΔ3, and 2AΔ4) were constructed from the corresponding pGex vectors (Hata et al., 1993) by subcloning the *BamHI*-*HindIII* fragment from the pGEX plasmids into the same sites of pMalC2. DNA sequencing was performed by the dideoxy nucleotide chain termination method using fluorescently labeled primers and an ABI370A DNA sequencer. The nucleotide sequences of the cDNA clones were deposited in the Genbank data bank (accession

number U47110). Plasmid DNA was transfected into COS cells using DEAE-dextran, and transfected COS cells were harvested 48–72 hr after transfection.

Antibodies. Antibodies were raised in rabbits immunized with purified recombinant GST-CASK fusion proteins. Proteins derived from the following pGEX vectors were used for the following antibodies: pGEX-Cash3–3, M735 and M736; pGEX-Cash3–9, N318 and N319; pGEX-Cash3–13, N392; pGEX-Cash3–16, N393; pGEX-Cash3–18, N394. The most frequently used antibodies (N319 and N393) were affinity purified for some applications using antigen immobilized on a CNBr-activated Sepharose column. Antibody specificity was tested using transfected COS cells.

Binding of recombinant CASK to neuexin fusion proteins with maltose binding protein (MBP). COS cells transfected with pCMVCASK581 were sonicated in 20 mM HEPES/NaOH pH 8.0, 10 mg/l APMSF and centrifuged at low speed to remove cell debris, and the supernatant was mixed with an equal volume of 20 mM HEPES/NaOH pH 8.0, 0.2 M NaCl containing either 5 mM Ca^{2+} or 5 mM EGTA. Maltose-binding fusion proteins encoded by pMalSNAP25A, pMalNe2a, and pMalNe2aΔ1 to Δ4 were immobilized on amylose resin and incubated at ~100 pmol per assay with 0.8 ml of COS cell extract for 3 hr at 4°C and washed five times with the incubation buffer. Final pellets were resuspended in 0.18 ml of sample buffer and analyzed by SDS-PAGE and immunoblotting.

Calmodulin binding. GST-fusion proteins of CASK encoded by pGexCASK-13, -15, -16, and -18 as well as GST alone and a control GST-fusion protein were separated on SDS-PAGE and blotted to nitrocellulose filters. Filters were blocked in 50 mM HEPES/NaOH pH 7.4, 0.15 M NaCl, 5% nonfat dry milk, with either 1 mM Ca^{2+} or 5 mM EGTA, washed repeatedly in the same buffer lacking dry milk, and incubated with 1.9 mg/l biotinylated calmodulin (gift of Dr. H. Schulman, Stanford University) in the same buffer without dry milk. After additional washing in the same buffer, signals were amplified using avidin and biotinylated peroxidase.

Subcellular fractionations. Subcellular fractionations of rat brain into membranes and cytosol and into membrane fractions enriched in synaptic plasma membranes, mitochondria, and myelin were performed essentially as described (Jones and Matus, 1974; Brose et al., 1995). Briefly, rat brain homogenates were centrifuged at 800 × g to remove nuclei and aggregates; the supernatant was centrifuged at 9000 × g to obtain a low-speed supernatant and a crude synaptosomal pellet that was lysed hypototically and centrifuged at 25,000 × g to prepare synaptosomal membranes. These were then subfractionated on a sucrose gradient into the different membrane fractions as described previously (Jones and Matus, 1974).

Miscellaneous procedures. SDS-PAGE and immunoblotting were performed using standard procedures and antibodies described previously (Johnston et al., 1989; Ushkaryov et al., 1992). Protein loads were equalized based on the Coomassie blue stains of the gels and on protein assays. Quantitation of immunoblotting signals was achieved by using ¹²⁵I-labeled secondary antibodies and PhosphorImager detection. RNA blots were purchased from CLONTECH (Palo Alto, CA) and hybridized with uniformly labeled cDNA probes (Ushkaryov et al., 1992).

