Localization of Ca^{2+} -dependent conformational changes of calretinin by limited tryptic proteolysis

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Calretinin is an EF-hand Ca^{2+} -binding protein expressed predominantly in some neurons. We have found that the tryptic digestion pattern of rat recombinant calretinin depends on Ca^{2+} concentration as determined by SDS/PAGE, amino-acid-sequence analysis and electrospray-ionization MS. Ca^{2+} -saturated calretinin was cleaved between amino acids 60 and 61 to yield two fragments, which accumulated during cleavage. Small amounts of the larger fragment (amino acid residues 61–271) were further cleaved from the C-terminal end. Ca^{2+} -free calretinin was also cleaved between residues 60 and 61; however, under the latter conditions the fragment 61–271 was further cleaved from the N-terminal end. Native rat calretinin was cleaved by trypsin

in a similar Ca^{2+} -dependent fashion. All identified fragments of recombinant calretinin bound $^{45}\text{Ca}^{2+}$ on nitrocellulose filters, although to a different extent. The 61–271 fragment was released by EGTA from an octyl-agarose column in a manner similar to intact calretinin, while fragment 61–233 was not eluted by EGTA. These observations show that there are trypsin cleavage sites in calretinin that are available regardless of Ca^{2+} binding, other sites that are completely protected against trypsin on Ca^{2+} -binding and sites which become partially available on Ca^{2+} -binding. Together these data show that calretinin changes its conformation on Ca^{2+} binding and identify the regions which are exposed in apo and Ca^{2+} -bound form.

INTRODUCTION

Calretinin is a Ca^{2+} -binding protein present mainly in a variety of brain neurons [1–6]. Recently calretinin has also been identified in interstitial cells of rat ovary [7], enteric nervous system [8,9] and in Leydig cells of testis [10], as well as in human cancer cells [11] and in early embryonic development [12]. Calretinin is a member of the EF-hand superfamily which includes, for example, calbindin D28k, calmodulin, troponin C, parvalbumin and S100 proteins (reviewed in [13–16]). Calretinin shows most similarity to calbindin D28k, sharing 58% identical residues [1, 17, 18]. Both proteins contain six putative EF-hand motifs (although the sixth is aberrant), each of which consists of a helix–loop–helix structure and represents potential Ca^{2+} -binding sites (reviewed in [19,20]). The function of calretinin is not yet known. Its predominantly cytosolic distribution and ability to bind Ca^{2+} ions would be consistent with a role in neurotransmission. This role could involve interaction with other proteins, facilitating Ca^{2+} diffusion, or providing short-term buffering of local Ca^{2+} concentrations in cytosol. A Ca^{2+} -buffering role has been previously suggested for calbindin D28k and parvalbumin (reviewed in [21,22]), but a neuroprotective action against Ca^{2+} overload for these Ca^{2+} -binding proteins has not been proved (reviewed in [16,23]). On the basis of the low evolutionary rate of calretinin and the strong sequence conservation with calbindin D28k in EF-hand regions and sequences that follow the even-numbered EF-hands, it has been suggested that calretinin may do more than simply bind Ca^{2+} for the purpose of lowering cytosolic concentrations [17,20,24]. Despite many studies of the distribution of calretinin in tissues, information about its biochemical properties is very limited. Calretinin's electrophoretic properties and its ability to bind Ca^{2+} were shown by several authors [1,2,24,25].

Calretinin was found to bind at least four Ca^{2+} ions with a K_d between 0.3 and 0.5 μM [24,25]. Recently, several groups have developed methods for the production and purification of recombinant calretinin from *Escherichia coli*, which will permit a structure–function analysis of this protein [17,24,25]. Using rat recombinant calretinin we have initiated studies of structure, conformational changes and a search for possible calretinin targets. Work on the hydrophobic properties and subcellular distribution of calretinin has been presented [26,27]. The aim of the present work was to examine the effect of Ca^{2+} binding on the calretinin structure, using limited proteolysis by trypsin. A different cleavage pattern was generated by Ca^{2+} -free and Ca^{2+} -bound forms of calretinin, indicating a characteristic conformational change on Ca^{2+} binding. The identification of cleavage sites by MS and amino-acid-sequence analysis allowed us to identify specific regions which were exposed in the apo and Ca^{2+} -bound form. The analysis of proteolytic fragments also allowed us to locate a region which may be responsible for the Ca^{2+} -dependent hydrophobicity of calretinin. These studies provide insights into the molecular design of this protein and its role as a Ca^{2+} buffer and/or a Ca^{2+} -dependent activator.

