Caldendrin, a Novel Neuronal Calcium-binding Protein Confined to the Somato-dendritic Compartment*

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Using antibodies against synaptic protein preparations, we cloned the cDNA of a novel Ca$^{2+}$-binding protein. Its C-terminal portion displays significant similarity with calmodulin and contains two EF-hand motifs. The corresponding mRNA is highly expressed in rat brain, primarily in cerebral cortex, hippocampus, and cerebellum; its expression appears to be restricted to neurons. Transcript levels increase during postnatal development. A recombinant C-terminal protein fragment binds Ca$^{2+}$ as indicated by a Ca$^{2+}$-induced mobility shift in SDS-polyacrylamide gel electrophoresis. Antisera generated against the bacterial fusion protein recognize a brain-specific protein doublet with apparent molecular masses of 33 and 36 kDa. These data are confirmed by in vitro translation, which generates a single 36-kDa polypeptide, and by the heterologous expression in 293 cells, which yields a 33/36-kDa doublet comparable to that found in brain. On two-dimensional gels, the 33-kDa band separates into a chain of spots plausibly due to differential phosphorylation. This view is supported by in situ phosphorylation studies in hippocampal slices. Most of the immunoreactivity is detectable in cytoskeletal preparations with a further enrichment in the synapse-associated cytomatrix. These biochemical data, together with the ultra-structural localization in dendrites and the postsynaptic density, strongly suggest an association with the somato-dendritic cytoskeleton. Therefore, this novel Ca$^{2+}$-binding protein was named caldendrin.

Neurons are structurally and functionally highly polarized cells consisting of an axonal sending and a somato-dendritic receptive compartment. Communication between neurons occurs at synapses, which are asymmetric cell-cell contact sites that typically consist of specialized membrane structures and the underlying cytoskeleton. These cellular specializations derive from the axon of the presynaptic neuron and a dendrite of the postsynaptic neuron. Normal synaptic transmission as well as synaptic plasticity, i.e. use-dependent modulation of synaptic strength, critically depend on intracellular signaling processes on either side of the synapse. Although many of these signaling pathways are mediated by few common messengers, including Ca$^{2+}$ or cAMP, local specificity is achieved by clustering protein components involved in these pathways at distinct subcellular sites. One such site is the postsynaptic density (PSD), an electron-dense proteinaceous structure of the postsynaptic cytoskeleton at excitatory synapses, which is thought to cluster together neurotransmitter receptors with components of signaling pathways (1–4).

Signaling by a great variety of external stimuli, including neurotransmitters, growth factors and hormones converges at the level of the Ca$^{2+}$ ion and is further mediated by diverse intracellular Ca$^{2+}$-binding proteins (CaBPs) that either can modulate enzymes or structural proteins of the cytoskeleton or are enzymes or cytoskeletal proteins themselves (5). Some of these CaBPs, like calmodulin, are ubiquitously expressed in all cells and mediate a plethora of intracellular responses to Ca$^{2+}$ signals. Others, e.g. members of the intracellular neuronal calcium sensor (NCS) family (6, 7) are restricted to the central nervous system and even to specific neural cell types.

Several neuronal CaBPs have been localized to the postsynaptic compartment. These include α-actinin-2 (8) and fodrin (9) as structural components, as well as the regulatory molecule calmodulin (10), the protein phosphatase calcineurin (11), and the NCS protein VILIP (12). Recent studies revealed an important role for α-actinin and Ca$^{2+}$/calmodulin in the Ca$^{2+}$-dependent attachment of NMDA receptors to the synaptic cytomatrix (8, 13, 14). Moreover, calmodulin in its Ca$^{2+}$-bound state is able to bind and activate calmodulin kinase II, a multifunctional kinase highly enriched in the PSD, which is engaged in several processes of neuronal plasticity including the induction of long term potentiation (15, 16). Calcineurin has been shown to dephosphorylate the NMDA receptor in a Ca$^{2+}$-dependent manner, thereby shortening the channel openings (17). Calcineurin overexpression affects the transition of short term to long term memory and reveals a novel intermediate

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) Y17048.

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‡ The abbreviations used are: PSD, postsynaptic density; CaBP, calcium-binding protein; DAB, diaminobenzidine; HER, human embryonic kidney; NCS, neuronal calcium sensor; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid

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phase of long term potentiation (18, 19). VLIP appears to be involved in the cross-talk of cyclic nucleotide- and Ca$^{2+}$-regulated signaling pathways (20).