RESULTS

Yeast two-hybrid screens for neuexin interacting proteins

One million, 4 million, and 6 million yeast transformants with a rat brain cDNA library were screened for proteins interacting with neuexins 1, 2, and 3, respectively. Only a single positive clone was identified from the neuexin 2 screen and 3 positive clones from the neuexin 3 screen. Restriction mapping and sequencing revealed that all four clones represented isolates of a single cDNA, pPreyCask600. Retransformations of yeast with the bait and prey plasmids singly or in combination demonstrated that β-galactosidase activation occurred only when pPreyCask600 was cotransformed with one of the three neuexin bait constructs but not with unrelated bait constructs, suggesting that the interaction observed was specific (data not shown). Because of its homologies (see below), we named the protein encoded by the prey vector CASK.

MADDDVLFEDVYELCEVIGKGPFSVVRRCINRETGQQFAVKIVDAKFTSSPGLSTEDLKR	61
EASICHMLKHPHIVELLEITYSSDGMVMVFEMDGLCFEIVKRADAGFVYSEAVASHYM	122
RQILEALRYCHDNNIIHRDVKPHCVLLASKENSAPVKLGFGVAIQLGESGLVAGGRVGT	183
HMAPEVVKREPYGKPVVDVWGCGVILFILLSGCLPFYGTGERLFEIGIKGYKMNPRQWSH	244
ISESAKDLVRRMLMLDPAERITVYEALNHPWLKERDRYAYKIHLPETVEQLRKFNARRKLK	305
GAVLAASVSSHKFNSFYGDPPEELPDFSEDPTSSGLLAAERAVSQVLDSEELIHALTDCSEK	366
DLDFLHSVFQDQHLHTLLDLYDKINTKSSPQIRNPPSDAVQRAKEVLEEISCYPENNDKE	427
LKRILTQPHFMALLQTHHVVAHEVYSDEALRVTPPTSPYLNQDSPESANGMDMENVT	488
RLVQFQKNTDEPMGITLKMNELNHCIVARIMHGGMIHRQGT	549
EQQLKMLREMRGSITFKIVPSYRTQSSSCERDSPSTSRQSPANGHSSTNNSVSIYVRAQFE	610
-DLPSTT-Q-P-KGRQ-----	599
YDPAKDDLIPCKEAGIRFRVGDI IQIISKDDHNWWQKLENSKNGTAGLIPSELPQEWVVA	671
CIAMEKTKQEQASCTWFGKKKKQYKDKYLAKHNADLVTYEEVVKLPFAFRKTLVLLGAHG	732
VGRRHINKNTLITKHPERFAYPIPHTRPPKKDEENGKNYYFVSHDQMMQDISNNEYLEYGS	793
HEDAMYGTKLETIRKIEHGLIAILDVEPQALKVLRATAEFPPFVVFIAAPTITPGLNEDES	854
LQLQKESDVLQRTYAHYFDLTIINNEIDETIRHLEEAVELVCTAPQWVPVSWVY*	909

Figure 1. Primary structure of CASK. The amino acid sequence of rat CASK as determined from multiple full-length cDNA clones is shown in single-letter amino acid code and numbered on the right. The putative alternatively spliced sequences are shown in *bold*, with *dashes* indicating gaps. The prey clone independently isolated in multiple neuexin Y2 H screens encodes residues 218–909.

Structure of CASK

Using the cDNA insert of pPreyCask600, we isolated full-length clones from a rat brain cDNA library. Their translated amino acid sequence is assembled in Figure 1. Multiple overlapping cDNAs were characterized that contained identical sequences except for a short region in which two sequence variants were observed. Of four clones, three were identical but one clone contained a distinct in-frame sequence that was homologous to that present in the other clones (*bold* in Fig. 1). The homology between the two sequences suggests that its presence does not represent a cloning artifact but is attributable to differential splicing similar to that observed in dynamin and other neural proteins where distinct but homologous sequences are also differentially inserted at positions of alternative splicing (Sontag et al., 1994).