EXPERIMENTAL

Purification of rat calretinin

Rat calretinin was expressed in *E. coli* as a fusion protein with glutathione S-transferase and purified as described by Strauss et al. [25]. Essentially the fusion protein bound to glutathione–Sephacrose was cleaved by thrombin to yield calretinin. Separation from thrombin was achieved using Ca^{2+} -dependent elution of calretinin from DEAE-cellulose. If necessary, an additional purification step was performed using an FPLC system and a

Abbreviations used: C1–C5 and E1–E6, tryptic fragments generated from recombinant calretinin in the presence of Ca^{2+} and EGTA respectively; PVDF, poly(vinylidene difluoride); ESI, electrospray ionization.

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Superose 12 column (Pharmacia) equilibrated with 150 mM NaCl/1 mM EGTA/50 mM Tris, pH 8.0. Native rat calretinin was purified from the cerebellum of male rats (Sprague-Dawley; Zivic-Miller, Zelienope, PA, U.S.A.) weighing 200–350 g. Fresh rat cerebella were homogenized (4 °C) in 10 vol. of 0.32 M sucrose/5 mM Hepes (pH 7.4)/0.1 mM CaCl_2 /25 $\mu\text{g}/\text{ml}$ leupeptin, and subjected to centrifugation at 800 *g* for 10 min. The final soluble extract (cerebellar cytosol) was obtained by ultracentrifugation of the supernatant at 165000 *g* for 1 h. Aliquots were stored (–80 °C) until use. Native rat calretinin was bound to DEAE-cellulose equilibrated with 20 mM NaCl/1 mM EGTA/50 mM Tris, pH 8.0. It was then eluted with 20 mM NaCl/2 mM CaCl_2 /50 mM Tris, pH 8.0, and applied on to a Superose 12 column as described above for recombinant calretinin. The fractions containing calretinin were desalted on PD-10 columns (Pharmacia) equilibrated with 50 mM NH_4HCO_3 and concentrated by partial freeze-drying. Protein concentration was measured using the Bio-Rad protein assay with BSA as a standard.

Tryptic digestion of rat calretinin

Rat recombinant calretinin (0.18–0.31 mg/ml) or native calretinin (0.1 mg/ml) were incubated (37 °C) with sequencing-grade trypsin (Boehringer-Mannheim Biochemica) at an enzyme/substrate ratio of 1:100 (w/w) in the presence of 150 mM NaCl/50 mM Tris, pH 8.0, and either 0.1 mM CaCl_2 or 1 mM EGTA. The digestion was stopped at various times by transferring sample aliquots to tubes containing soybean trypsin inhibitor (Sigma) (enzyme/inhibitor ratio 1:2, w/w). The fragments generated from calretinin digestion were subsequently analysed by SDS/PAGE, and either stained with silver or transferred on to nitrocellulose filters (for $^{45}\text{Ca}^{2+}$ overlay and immunoblotting) or poly(vinylidene difluoride) (PVDF) membranes (for amino acid sequencing).

$^{45}\text{Ca}^{2+}$ overlay and immunoblotting

SDS/PAGE was performed on 15% precast gels (Bio-Rad) in Tris/glycine buffer, pH 8.3. The Ca^{2+} binding of calretinin fragments was analysed on nitrocellulose filters (0.45 μm pore size; Schleicher and Schuell) using the method described by Maruyama et al. [28]. Immunoblotting was performed using a chemiluminescence kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD, U.S.A) with anti-calretinin serum diluted 1:7000.

Amino-acid-sequence analysis

Proteins were transferred from SDS/page gels on to PVDF membrane (Millipore). The membranes were stained with 0.1% Coomassie Blue in 50% methanol, destained in 50% methanol, and washed in deionized water. The protein bands were excised from the membrane and sequenced using an Applied Biosystems 470A gas-phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyser. Four to ten cycles were run for each peptide band.

