Association of calcium/calmodulin-dependent kinase II with developmentally regulated splice variants of the postsynaptic density protein densin-180*

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*This work was supported by NIH grant NS37508, and AHA Grants-in-Aid 96010040 (National) and 9950865V (Southeast affiliate) to RJC, an Established Investigator of the AHA. AJR holds a Molecular Endocrinology Training Program stipend (5T32DK07563). Confocal microscopy was performed using the VUMC Cell Imaging Resource (supported by CA68485 and DK20593).

Running title: CaMKII binding to densin-180 splice variants.
Abstract

In a continued search for proteins that target calcium/calmodulin-dependent protein kinase II (CaMKII) to postsynaptic density (PSD) substrates important in synaptic plasticity, we identify the PSD protein densin-180 as a CaMKII-binding protein. Four putative splice variants (A-D) of the cytosolic tail of densin-180 are shown to be differentially expressed during brain development. Densin-180 splicing affects CaMKII phosphorylation of specific serine residues. Forms A, B, and D, but not C, bind CaMKII stoichiometrically and with high affinity, mediated by a differentially spliced domain. Densin-180 differs from the previously identified CaMKII-binding protein NR2B in that binding does not strictly require CaMKII autophosphorylation. Binding of densin-180 and NR2B to CaMKII is non-competitive, indicating different interaction sites on CaMKII. Expression of the membrane-targeted CaMKII-binding domain of densin-180 confers membrane localization to coexpressed CaMKII without requirement for calcium mobilization, suggesting that densin-180 plays a role in the constitutive association of CaMKII with PSDs.
INTRODUCTION

CaMKII is a broad-specificity, calcium/calmodulin-dependent kinase with central roles in hippocampal long-term potentiation (LTP), a widely studied cellular model of learning and memory (1-3). Critical for LTP and spatial learning (4,5), autophosphorylation at T286 confers calcium-independent activity to the kinase and underlies its ability to decode the frequency of calcium transients (6). While CaMKII is an abundant soluble protein, it is also a major component of postsynaptic densities (PSDs), cytoskeletal scaffolds for neurotransmitter receptors and their regulators (7,8). The localization of CaMKII to the PSD is believed to be important in regulating the phosphorylation state of neurotransmitter receptors, such as the AMPA-type glutamate receptor (9,10), and CaMKII has been shown to translocate to PSDs following synaptic stimulation (11,12). We have recently identified the NR2B subunit of the N-methyl-D-aspartate (NMDA)-receptor as a targeting protein for T286-autophosphorylated CaMKII (13,14). Here, we characterize developmentally regulated, putative splice variants of the postsynaptic density protein densin-180 as additional PSD-anchoring proteins that bind CaMKII by a mechanism different from NR2B.
EXPERIMENTAL PROCEDURES

Cloning and expression of densin-180 fusion proteins. Rat forebrain RNA was isolated with Trizol reagent (Gibco BRL) according to supplied instructions. Sense and antisense primers corresponding to the borders of the putative cytosolic domain of densin-180 including BamHI and EcoR1 adapters (sense: 
\[
CGGGATCCGACAAGACATCAGATAACAGTG
\]
antisense: 
\[
GGAATTCCTTAGACAGTGAGCTCACGTTG
\]
were used clone densin-180 by reverse transcriptase polymerase chain reaction (RT-PCR) according to instructions for the Access RT-PCR kit (PROMEGA). RT-PCR products were gel-purified and ligated into the prokaryotic glutathione S-transferase (GST)-fusion protein expression vector pGEX-2T (Pharmacia) and sequenced on both strands. The sequence of the D variant cytosolic tail containing a novel 141 bp insert was submitted to GenBank (accession number AF266164). Truncation constructs were generated by PCR or utilizing endogenous restriction sites. Site-directed mutagenesis was carried out by separately PCR-amplifying densin-180 halves 5' and 3' of the point mutation utilizing complementary mutagenic primers that also incorporate a unique silent restriction site to permit re-assembly of the two PCR products. Fusion protein were expressed and purified on glutathione-agarose by standard methods.