Using a combined biochemical and molecular approach to identify new synapse-associated proteins (21, 22), we cloned a cDNA encoding a novel neuron-specific polypeptide that was named caldendrin because it binds Ca$^{2+}$ and is primarily localized in dendrites and neuronal somata. The protein was formerly called calp (calmodulin-like protein), indicating its similarity to calmodulin (22). The present study describes the cloning of the full-length caldendrin cDNA, the tissue distribution of the corresponding transcript, and the biochemical characterization and subcellular localization of the encoded protein.

**MATERIALS AND METHODS**

**Transcript Analysis**—Northern analysis was performed as described previously (23). For in situ hybridization, the following oligodeoxynucleotides (36–43 mers) were derived from the caldendrin cDNA: 5′-CAA TTC GTA AGT CCA GTC CAT CTC CAT GGT C-3′ (nucleotides 962–923 of caldendrin cDNA); 5′/3′-TTG TTC GTA CAT CAA AGG AGG CAG GTA GGC A-3′ (nucleotides 1385–1346 of caldendrin cDNA); 5′/5′-GAC CTC AAT GGT TTC GGT GAC TTC CCA AGA TTT G-3′ (nucleotides 923–962 of caldendrin cDNA) sense control. Labeling and hybridization were performed exactly as described recently (24).

**In Vitro Transcription/Translation**—A coupled cell-free transcription/translation system (TNT T3, Promega) was used to generate the primary caldendrin translation product in vitro. Translation products were analyzed by SDS-PAGE and Western blotting.

**Isolation of Subcellular Protein Fractions and Western Blotting**—Tissue from adult rats (total brain, neocortex, heart, or liver) was homogenized in 20 mM Tris buffer, pH 7.4, containing either 2 mM CaCl$\textsubscript{2}$ or 1 mM EDTA and protease inhibitor mixture (Boehringer, Mannheim, Germany). Soluble proteins were obtained as the supernatant after centrifugation for 30 min at 32,800 $g$ at 4°C. The suspension was kept on ice for 15 min and centrifuged at 100,000 $g$ for 20 min. Each pellet was then resuspended in 0.5 ml of lysis buffer (zinc precipitation buffer A (10 mM Tris/HCl, 0.5 M NaCl, 1% Triton X-100, pH 7.4), 95/60, 5′-GAC CTC AAT GGT TTC GGT GAC TTC CCA AGA TTT G-3′ (nucleotides 962–923 of caldendrin cDNA) sense control. Labeling and hybridization were performed exactly as described recently (24).

**Results**

**Preparation of Hippocampal Slices, in Situ Phosphorylation, and Immunoprecipitation**—For in situ phosphorylation studies, hippocampal slices were preincubated in 100 mCi of $^{33}$P (final concentration in the incubation medium 50 $\mu$Ci/ml) as described previously (25). After 90 min of labeling time, slices were rinsed with homogenization buffer A (10 mM Tris/HCl, 0.5 mM NiCl$\textsubscript{2}$, 1% Triton X-100, pH 7.4), homogenized, and spun at 100,000 $g$ for 1 h. The supernatant was immunoprecipitated with 10 ml of polyclonal rabbit antisemur preabsorbed to GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden). The remaining polyclonal antisemur preabsorbed to GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden). The remaining polyclonal antisemur preabsorbed to GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden). The remaining polyclonal antisemur preabsorbed to GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden).

**RESULTS**

**Identification and Cloning of Caldendrin cDNAs**—From a collection of cDNA clones isolated by expression screening with antisera against a rat brain synaptic protein preparation (22), one cDNA clone, termed sap89, encoded a protein fragment that displayed distinct sequence similarity to rat calmodulin. The clone was used to isolate a set of seven overlapping cDNAs ranging in size from 1 to 1.4 kilobases. They harbor an open reading frame of 298 amino acids (Fig. 1). The translation initiation site was assigned to nucleotides 86–88 encoding the first in-frame methionine. This region fits well with the consensus pattern for eukaryotic translation initiation (AAG/UCC AUG (GA); Ref. 31), including a 5′ GC-rich region with only one mismatch at the −1 position (G instead of C). In vitro translation and heterologous expression studies confirmed the use of this initiation site (see below). A putative polyadenyl-
The deduced protein caldendrin has a calculated Mr of 33,071 and a theoretical isoelectric point of 7.42. Whereas primary structure analysis revealed no identifiable structural features in the N-terminal half of the protein, the C-terminal part displays a high degree of similarity to the entire reading frame of rat calmodulin. As shown in an alignment of both proteins (Fig. 1B), two of the four Ca\(^{2+}\)-binding EF-hand structures found in calmodulin are conserved in the novel protein. However, the remaining two miss several essential residues in the consensus pattern. Within the aligned region sequence, identity and similarity between caldendrin and calmodulin are 42% and 57%, respectively. The N-terminal half of caldendrin contains seven putative protein kinase C phosphorylation sites (arrows in Fig. 1A) and an unusually high proline content (13%).