Electrospray-ionization (ESI) MS

Analyses of native calretinin, recombinant calretinin and mixtures of tryptic fragments were performed by ESI-MS using a Hewlett-Packard electrospray source mounted on an Hewlett-Packard 5989 mass spectrometer. The capillary, end plate and cylinder potentials were set at –4500, –3500 and –3000 V respectively. The capillary exit potential was set at 200 V. The

analyses of tryptic digests of recombinant calretinin (83.3 μM) by MS were performed in 50 mM $(\text{NH}_4)_2\text{CO}_3$, since Tris is incompatible with ESI-MS analyses. The digestions were stopped at 50 min (in the presence of Ca^{2+}) and 20 min (in the presence of EGTA) by the addition of the electrospray solvent mixture and analysed immediately.

Hydrophobic chromatography using centrifugal filtration

All steps were performed at room temperature as previously described [26]. Suspensions (0.2 ml each) of octyl-agarose (Sigma; CNBr-activated; O-6376) were added to Ultrafree-MC filter units (0.45 μm pore size); Millipore (UFC3 OHV 00) sitting inside 1.5 ml Eppendorf collection tubes. The suspensions were centrifuged for 10 s in a Tomy HF-120 instrument, and the fluid present in the collecting tubes was discarded. The resin in each sample was washed five times by addition of 0.2 ml of the appropriate buffer, mixed, centrifuged, and the fluid present in the collecting tubes was removed. Samples of recombinant calretinin, or calretinin tryptic fragments, were equilibrated with initial resin buffer on Sephadex G-25 column (PD-10 or NAP-5; Pharmacia) and added to the resin in 0.2 ml portions (60–105 μg of recombinant protein or 50 μg of tryptic fragments). The contents were then vortex-mixed and incubated for 10 min. Filter units were centrifuged, and the fluid containing the unbound fractions was collected. The samples of resin were washed twice with 0.2 ml of the appropriate solutions (the second wash contained practically no protein), and an elution buffer containing 2 mM EGTA, and then 1% SDS in water (0.2 ml), were sequentially applied to the resin. Eluates were collected by centrifugation after each addition. The applied, unbound and eluate fractions were examined on SDS/PAGE gels stained with silver.

RESULTS

Tryptic digestion of rat recombinant calretinin

Examinations of proteins separated on SDS/PAGE gels and stained with silver (not shown) or transferred to blots and stained with Ponceau Red (Figure 1) revealed that the pattern of tryptic digestion of calretinin was dependent on the presence of Ca^{2+} . Calretinin saturated with Ca^{2+} yielded two major fragments which could be seen after 1 min exposure to trypsin. The larger fragment was partially cleaved at additional sites to yield three smaller peptides. Apo-calretinin was initially more resistant to tryptic digestion than the Ca^{2+} -bound form, since after 20 min of incubation with trypsin, undigested apo-calretinin was still present, while Ca^{2+} -bound calretinin was totally cleaved (Figure 1). However, more fragments of lower molecular masses were formed from apoprotein than from Ca^{2+} -bound calretinin (Figure 1; 20 min). Native rat calretinin was cleaved by trypsin in a similar Ca^{2+} -dependent fashion (results not shown).

Ability of tryptic fragments to bind $^{45}\text{Ca}^{2+}$

Major calretinin tryptic fragments were labelled either C1–C5 (Figure 1, top) or E1–E6 (Figure 1, bottom) after digestion of calretinin in the presence of Ca^{2+} and of the apoprotein respectively. The calretinin fragments generated by trypsin were separated by gel electrophoresis, transferred to nitrocellulose filters, and incubated with $^{45}\text{Ca}^{2+}$. Autoradiograms of the blots revealed that all fragments stained with Ponceau Red were able to bind Ca^{2+} (Figure 1). The amount of radioactivity associated with each band varied, but binding was detectable even for small fragments such as C5 and E6. A strong signal, similar to that of

Figure 1 Digestion of recombinant calretinin (CR) by trypsin at indicated time points (min) in the presence of Ca²⁺ (top) or EGTA (bottom)

Samples were separated by SDS/15%-PAGE and blotted on to nitrocellulose. Proteins were stained with Ponceau Red ('Protein staining') and next incubated with ⁴⁵Ca²⁺ (⁴⁵Ca²⁺ autoradiogram). M_r, molecular masses of markers: H, 30, 20.1 and 16.9 kDa; L, 16.9, 14.4, 8.1, 6.2 and 2.5 kDa. Calretinin fragments generated in the presence of Ca²⁺ are labelled C1–C5 (see Table 1) and those generated in the presence of EGTA are labelled E1–E6 (Table 1).