Databank searches revealed that the protein encoded by the CASK cDNAs is composed of a series of defined domains that were not previously observed in this combination (Figs. 2A,B). The N-terminal third of CASK is highly homologous to Ca^{2+} , calmodulin-dependent protein kinases (CaM kinases). The strongest homology is found with Ca^{2+} , calmodulin-dependent protein kinase II (CaMK II; 45.0% identity over 322 amino acids). This homology includes the autoinhibitory and calmodulin binding domain of CaMK II which extends from residues 281–310 (Hanson and Schulman, 1992; Haribabu et al., 1995). Of the 18 residues of the CaM binding sequence of CaMK II (residues 293–310), 11 are identically present in CASK (Fig. 2A). The threonine of the autophosphorylation site of CaMK II (Thr¹⁸⁶) in the autoinhibitory domain is also conserved in CASK (Thr¹⁹¹). However, immediately preceding the threonine, CASK contains the sequence ¹⁸⁷HLPET¹⁹¹ as opposed to ¹⁸²HRQET¹⁸⁶ in CaMK II. The substitution of the arginine in CaMK II for leucine in this sequence in CASK suggests that this is not a good CaMK substrate and that Thr¹⁹¹ in CASK may not be an autophosphorylation site.

Immediately after the calmodulin binding domain, the CASK sequence diverges from that of other CaM kinases. After a short linker sequence that is homologous to a human *dlg-A* homolog (DLG2; Mazoyer et al., 1995), the C-terminal half of CASK assumes a high degree of similarity to a set of proteins that includes the erythrocyte plasma membrane protein p55, the syn-

aptic junction proteins PSD95/SAP90 and SAP97, the *Drosophila* septate junction protein *dlg-A* and its human homologs, and the tight junction proteins ZO-1 and ZO-2 (Fig. 2; Ruff et al., 1991; Cho et al., 1992; Itoh et al., 1993; Kistner et al., 1993; Tsukita et al., 1993; Willott et al., 1993; Jesaitis and Goodenough, 1994; Müller et al., 1995). All of these proteins are characterized by the presence of three separate domains: N-terminal DHR repeats that are present in a single copy in CASK, DLG2, and p55; a middle SH3 domain; and a C-terminal guanylate kinase-like sequence that lacks several residues thought to be essential for enzyme activity. The unusual combination of CaM kinase and *dlg*-like domains observed in CASK is not a cDNA cloning artifact because the same combination of domains was observed in multiple independent cDNA clones with different sequence boundaries. Thus, CASK is composed of a mosaic of domains that include signaling modules (CaM kinase and SH3 domains) and putative intercellular junction and cytoskeletal attachment domains (the *dlg-A* homologous set of domains).

Properties and tissue distribution of CASK

To study the tissue distribution of CASK, we performed RNA and protein blotting experiments. For this purpose, antibodies were raised to recombinant GST-CASK fusion proteins. The specificity of the antibodies was confirmed in immunoblots comparing COS cells transfected with control DNA and with the CASK expression vector (see below). Both RNA blots and immunoblots revealed that CASK is primarily expressed in brain (Fig. 3). However, in contrast to the brain-specific neuexins, lower levels of CASK are ubiquitously present in all cells and tissues tested. Quantitative immunoblots using ¹²⁵I-labeled secondary antibodies demonstrated that brain expresses approximately fourfold more CASK than kidney, the highest peripheral tissue, whereas other tissues contain lower levels (5–20% of brain levels; data not shown).