Several human expressed sequence tags (GenBank accession nos. N48250, H93147, AA363865, AA364517, H92751, AA349351, and AA364942) have a high degree of nucleotide identity (81–89%) with corresponding stretches in the caldendrin cDNA, strongly suggesting that they encode human caldendrin.

**Spatial and Temporal Expression Pattern of the Caldendrin Transcript**—Northern blot analysis revealed a single hybridizing band of 1.8 kilobases that is detectable in various brain regions with strongest expression in the cerebral cortex. No hybridization is detected in heart, muscle, liver, and C6 glioma cells (Fig. 2A). During brain development, hybridization signals were first observed in the second postnatal week, i.e. during period of synapse formation and terminal differentiation of the brain. After the initial period of increase caldendrin, mRNA levels remain essentially unchanged throughout later...
ontogenetic stages (Fig. 2B).

To examine the transcript distribution at the cellular level, we performed in situ hybridization studies in horizontal brain sections (Fig. 2, C and D). With this more sensitive method the transcript can be clearly localized first at day P10 to distinct brain regions including cerebral cortex, developing cerebellar cortex, and hippocampal formation (Fig. 2C). In adult rats, the hybridization pattern is qualitatively the same, while the labeling intensity has increased (Fig. 2D). Silver grains were mainly found in cortically organized parts of the brain. Interestingly, the hippocampal subfields are not uniformly labeled. In contrast to a very prominent hybridization in the CA3 pyramidal cell layer, labeling of the CA1 region and the dentate gyrus is much less intense (Fig. 2E). Thus, caldendrin expression is restricted to certain brain regions and the transcript seems to be differentially expressed, at least in the hippocampus, in different subsets of cells.

In control experiments, the use of a sense oligonucleotide, competition with 100-fold excess of unlabeled oligonucleotide as well as washing steps above the calculated melting temperature of the hybrid yielded no significant labeling. Specificity of the hybridization was further confirmed by use of two independent oligonucleotide probes that produced virtually identical results (data not shown).

**Calcium Binding Activity of Recombinant Caldendrin Fragments**—A caldendrin cDNA fragment comprising nucleotides 500–979 (see Fig. 1) was generated via polymerase chain reaction and subcloned into the pQE30 vector to generate a bacterially expressed fusion protein including amino acids 139–298 and six histidine residues at the N terminus. This fusion protein has a calculated Mr of 19.386 kDa. In the presence of 1 mM Ca\(^{2+}\), a clear mobility shift in the PAGE gel can be observed, indicating the ability of the caldendrin C terminus to bind Ca\(^{2+}\) (Fig. 3). This shift is most plausibly based on conformational changes that occur in caldendrin upon Ca\(^ {2+}\) binding as has been shown for other CaBP, e.g. calmodulin (32, 33) or VILIP (34).

**Characterization of Caldendrin**—The fusion protein was used to raise polyclonal caldendrin antisera in mice and rabbits. Three independent antisera specifically detect a protein doublet of 33/36 kDa in brain protein preparations (Fig. 4). This doublet is not recognized by either of the pre-immune sera (data not shown). To get a first clue to the biochemical nature of the two polypeptides, we tested their solubility in low salt buffer and detergent. The 33-kDa protein isomer is partly found in soluble protein fractions. Heart and kidney protein preparations obtained from 8-week-old rats are devoid of caldendrin immunoreactivity (data not shown).

To verify the proposed ORF, a coupled cell-free transcription/translation system was used to generate the primary calden-
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Subcellular Localization of Caldendrin—Subcellular fractionation by differential centrifugation of brain proteins demonstrated that Caldendrin immunoreactivity is associated with nearly all particulate fractions including light and heavy membrane fractions and several synaptic fractions, like synaptosomes, synaptic membranes and the PSD fraction (Fig. 6A). The immunoreactivity in particular of the 36-kDa band is clearly enriched in the PSD fraction after the second washing step with Triton X-100 (T2 in Fig. 6A), indicating a tight association with the synaptic cytomatrix. However, Caldendrin appears not to be an exclusively synaptic protein.