Figure 2 Spectra obtained by ESI-MS before (insets) and after deconvolution

Recombinant rat calretinin (1.3 μM) (a); native calretinin (< 1 μM) (b). Alternate charge states are labelled and indicate that recombinant calretinin prefers a +34 charge state, whereas native calretinin exhibits a +29 charge-state maximum.

the uncleaved protein, was observed for the C1 fragment formed in the presence of Ca²⁺ (Figure 1).

MS analysis of calretinin

Figure 2 shows the computer-deconvoluted mass spectra of recombinant (Figure 2a) and native (Figure 2b) calretinin. The ESI mass spectra are shown in the insets to Figure 2, with the charge states of the ions labelled. The best spectrum of recombinant calretinin was obtained after 20–200-fold dilution of the stock sample (83.3 μM) in acetonitrile/water/acetic acid (99:99:2, by vol.) The mass measurement accuracy over this concentration range was found to be within 0.006% (i.e. ±1.9 atomic mass units). The ESI mass spectrum for native calretinin (< 1 μM) in the same solvent mixture is shown in the inset of Figure 2(b). The deconvoluted mass spectrum shows a molecular mass of 31 428 Da. However, the charge state ions exhibit broader peak widths than in the spectrum of recombinant calretinin.

Identification of tryptic cleavage sites

Amino-acid-sequence analysis (not shown) enabled us to determine the N-terminal ends of the calretinin fragments which were stained on the blots (Figure 1). These data were useful in identifying cleavage sites which yielded C1 and C5 fragments. The fragments C2–C4 had the same N-terminal end as C1, but a different molecular mass as estimated by their mobility on SDS/PAGE. This indicates that the smaller fragments were generated from bigger ones by removing the C-terminal amino acids. In contrast, except for E1, which had the same N-terminal end as C1, all other fragments generated from the apo form had different N-terminal sequences. To identify the C-termini of all fragments, their exact masses were measured by ESI-MS. The spectrum of the Ca²⁺ digest of recombinant calretinin was

Figure 3 Spectrum of calretinin fragments generated by trypsin digestion (50 min) in the presence of Ca^{2+} (see Figure 1 and Table 1) before (inset) and after deconvolution

The sample (2.6 μM) did not contain soybean trypsin inhibitor.

Table 1 Molecular mass and amino acid composition of rat calretinin and its tryptic fragments

Calretinin or fragment	Molecular mass (Da) determined by MS	Calculated from amino acid sequence	Amino acid sequence
Native	31428–31431	31404*	1–271
Recombinant	31547–31550	31549	Gly-Ser-1–271
Tryptic fragment of recombinant			
C1	24579	24579	61–271
C2	20208	20210	61–233
C3	15055	15058	61–189
C4	ND	12888	†61–170
C5	6987	6988	Gly-Ser-1–60
C5'	6180	6181	Gly-Ser-1–52
E1	24619	24579	†61–271
E2	ND	23801	†68–271
E3	23539	23542	70–271
E4	22879	22878	75–271
E5	10834	10835	179–271
E6	6987	6988	Gly-Ser-1–60
E6'	ND	6181	†Gly-Ser-1–52
CX1‡	21588	21589	61–244
CX2	21719	21717	61–245
EX1‡	23687	23686	27–230
EX2	16209	16204	40–178
EX3	15053	15058	61–189
EX4	11909	11911	61–161

* With methionine.

† means a possible calcium adduct formation, or when mass of the fragment was not detected (ND).