CaMKII-binding assays. Expression and purification of the murine α isoform of CaMKII from Sf9 cells, site-specific autophosphorylation of CaMKIIα at T286 in the presence, and at T305/306 in the absence of calcium/calmodulin, and \([^{32}\text{P}]\text{CaMKIIα-}
\]
overlay binding assays were performed as described (15). Glutathione-microtiter plate binding and glutathione-agarose sedimentation assays were described previously (14).
Colocalization experiments. A membrane-targeted fusion protein cDNA was constructed as described for a similar protein targeted to mitochondria (14). Briefly, this construct encodes, from N- to C-terminus, a 10 amino acid membrane-targeting sequence from the lck tyrosine kinase with myristoylation and dual palmitoylation sites (16,17), a myc-epitope tag, and the GST and green fluorescent protein (GFP) coding sequences. Domain 4 of the densin-180 cytoplasmic tail was ligated into BamHI and EcoRI sites between the GST and GFP sequences. HEK293 cells were cotransfected with membrane-targeted fusion protein and CaMKII expression plasmids, and colocalization was assayed by double-immunofluorescence confocal microscopy as described (13,14).
RESULTS AND DISCUSSION

In an effort to identify proteins that target CaMKII to PSDs, we have previously characterized a p190 band enriched in postsynaptic densities that binds T286-autophosphorylated CaMKII\(\alpha\) by overlay (15). The NR2B subunit of the NMDA receptor was shown to be a component of this 190 kDa binding activity (13,14). To reveal other potential CaMKII-binding proteins that comigrate with NR2B, PSD proteins were treated with N-glycanase to remove N-linked carbohydrates. Deglycosylation lead to a quantitative increase in electrophoretic mobility of NR2A and NR2B subunit immunoreactivity, while only a fraction of the CaMKII-binding activity was shifted to a lower molecular weight (data not shown), implicating the existence of at least one other, ~190 kDa CaMKII-binding protein in PSDs.

**Developmental splicing of the densin-180 cytosolic tail**

Densin-180 was discovered in a systematic effort by the Kennedy group to identify major PSD proteins (18). Densin-180 is an O-sialoglycoprotein of 180 kDa with a postulated role in cell-cell adhesion, whose sequence predicts a large N-terminal extracellular domain, a single transmembrane domain, and a short cytosolic tail ending in a PDZ domain of unknown ligand specificity. To find out whether densin-180 is the missing CaMKII-binding protein, its cytosolic domain was isolated by RT-PCR. Surprisingly, using RNA from adult rats as a template, we consistently observed four products ranging in length from about 940 to 570 bp, with 790 and 670 bp representing the major products. Sequencing of individually subcloned RT-PCR products revealed that the 790 bp product corresponds to the previously published densin-180 sequence (18), whereas the two shorter variants (termed B and C) feature in-frame deletions of amino acids 1291-1330 and 1331-1404, respectively. The
longest, D variant has a novel 47 amino acid insert after R1290 (Apperson et al., 1996 clone numbering), without homology to other proteins in databases (Fig. 1A). The inserted and deleted sequences define five domains, presumably corresponding to differentially spliced exons, with domain 1 and 5, the PDZ domain, common to all four variants (Fig. 1A). A presumed splice variation in the extracellular domain of densin-180 was previously described (18), implying even greater structural diversity in densin-180 gene products.

To examine the developmental expression pattern of densin-180 cytosolic tail variants, we carried out RT-PCR with forebrain RNA from rats of different ages (Fig. 1B). The D variant was the major splice form in embryonic brain and decreased in abundance during early postnatal development. A steady increase in A and B variant expression was observed over the same time course. C variant expression was variable, but most consistently observed in embryonic and early postnatal RNA samples. Thus, differential splicing of densin-180 appears to be developmentally regulated. Since most synapses (and PSDs) develop postnatally, embryonic forms of densin-180 may be involved in early developmental processes, such as process outgrowth and path finding.

**Differential phosphorylation of densin-180 variants**

Densin-180 can be phosphorylated by CaMKII in PSDs at unidentified sites (18). Phosphoamino acid analysis following exhaustive phosphorylation of cytosolic tail fusion proteins by CaMKII in vitro revealed 90% serine phosphate and 10% threonine phosphate, indicating phosphorylation at multiple sites (data not shown). Intriguingly, a sequence matching a consensus CaMKII phosphorylation site ([I/L]XRXX[S/T], (19)) bridges domain boundaries and is altered in some densin variants (Fig. 2A). Deletion of domain 3 in variant B removes S94 as a potential
phosphate acceptor (numbering starting with the first residue of the D variant cytosolic tail), but reconstitutes the consensus motif with S134 in domain 4. Insertion of domain 2 of the D variant replaces the terminal LDR sequence of domain 1 with LQK, which differs only in a conservative substitution (R to K) from the phosphorylation consensus. S94 (or the positionally equivalent S134 in the B variant) was mutated to Asp, and CaMKII phosphorylation of GST-cytosolic tail fusion proteins was compared to wild-type sequences (Fig. 2B). Replacing this serine in the A, B, and D variants reduced [32P]-incorporation by about half, and abolished phosphorylation of the C variant completely, demonstrating that differential splicing conserves phosphorylation of this residue. These data also imply the existence of an additional phosphorylation site in domain 4, missing in the C variant. Phosphoamino acid analysis of truncation constructs narrowed down the candidate residue to the single serine within residues 180-205, S198 (data not shown), even though the context of this residue (TKGQRS) does not conform to the CaMKII phosphorylation consensus (19). Indeed, the S198D mutant A variant displayed 40-50% reduced phosphorylation, and the S94/198D double mutant could be phosphorylated to less than 10% of wild-type (Fig. 2B), indicating that these two residues are the major CaMKII phosphorylation sites, at least in vitro.