To determine if CASK is a soluble protein, tissues were separated into membranes and cytosol. CASK was associated with the membrane pellet and could be partially solubilized in detergents but not with high salt (data not shown). This is consistent with the notion that CASK may be a peripheral membrane protein. To investigate the subcellular localization of CASK, we subfractionated brain homogenates into different membrane fractions. CASK

Figure 3. Tissue distribution of CASK expression. Homogenates from the indicated rat tissues were analyzed by immunoblotting using affinity-purified CASK antibodies. Immunoreactive bands were reacted with ^{125}I -labeled secondary antibodies and exposed to film. Numbers on the left indicate positions of molecular weight standards. PhosphorImager quantitation suggests that the levels of CASK in brain are ~ 4 times higher than in kidney, and 5–20 times higher than in other non-neural tissues.

was highly enriched in the so-called synaptic plasma membrane fraction whereas the myelin and mitochondrial fractions contained virtually no CASK in spite of equal protein loads (Fig. 4). The synaptic plasma membranes represent a fraction that contains synaptic junctions but also other parts of neuronal and glial plasma membranes, allowing no definitive conclusion about the part of the plasma membrane CASK may be associated with (Jones and Matus, 1974).

Because the N-terminal domain of CASK is highly homologous to CaM kinases, we tested whether it possesses kinase activity or binds calmodulin. No kinase activity could be demonstrated with recombinant protein, possibly because an additional regulatory step similar to CaMKI and IV is required (Haribabu et al., 1995; Tokumitsu et al., 1995). In addition, no autophosphorylation was observed with CASK kinase domains expressed in bacteria, COS cells, or baculovirus (data not shown). However, recombinant fragments of CASK containing its putative calmodulin binding site bound calmodulin efficiently in a Ca^{2+} -dependent manner, supporting the notion that calmodulin regulates CASK activity (data not shown).

Specificity of the interaction of CASK with the cytoplasmic domains of neuroligins

Deletion mutants of CASK and of neuroligins were tested for interactions in the Y2 H assay to determine their specificity. Deletions in CASK that were smaller than the originally isolated Y2 H prey clone (containing residues 218–909) abolished the interaction whereas longer sequences of CASK were active (data not shown). This suggests that several domains in CASK may be required for the interaction with neuroligins. By contrast, deletion analysis of the cytoplasmic domain from neuroligin 1 revealed that deletions from the N terminus of the cytoplasmic domain of neuroligin 1 weakened but did not abolish the interaction (Table 1). Deletions from the C terminus, however, severely inhibited the interaction, with a deletion of only three amino acids being sufficient to abolish binding in the Y2 H assay.

To confirm the interaction of CASK with neuroligins biochemically, full-length and deleted sequences from the cytoplasmic domain of neuroligin 1 identical to those used in the Y2 H assays (Table 1) were produced as recombinant MBP-fusion proteins in bacteria and immobilized on amylose resin. The immobilized cytoplasmic domains were incubated with extracts from COS cells

Figure 4. Analysis of the distribution of CASK in brain using subcellular fractionation. Rat brain homogenates (left lane) were fractionated into a crude synaptosome pellet (Synaptosomes) and low-speed supernatant (LS Supernatant; see Materials and Methods). Synaptosomal membranes (Syn. Plasma Membranes) were isolated from hypo-osmotically lysed synaptosomes by centrifugation and subjected to sucrose gradient centrifugation, yielding fractions enriched in synaptic plasma membranes (Syn. Plasma Membrane), mitochondria, and myelin (Jones and Matus, 1974). Equivalent amounts of the fractions were analyzed by SDS-PAGE and immunoblotting for CASK (A) and neuroigin (B; antibody used recognizes all three neuroligins) as plasma membrane markers, and for synaptophysin as a synaptic vesicle marker and complexins as cytosolic proteins (C). The smaller bands observed in the CASK immunoblots probably represent proteolytic products generated during membrane purification because such bands are never observed in fresh total brain samples (Fig. 3).

transfected with CASK, the resin was washed extensively, and bound proteins were analyzed by immunoblotting (Fig. 4). As a control, an MBP-fusion protein with SNAP-25 was used.