To investigate the association of Caldendrin with the cytoskeleton, several attempts were made to solubilize the 36-kDa isoform from the crude membrane fraction (14,000 x g pellet P2) as shown in Fig. 6B. A complete extraction was successful with a combination of high salt and detergent (1 M NaCl, 2.5% Chaps), with chaotropic agents (3 M KSCN) and under fully denaturing conditions (8 M urea). Treatment with 1 M Tris, even in combination with 1% Triton X-100 as well as alkaline conditions (pH 11.5), did not lead to a release of substantial amounts of 36-kDa Caldendrin. Interestingly, alkaline treatment results in a complete extraction of 33-kDa Caldendrin, thereby fully separating both isoforms. These solubilization characteristics of the protein indicate an association, particularly of the larger Caldendrin isoform, with the neuronal cytomatrix.

FIG. 4. Association of Caldendrin isoforms with cellular compartments and appearance during postnatal development. Protein extracts were obtained from cerebral cortex of rats at the ages of 2, 4, 6, or 8 weeks as indicated or from heart (H) and kidney (K) of 8-week-old rats. Western blots were loaded with 50 µg per slot of soluble protein (SP; upper panel) or detergent-insoluble cytoskeletal protein (CP; lower panel). The apparent sizes are indicated.

FIG. 5. Caldendrin expression in a cell-free system (A) and transiently transfected 293 cells (B). A, in vitro transcription/translation of the total Caldendrin ORF using the coupled cell-free TNT-T3 system (Promega). Western blot loaded with in vitro translation products obtained in the absence (control, lane 1) or presence (lane 2) of Caldendrin cDNA in comparison to Caldendrin immunoreactivity in a brain cytoskeletal preparation (lane 3). Apparent molecular masses are indicated. Note that only the 36-kDa band is generated under in vitro conditions. B, transient heterologous expression of Caldendrin in HEK293 cells. Western blot with HEK cell lysates from untransfected controls obtained in the absence (control, lane 1) or from heart (lanes 2, 4, 6, or 8 weeks as indicated or from heart (H) and kidney (K) of 8-week-old rats. Western blots were loaded with 50 µg per slot of soluble protein (SP; upper panel) or detergent-insoluble cytoskeletal protein (CP; lower panel). The apparent sizes are indicated.
This assumption is supported by light and electron microscopic localization of caldendrin immunoreactivity in the brain. A representative light micrograph (Fig. 7A) shows several immunopositive neurons of the cerebral cortex. Within the neurons, caldendrin is found only in the somato-dendritic compartment. No immunostaining is found associated with axonal processes of neurons or in glial cells. Staining is most intense underneath the membranes of the neuronal cell bodies as well as within the dendrites as depicted at higher magnification in Fig. 7B. In the CA3 region of the hippocampal formation, the pyramidal neurons as well as some hilus neurons are intensely stained (Fig. 7C). The picture also reveals immunoreactivity in...
pyramidal cell primary dendrites. Ultrastructural localization studies in the hippocampal CA3 region confirm the dendritic and postsynaptic localization (Fig. 7, D and E). Interestingly, accumulation of the reaction product is not evenly distributed inside the dendrites but concentrates along intracellular membranes and cytoskeletal structures like tubular bundles as depicted in Fig. 7D. This is in good agreement with the biochemical data. We never observed any immunolabeling of glial cells, axons or axon terminals at the ultrastructural level (AT in Fig. 7E). Consistent with the subcellular fractionation studies caldendrin appears highly enriched at PSDs (Fig. 7E).

Caldendrin as a Putative Substrate for Protein Kinases—The primary structure of caldendrin contains seven putative protein kinase C phosphorylation sites (see Fig. 1). Therefore, we tested whether caldendrin is a possible substrate for protein kinases. Two-dimensional gel electrophoresis of immunoprecipitated caldendrin demonstrates that both isoforms differ in their migration behavior along the pH gradient (Fig. 8). Whereas the 36-kDa isoform appears as a single spot at a pI of 6.8–6.9 (calculated pI of 7.42), the 33-kDa band separates into a chain of at least three spots within the range of pH 5.9–6.8. This could be a consequence of phosphorylation, because a phosphate group added to a protein shifts the pI for 0.1 to 0.3 units (35).