**CaMKII binding to densin-180 variants**

To determine if CaMKII can stably interact with the cytosolic tails of densin-180, fusion proteins were probed with CaMKIIα, [32P]-labeled by autophosphorylation at T286 ([32P-T286]CaMKIIα). Specific binding was detected to the full-length cytosolic tails of the A, B, and D, but not the C variant (Fig. 3A); signal intensities were similar to CaMKII binding to NR2B fusion proteins on the same blots (not shown). The failure of
CaMKII to bind to the C variant suggested that domain 4 contains important binding determinants. This was further supported by overlay analysis of truncation constructs, demonstrating that domain 4 is sufficient for CaMKII binding (Fig. 3B). Residues 155-182 within domain 4 were found to be necessary for binding, but supported only reduced interaction with CaMKII when expressed in isolation, suggesting that flanking regions may stabilize the interaction of CaMKII with densin-180 residues 155-182. Interestingly, the sequence of this domain bears no resemblance to that of the CaMKII-binding domain in NR2B (residues 1290-1309, (14)). Whereas NR2B-1290-1309 contains a high-affinity CaMKII phosphorylation site which modulates CaMKII binding (14), residues 155-182 in densin-180 are not phosphorylated and the presumed phosphomimetic S94/134D and S198D mutations do not affect CaMKII binding by overlay (not shown).

To determine the affinity of the CaMKII:densin-180 interaction, $[^{32}P]$-T286CaMKII$\alpha$ was incubated with densin-180 cytosolic tail (A variant) GST-fusion proteins immobilized on glutathione-coated microtiter plates (Fig. 4A). CaMKII bound saturably with an affinity of 150-250 nM (N=2), and Scatchard analysis indicated a simple bimolecular event (not shown). This $K_d$ is similar to the $K_d$ for the CaMKII:NR2B interaction (13) and two orders of magnitude lower than the average concentration of CaMKII in neurons (15,20), suggesting that both complexes can exist in neurons.

NR2B only binds to the T286-autophosphorylated, calcium/calmodulin-independent form of CaMKII (13,14). In contrast, densin-180 bound significant amounts of inactive CaMKII, $[^{32}P]$-labeled by T305/306-autophosphorylation in the absence of calcium/calmodulin (15), in glutathione-plate assays (Fig. 4A inset), a finding that was confirmed in overlay experiments (not shown). Furthermore, in glutathione-agarose cosedimentation experiments T286 autophosphorylation increased the amount of CaMKII copurifying with densin-180 to stoichiometric levels, even though some CaMKII copurified without prior autophosphorylation (Fig. 4B).
The finding that the densin-180 and NR2B interactions differ in their degree of dependence on CaMKII T286 autophosphorylation combined with the lack of conservation between the CaMKII-binding domains in NR2B and densin-180 suggests different mechanisms of binding. Competition experiments support this conclusion. Whereas soluble NR2B cytosolic tail protein inhibits CaMKII binding to immobilized GST-NR2B fusion protein in a concentration dependent manner, it has no effect on CaMKII binding to GST-densin-180 (Fig. 4C), indicating that densin-180 and NR2B interact with different and independent sites on CaMKII and suggesting that ternary complexes may exist in neurons.