‡ CX and EX refer to the fragments which were not detected on gels and blots.

obtained after 32-fold dilution of the digestion mixture (inset to Figure 3). The spectrum from the EGTA digest was more complicated than Ca^{2+} -digest spectrum and required several processes of deconvolution (not shown). The molecular masses of calretinin fragments generated both in the presence of Ca^{2+} (Figure 3) or EGTA (not shown) are shown in Table 1. These

Figure 4 Schematic structure of calretinin and its major tryptic fragments generated in the presence of Ca^{2+} (a) and in the absence of Ca^{2+} (EGTA) (b)

The tryptic cleavage sites are shown by arrows. Arabic numbers refer to the position of lysine or arginine at the cleavage sites, and Roman numerals show EF-hand motifs. Asterisks indicate the fragments whose mass determined by MS did not match the mass derived from the amino acid sequence.

masses were compared with the theoretical masses of the fragments having known N-terminal ends and possible C-terminal ends. The sequences that matched the experimentally determined molecular masses revealed some unknown cleavage sites and confirmed those identified by amino acid sequencing (Table 1). These data enabled us to identify trypsin cleavage sites of Ca^{2+} -bound and apo-calretinin as summarized in Figure 4. The bond between amino acid residues 60 and 61 was most readily cleaved by trypsin in both the presence and absence of Ca^{2+} . Thus fragments C1 and E1 were equivalent, as were C5 and E6. The 60–61 site is located in the region connecting EF-hand segments I and II. The C1 fragment (61–271) accumulated in the presence of Ca^{2+} , even though it was susceptible to further partial cleavage at sites 189–190 (the region connecting EF-hand motifs IV and V), 233–234 (the region connecting EF-hand motifs V and VI) and possibly between 170 and 171 (Figure 4). This cleavage yielded fragments C3, C2 and C4 respectively. It is clear that the C-terminal half of the 61–271 fragment (C1) became partly exposed to trypsin in the presence of Ca^{2+} ions, whereas the N-terminal end was very resistant to proteolysis.

When the Ca^{2+} -free 61–271 (E1) fragment was incubated with trypsin different sites became exposed and cleaved than those seen for the Ca^{2+} -bound equivalent (C1). Three of the most susceptible sites (67–68, 69–70, 74–75) were located at the N-terminal end of this fragment, i. e. in the N-terminal α -helix of the EF-hand II, whereas the fourth site (178–179) was located in the C-terminal α -helix of the EF hand IV (Figure 4). Thus fragments E2–E4 were generated by cleavage at sites which were not exposed in the presence of Ca^{2+} (Figure 4). Additional

Figure 5 Hydrophobic interaction of recombinant calretinin (CR) and tryptic fragments of calretinin with octyl-agarose

The fragments generated from Ca²⁺-bound calretinin after 20 (a) and 60 (b) min incubation with trypsin were added to octyl-agarose ('Applied'). Calretinin and all fragments (C1–C5, see Table 1) bound to the resin. The EGTA-containing solution eluted calretinin, C1 and C5 ('EGTA-eluted'), whereas the SDS-containing solution eluted C2, C3, C4 and the remainder of calretinin, C1 and C5 ('SDS-eluted').

cleavage sites found by MS analysis also were different in the absence and presence of Ca²⁺ (Table 1).

Ca²⁺-dependent binding of tryptic fragments to octyl-agarose

Intact calretinin has been shown to exhibit complex hydrophobicity. It binds to hydrophobic resins such as octyl-agarose in the presence of Ca²⁺, but is only partly released when the Ca²⁺ concentration is lowered by EGTA or EDTA [26]. All fragments generated by tryptic digestion in the presence of Ca²⁺ after 20 min (Figure 5a) or 60 min (Figure 5b) bound to octyl-agarose. On addition of the EGTA-containing solution, less than half of the recombinant calretinin and a comparable amount of the C1 fragment generated after 20 min digestion were released from the resin (Figure 5a). However, the smaller fragments (C2–C4) generated during 1 h digestion were not eluted by EGTA, but were released from the resin by the addition of SDS (Figure 5b). These results indicate that fragment 61–271 has Ca²⁺-dependent hydrophobic properties similar to those of intact protein, while fragment consisting of amino acids between 61 and 233 exhibit primarily Ca²⁺-independent hydrophobicity. Therefore it may be concluded that the region between amino acid residues 234 and 271, which encodes EF-hand motif VI, might be important for Ca²⁺-dependent elution from the hydrophobic resin. However, it is not clear if this region has a Ca²⁺-sensitive hydrophobic patch itself (EF-hand VI is aberrant and should not bind Ca²⁺) or if it interacts with another part of the molecule to induce Ca²⁺-sensitive hydrophobicity.