CASK binding was only observed to MBP-fusion proteins containing neuroligin sequences. It was independent of Ca^{2+} . A 16 amino acid deletion from the N terminus of the cytoplasmic domain of neuroligin 1 resulted in decreased binding; a deletion of 25 residues completely inhibited it. On the C-terminal side, all deletions abolished binding, even the removal of only three amino acids was sufficient to prevent the interaction (Fig. 4). These results are similar to those obtained in the Y2 H assays with the same deletion mutants (Table 1) except that in the Y2 H assay the 25 residue N-terminal deletion still interacted whereas biochemically no binding was observed. Together these results suggest that CASK interacts with neuroligins in a sequence-specific manner. However, this interaction was very sensitive to salt and no co-immunoprecipitation of neuroligins with CASK could be observed under standard conditions (data not shown).

DISCUSSION

Neuroligins are putative recognition molecules displayed on the neuronal cell surface (Ushkaryov et al., 1992). As such, they are

Figure 5. Binding of CASK to immobilized cytoplasmic domains of neuexin 1. Extracts from COS cells transfected with the CASK expression vector (lane 2; lane 1 shows a control COS cell extract for comparison) were reacted with immobilized fusion proteins of MBP with SNAP25A (lane 3), the full-length cytoplasmic domain of neuexin 1 (lane 4), or cytoplasmic domains of neuexin 1 with N- or C-terminal deletions (lanes 5–8). Ne1Δ1 and Ne1Δ2 carry 16 and 25 residue deletion from the N terminus of the cytoplasmic domain of neuexin 1, respectively, and Ne1Δ3 and Ne1Δ4 deletions of 10 and 3 amino acids, respectively, from the C terminus of the cytoplasmic domain. Incubations were performed in the presence of either Ca^{2+} (top) or EGTA (bottom). Immobilized MBP-fusion proteins were washed repeatedly in the incubation buffer, and bound proteins were analyzed by SDS-PAGE and immunoblotting for CASK.

thought to interact extracellularly with other cell surface proteins such as neuroligins (Ichtchenko et al., 1995, 1996), thereby mediating cell–cell interactions. By analogy with other cell–cell interaction molecules, neuexins might be expected to transduce extracellular binding events into intracellular signals. This could be achieved by binding of neuexins to signal transduction molecules, to proteins interacting with the cytoskeleton, or to junction proteins. In the current study we have identified such a protein molecule by the Y2 H assay. We found a novel protein called CASK that interacts with the intracellular domains of all neuexins. The structure of CASK suggests functions in signal transduction events involving Ca^{2+} , in organizing plasma membrane domains such as intercellular junctions, and in mediating the attachment of the cytoskeleton to intrinsic membrane proteins. These findings are compatible with the notion that neuexins transduce extracellular recognition signals into intracellular responses at the plasma membrane.

Three lines of evidence support the hypothesis that CASK specifically interacts with neuexins (1) CASK was independently isolated in multiple screens using different neuexin tails. (2) The interaction of CASK with neuexins could be reproduced with recombinant proteins *in vitro*. (3) Binding of CASK to the neuexin tails was sequence dependent. Small deletions from either CASK or the neuexin tail domains abolished the interaction in both the Y2 H assay and in the *in vitro* binding assay. However, although CASK is firmly associated with membranes in a salt-resistant manner, the interaction between CASK and neuexins is salt-labile and was disrupted by 0.5 M NaCl. Furthermore, the interaction could not be demonstrated by immunoprecipitations, suggesting that the interaction is weak. Together with the observation that CASK is present outside of brain where neuexins are absent, these data indicate that CASK is not primarily membrane associated via its interaction with neuexins but may also interact with other proteins.