**Densin-180 sequences target CaMKII in cells**

Data by Apperson et al. (18) showing that densin-180 is highly enriched in PSDs and colocalizes with CaMKII at synapses of hippocampal neurons are consistent with our proposed function of densin-180 as a CaMKII-targeting protein. To demonstrate directly that densin-180 can alter the subcellular localization of CaMKII, we cotransfected HEK293 cells with CaMKII and a synthetic fusion protein targeted to the membrane via multiple fatty acid modifications (see EXPERIMENTAL PROCEDURES). This fusion protein was expressed in large patches and in small punctate or ring-shaped profiles (Fig. 5). The small profiles were shown to be on the cell surface by optical sectioning and likely correspond to caveolae, where the tyrosine kinase lck from which the membrane-targeting sequence was derived is localized (21). Coexpression of the membrane-targeted fusion protein by itself had no effect on the localization of CaMKII, which remained diffusely localized in the cytoplasm (Fig. 5). When the CaMKII-binding domain 4 of the densin-180 cytosolic tail (residues 132-205, Fig. 3B) was inserted into the membrane-targeting vector, CaMKII localization
changed dramatically, resulting in strong colocalization in both large and small patches (Fig. 5).

Colocalization of CaMKII with artificially targeted NR2B sequences and NMDA receptors containing the full-length NR2B subunit requires calcium mobilization and is not absolute, presumably because CaMKII:NR2B complex formation requires T286 autophosphorylation of CaMKII but is antagonized by NR2B phosphorylation (13,14). In contrast, CaMKII and densin-180 colocalization was near perfect under basal conditions, and was not detectably altered by calcium ionophore treatment (2 µM A23187 for 5 and 15 min, data not shown).

In summary, data presented here indicates that densin-180 may be responsible for constitutively anchoring CaMKII to the PSD, where it is poised to respond to localized calcium influx by phosphorylating postsynaptic substrates important in synaptic plasticity. The highly regulated CaMKII:NR2B interaction (13,14), on the other hand, may underlie the dynamic association of CaMKII with PSDs following synaptic activation (11,12). Although phosphorylation of densin-180 itself does not appear to affect CaMKII binding, the careful reconstitution of one of the phosphorylation sites during alternative splicing suggests phosphorylation could have other important roles, perhaps in regulating association of densin-180 with other proteins. Lastly, the existence of the C variant of densin-180 that lacks the CaMKII-binding domain suggests alternative splicing as a potential mechanism for regulating the association of CaMKII with PSDs.
Footnotes

1 The abbreviations used are: CaMKII, calcium/calmodulin-dependent protein kinase II; [P-T286]CaMKIIα, CaMKIIα autophosphorylated at threonine-286; PSD, postsynaptic density; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; GST, glutathione S-transferase; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis.
References


Figure legends

Fig. 1. **Identification of developmentally regulated densin-180 cytosolic tail variants.** A, domain diagram of densin-180. The cytosolic tail is expanded, showing putative exon boundaries defined by the sequence of the indicated cDNA variants isolated by RT-PCR. The sequence of the novel insert (domain 2) in the D variant is listed (GenBank accession number AF266164). B, developmental expression of densin-180 tail variants. Total RNA from rat brains of the indicated ages (in days; E, embryonic; P, postnatal) and adult (> 6 weeks old) was analyzed by RT-PCR. A gray-scale inverted image of an ethidium bromide-stained gel is shown. Bands corresponding to the A-D isoforms are indicated by solid arrowheads. The identity of additional bands below A and D forms (open arrowheads) is unknown, since they could not be recovered by ligation into plasmids. Similar results were obtained in 6 RT-PCR reactions with 3 sets of RNA samples.

Fig. 2. **Identification of CaMKII-phosphorylation sites in densin-180.** A, partial domain diagram of densin-180 cytosolic tails centering on a consensus CaMKII phosphorylation motif that bridges domain boundaries. The phosphorylated serine (S94 in A, C, D; S134 in B) is highlighted. B, in vitro phosphorylation of densin-180 cytosolic tail GST-proteins. CaMKII was T286-autophosphorylated (14) and incubated (30 min, 30°C) at 2.5 nM in the absence of calcium/calmodulin with 200 µM [γ-32P]ATP and ~2 µM of the indicated wild-type (w.t.) or mutant densin-180 cytosolic tails expressed as GST-fusion proteins. Reactions were analyzed by SDS-PAGE, followed by Coomassie-Blue protein staining (top) and autoradiography (32P, bottom). Arrowheads indicate full-length fusion protein, asterisks a major proteolytic fragment. The position of molecular weight standards is shown between panels. Full-length w.t.
proteins were phosphorylated to 0.1-0.2 mol/mol stoichiometry in these experiments, which are representative of 4.