To test this hypothesis more directly, in situ phosphorylation experiments were performed in acute hippocampal slices. Phosphorylation was carried out for 90 min. Afterward, slices were homogenized and fractionated and the protein preparations used for caldendrin immunoprecipitation. The autoradiograph in Fig. 9 shows immunoprecipitation of radioactivity with polyclonal rabbit caldendrin antiserum from soluble proteins and in Fig. 9 shows immunoprecipitation of radioactivity with polyclonal mouse antiserum. Apparent molecular masses and isoelectric points as deduced from marker proteins are indicated.

In vitro phosphorylation of caldendrin was shown under in vitro conditions in hippocampal slice preparations. Interestingly, only the 33-kDa isoform incorporated 32P. This could point to an important role for phosphorylation in modulating biochemical properties of caldendrin. It is possible that an initial phosphorylation step of the 36-kDa isoform induces conformational alterations, which result in the observed change in migration behavior and solubility of the protein.

The potential of caldendrin to bind Ca2+ was shown in vitro with a bacterially expressed fusion protein. The electrophoretic mobility shift observed with this 18-kDa caldendrin fragment upon Ca2+ binding is not detectable with the full-length brain protein. A possible explanation may be that the conformational changes are compensated in the much larger full-length pro-
tein which exhibits a clear bipartite structure. The two parts are predicted to differ significantly in their physico-chemical features. Whereas the N-terminal half is highly basic (pI = 11.9; 13 positively charged amino acid residues), the Ca\(^{2+}\)-dependent C-terminal half is acidic (calculated pI = 4.5; 13 negatively charged residues). Therefore, intramolecular interactions are likely, which may be subject to regulation by Ca\(^{2+}\) binding and phosphorylation.

On the basis of their postulated function, the EF-hand CaBPs have usually been classified as "trigger" or "buffer" proteins. Whereas trigger proteins like calmodulin (for review, see Ref. 36) or the members of the NCS family change their conformation upon Ca\(^{2+}\) binding and then modulate a variety of channels or enzymes, buffer proteins like parvalbumin (reviewed in Ref. 37) are thought to play a more passive scavenger role to limit the rise in intracellular Ca\(^{2+}\) concentration. The modular organization of caldendrin supports a postulated trigger function. Analysis of the caldendrin N terminus, which appears to be structurally related to any known protein, may help to understand the functional significance of this bipartite molecular organization.

Caldendrin seems to be preferentially expressed in brain regions with a laminar organization (i.e. cortex, hippocampus, and cerebellum) and in neuronal cells with a broad dendritic tree. So far, we have found no evidence for expression of the protein in neuroglia. Its distribution within the neuron appears to be restricted to the somato-dendritic compartment, a feature that it shares for example with MAP2, a cytoskeletal protein associated with microtubules (38). Both the biochemical solubilization characteristics and the subcellular fractionation studies lead us to conclude that caldendrin is associated with the cortical cytoskeleton. Caldendrin seems to be localized to the subplasmalemmal cortex of cell soma and dendrites, and both biochemical fractionation and electron microscopic investigation clearly demonstrate a synaptic localization of caldendrin. Although caldendrin is not exclusively localized to synapses we found that, in contrast to many other proteins of the dendritic cytoskeleton, immunoreactivity is enriched in the postsynaptic density fraction.

Several other synaptic proteins display similar intramolecular dichotomies and therefore are good candidates to confer Ca\(^{2+}\)-dependent regulation to subcellular structures or for the coupling to different signaling pathways. Examples include the cytoskeletal proteins α-spectrin, non-muscle α-actinins (39), and myosin. In the latter case, the calmodulin-like domain represents the regulatory light chain (40). The Ca\(^{2+}\)-dependent Cys endopeptidase calpain also contains EF-hand domains at the C terminus of each subunit that confers Ca\(^{2+}\) regulation to the proteolytic action of the holoenzyme (41). Another example is calcineurin, a Ca\(^{2+}\)-calmodulin-dependent protein phosphatase that is composed of a catalytic subunit and a calmodulin-like regulatory subunit (42). Based on gene structure analyses, it has been suggested that these modular molecules, which are composed of one or more Ca\(^{2+}\)-binding EF-hand domains combined with other functional domains, have evolved from a single genetically mobile ancestral EF-hand motif (43).

The relatively late onset of transcription of the caldendrin gene during development is shared with several other neuronal CaBPs (44). This suggests that caldendrin expression is not crucial for synaptogenesis. However, in contrast to some other CaBPs, like hippocain (45) or calbindin-D\(_{28K}\) (46), no decrease in protein levels in mature neurons was observed. The ontogenetic expression profile, therefore, suggests a functional role for caldendrin in the dendritic tree of terminally differentiated neurons.

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