CASK is composed of a mosaic of distinct domains that were described previously in a number of separate proteins. Its N-terminal third is comprised of a CaM kinase-like domain that

Table 1. Quantitation of yeast two-hybrid interactions using measurements of β -galactosidase activity in strains harboring bait and prey vectors

Interactions of CASK with sequences from the cytoplasmic domains of neuexins

Bait vectors	Prey vector pPrey600CASK
pBTM116Ne1a	34.3 $\pm$ 0.0
pBTM116Ne1Δ1	11.0 $\pm$ 0.2
pBTM116Ne1Δ2	11.0 $\pm$ 0.6
pBTM116Ne1Δ3	1.6 $\pm$ 0.1
pBTM116Ne1Δ4	1.6 $\pm$ 0.1
pBTM116Ne2a	15.0 $\pm$ 0.2
pBTM116Ne3a	20.0 $\pm$ 0.6

The β -galactosidase activity of cell extracts from yeast harboring both plasmids was measured in triplicates and is shown as mean $\pm$ SD in arbitrary units (Hata and Südhof, 1995). The bait vectors containing the neuexin tails encode the full-length cytoplasmic domains of neuexins 1, 2, and 3 (pBTM116Ne1a, 2a, and 3a, respectively), or parts of the cytoplasmic tail from neuexin 1. pBTM116Ne1Δ1 and Δ2 contain deletions of 16 or 25 residues, respectively, from the N terminus of the cytoplasmic domain, and pBTM116Ne1Δ3 and Δ4 contain deletions of 10 and 3 amino acids, respectively, from the C terminus of the domain (Hata et al., 1993).

binds calmodulin as a function of Ca^{2+} . Although homologous to all CaM kinases described, it is most homologous to CaM KII, with which it shares almost 50% sequence identity (Bennett and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987; Hanson and Schulman, 1992). C-Terminally to the CaM kinase module, CASK contains a set of domains that was previously identified in a family of intercellular junction proteins homologous, first in the *Drosophila* *dlg-A* gene product. This family includes the synaptic junction proteins PSD95/SAP90 and SAP97, the tight junction proteins ZO-1 and ZO-2, the erythrocyte membrane protein p55, and the mammalian homologs of the *Drosophila* *dlg-A* gene hDLG and DLG2 (Woods and Bryant, 1991; Cho et al., 1992; Itoh et al., 1993; Kistner et al., 1993; Tsukita et al., 1993; Willott et al., 1993; Jesaitis and Goodenough, 1994; Lue et al., 1994; Mazoyer et al., 1995; Müller et al., 1995).

All of these proteins are peripheral membrane proteins that are composed of N-terminal DHR repeats, a single SH3 domain, and a noncatalytic guanylate kinase domain. Except for p55, these proteins are localized to a variety of intercellular junctions where they may organize plasma membranes into junctional domains. By contrast, p55 is uniformly distributed over the erythrocyte plasma membrane where it probably mediates the interaction of an intrinsic membrane protein, glycophorin C, with protein 4.1 and thereby contributes to determining the unusual shape of erythrocyte (Ruff et al., 1991; Allosio et al., 1993; Marfati et al., 1994). The high degree of sequence similarity between the *dlg-A* like proteins, p55, and CASK raises the possibility that CASK is also involved in organizing special domains of the plasma membrane by binding to intrinsic membrane proteins such as the neuexins and to protein 4.1 or its homologs ezrin, meosin, merlin, or radixin. This hypothesis is supported by the tight association of CASK with the synaptic plasma membrane.

The combination of domains found in CASK has not been observed previously in a single protein. Although many *dlg-A* homologs have been described, none of them is fused to a signal transduction module. Conversely, in spite of the presence of several CaM kinases in eukaryotic cells, these are not fused to a putative plasma membrane organizer as CASK is

(Hanson and Schulman, 1992). The fusion of a signal transduction domain to an intercellular junction/cytoskeletal insertion domain could have multiple advantages: it could localize a protein kinase to a specific compartment, it could bring the protein kinase in proximity to its target and restrict its number of substrates, and it could allow selective modulation of a subset of structures after excitation. In addition, the interaction of CASK with neurexins could potentially allow activation of CASK after ligand binding and initiate a signal transduction cascade.

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