Fig. 3. **Differential binding of CaMKII to densin-180 variants and binding-domain mapping.** A, GST-fusion proteins of the indicated densin-180 cytosolic tail variants (~2 µg/lane) were separated by SDS-PAGE, blotted and stained for protein with Ponceau-S (top), followed by [\(^{32}\)P-T286]CaMKII\(\alpha\) overlay (200 nM) and autoradiography (bottom). Molecular weight markers are indicated on the right. B, GST-fusion proteins of the full-length A variant cytosolic tail and the diagrammed truncations (D variant numbering starting after transmembrane domain) (~0.5 µg/lane) were analyzed for [\(^{32}\)P-T286]CaMKII\(\alpha\) binding as in A. The region identified as necessary for binding is cross-hatched with its sequence listed on top. Data is representative of 6 similar experiments.

Fig. 4. **Characterization of CaMKII binding to densin-180.** A, graph, [\(^{32}\)P-T286]CaMKII\(\alpha\) at the indicated concentrations was allowed to bind to GST-densin-180 A variant (10 µg/well) immobilized in glutathione-coated microtiter wells and bound CaMKII was quantified by liquid-scintillation counting. Inset, binding of 50 nM [\(^{32}\)P]CaMKII\(\alpha\) phosphorylated at T286 or T305/6 to GST-densin-180 A variant (densin) or GST-NR2B-1260-1316 (NR2B) by glutathione-plate assay. Means of duplicates ± SD are plotted. B, glutathione-agarose cosedimentation assay showing stoichiometric association of densin-180 with [P-T286]CaMKII\(\alpha\) (densitometric ratio 1.0 ± 0.1, N=2). CaMKII\(\alpha\) was incubated under calcium/calmodulin-dependent autophosphorylation conditions without (-) or with (+) ATP. After stopping phosphorylation, incubation was continued with equimolar (1 µM) GST-densin-180 A variant, residues 1-205 (densin) or GST alone, followed by purification of complexes on glutathione-agarose (14). Aliquots of the incubation mixture (input) and glutathione-
agarose pellet were analyzed by SDS-PAGE, blotted, and stained for total protein with Ponceau-S (top), followed by immunoblotting with a phospho-T286-specific CaMKII antibody (α-pT286, Promega, bottom). Full-length densin-180 GST-fusion protein and GST are indicated by solid arrowheads, CaMKIIα with open arrowheads. Densin-180 fragments are labeled with asterisks. Positions of molecular weight markers are shown on the right. C, failure of NR2B to compete for densin-180 binding to CaMKII. [32P-T286]CaMKIIα (100 nM) in the presence of the indicated concentrations of a soluble bacterial protein extract expressing His6-NR2B-1185-1482 (supplemented to 400 µg/ml with control bacterial extract) was allowed to bind to GST-densin-180 A (densin) or GST-NR2B-1260-1316 (NR2B) on glutathione-plates. Means of duplicates ± SD are plotted. Data are representative of 2 to 3 experiments.

Fig. 5. **Densin-180 sequences target CaMKII to the membrane.** A membrane-targeted fusion protein with myc-epitope tag was coexpressed with CaMKII in HEK293 cells and localized by double-immunofluorescence microscopy with antibodies to CaMKII (red) and myc epitope (green). CaMKII was diffusely cytoplasmic when cotransfected with the fusion protein without densin-180 insert (control, top), but perfectly colocalized with a fusion protein containing densin-180 domain 4 (residues 132-205, bottom) apparent as yellow in the merged image. Arrows point to circular profiles, presumably caveolae. Identical results were obtained in 3 transfections. Scale bars = 10 µM.
Fig. 1, Strack et al.

A

extracellular

intracellular

1 2 3 4 PDZ

A

B

C

D

NAAYKHNTVNLGMLPYGGISAMHAGRSMTNLQTKSKFDLQDLPLQK

B

E18  P1  P7  P14  P21  Adult

bp

1000

900

800

700

600

500

A

B

C

D

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Fig. 2, Strack et al.

A

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domain key: 1 2 3 4

B

A  B  C  D  A

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<th>S94D</th>
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protein

\[^{32}P\]

Mr (K)

45 31

w.t. S198D S94/198D
Fig. 3, Strack et al.

A

B

protein

CaMKII overlay

YEGNINKVTIQQFQSPLPIQIPSSQAT

1-182
1-155
132-205
155-205
155-182

ABCD

45
31
66

Mr (K)

protein

CaMKII overlay

Fig. 3, Strack et al.
Fig. 5, Strack et al.
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J. Biol. Chem. published online May 25, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000319200

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