DISCUSSION

The studies described here provide strong evidence that calretinin changes conformation on Ca²⁺ binding and give some insights into its structure. Rat calretinin has 26 lysine and nine arginine residues which are potential cleavage sites for trypsin. Among these, only one, a bond between residues 60 and 61, is preferentially cleaved by this proteinase. This bond is cleaved in both Ca²⁺-free and Ca²⁺-bound forms of the protein, although at a slightly lower rate in the Ca²⁺-free form. These observations

suggest that the linking region between EF-hand motifs I and II of calretinin is exposed regardless of Ca²⁺ binding. However, different dynamics seems to occur in this region on Ca²⁺ binding, resulting in different rates of cleavage at this site. As in calbindin D28k, the linking region between EF-hand motifs I and II of calretinin is longer than the linking regions connecting other EF-hand motifs [1,29].

A significant effect of Ca²⁺ binding is observed on the tryptic digestion pattern of the calretinin fragment 61–271. In general, Ca²⁺ bound to this fragment provides resistance to cleavage, since it accumulates during long exposure to trypsin. The N-terminal half of this fragment is resistant to trypsin, but some regions in the C-terminal half are not resistant. It should be pointed out that two out of three secondary digestion sites of Ca²⁺-bound calretinin are not in the Ca²⁺-binding loops, but rather in the linking regions connecting EF-hand motifs. Therefore, the different pattern of digestion cannot be explained by the direct effect of Ca²⁺; rather it should be explained by a Ca²⁺-induced conformational change.

In a Ca²⁺-free conformation, fragment 61–271 was cleaved predominantly at the N-terminal end. Three cleavage sites were located in the N-terminal α -helix of EF-hand motif II and one in the C-terminal α -helix of EF-hand motif IV. These observations indicate that dissociation of Ca²⁺ from calretinin exposes different regions to trypsin than those which were exposed in the Ca²⁺-bound state. Moreover, the fact that EF-hand site II shows Ca²⁺-sensitive tryptic degradation after separation from the EF-hand site I, may suggest that this site is capable of binding Ca²⁺, as proposed by Parmentier [30], and in contrast with the properties of this site in calbindin D28k. Recent data showing that calretinin binds more Ca²⁺ than calbindin D28k, is consistent with these observations [24].

The spectrum of native calretinin exhibits broad peaks which could affect the accuracy of the deconvoluted mass spectrum shown in Figure 2(b). The molecular mass of native calretinin obtained by ESI-MS is 31428. Since the N-terminal end of this protein is blocked, as found by amino-acid-sequence analysis, this value includes the mass of the blocking group. The theoretical mass calculated from the amino acid sequence of rat native protein is 31404 (if the initiator methionine is present), 31447 (if such a methionine is acetylated), or 31315 (if N-terminal alanine is acetylated and methionine is removed). Since these values do not match the mass obtained experimentally, we cannot say what is the N-terminal end of native rat calretinin and how it is modified. However, the N-terminal residues and post-translational modification have no effect on Ca²⁺-dependent behaviour of calretinin as indicated by the similar proteolytic cleavage (the present work), Ca²⁺ binding [25] and hydrophobicity [26] of native and recombinant proteins. Calbindin D28k was reported to be acetylated at the N-terminal alanine [31]. Despite the accumulation of a large amount of data on the tissue- and cell-specific distribution of calretinin [1,3–8,10–12], little is known about the function of this protein. It is possible that calretinin may act as a Ca²⁺-buffer protecting cells against Ca²⁺ overload, as was suggested for calbindin D28k [21,22,32,33]. On the other hand, it has also been speculated that calretinin may do more than simply bind Ca²⁺ ions [16,17,20,23,24], although there is no experimental evidence to support this hypothesis and no other function was proposed. The ability of calretinin to change conformation on Ca²⁺ binding (as evidenced by Ca²⁺-dependent susceptibility of calretinin to trypsin shown in the present study), and the existence of specific hydrophobic site(s) which are sensitive to Ca²⁺ (as shown by Ca²⁺-dependent elution from hydrophobic resins) [26], show that calretinin shares some of the properties of calmodulin, a Ca²⁺-dependent activator (reviewed

in [34–36]). These observations allow us to speculate that calretinin may function not only by Ca^{2+} -buffering, but also, like calmodulin, by hydrophobic interactions with as-yet-unidentified targets. The recent analysis of calretinin distribution in rat cerebellum suggests that such targets may exist in membrane preparations [27].

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