Brain Calbindin-D_{28k} and an M_r 29 000 Calcium Binding Protein in Cerebellum Are Different but Related Proteins: Evidence Obtained from Sequence Analysis by Tandem Mass Spectrometry†

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ABSTRACT: A calcium binding protein of M_r 29 000 which cross-reacts with antibodies raised against chick calbindin-D_{28k} was previously reported to be present in rat cerebellum [Pochet, R., Parmentier, M., Lawson, D. E. M., & Pasteels, J. L. (1985) *Brain Res. 345*, 251-254]. It was suggested that the M_r 29 000 protein represents another form of calbindin-D_{28k}. In our laboratory we were able to identify M_r 28 000 and 29 000 proteins in rat, human, and chick cerebellum by their ability to bind ^{45}Ca in a ^{45}Ca blot assay. Two calcium binding proteins of M_r 27 680 and 29 450 were isolated from rat cerebelli by the use of gel permeation chromatography and preparative gel electrophoresis. After reverse-phase high-performance liquid chromatography (HPLC) the proteins were sequenced. Sequence analysis by tandem mass spectrometry indicated only 52% identity between the rat cerebellar M_r 28 000 and 29 000 proteins. Thus they are not different forms of the same protein, as previously suggested. Eighty-nine percent identity was observed between the rate cerebellar M_r 29 000 protein and chick calretinin [Rogers, J. H. (1987) *J. Cell Biol. 105*, 1343-1353]. The difference in identity between the rat cerebellar M_r 29 000 protein and chick calretinin may be due to species differences, and thus this protein is most likely rat calretinin. However, barely detectable levels of mRNA and weak immunoreactivity have been reported for calretinin in cerebellum. Since we found the M_r 29 000 calcium binding protein to be an abundant protein in rat cerebellum, these results suggest either posttranscriptional regulation of calretinin in cerebellum or species differences. Our study also suggests that previous immunocytochemical mapping for calbindin using antisera which cross-reacted with both proteins detected brain regions that expressed not only calbindin but also calretinin or a calretinin-like protein.

A transient increase in free calcium levels within the nerve cell is required for the release of neurotransmitters (Erulkar & Fine, 1979). Although the exact mechanism by which calcium mediates neurotransmitter release is not known, it has been demonstrated that variations in the intracellular localization of calcium account for changes in brain activity seen under conditions of both normal excitability and hyperexcitability (Traub & Llinas, 1979). The regulation of calcium flux across the nerve membrane and in the cell is also important for such functions as transport of proteins across the axon, exocytosis of transmitter-laden vesicles, and maintenance of ATPase activity and mitochondrial function. A major advance in our understanding of the molecular mechanisms by which calcium mediates cellular events was the identification of calcium-modulated proteins that have dissociation constants in the range of 10^{-8}-10^{-5} M and are postulated to be major targets of biological calcium signals (Means, 1981; Van Eldik et al., 1982; Christakos et al., 1989). The mammalian brain contains a number of proteins that bind calcium including calmodulin (Means, 1981; Van Eldik et al., 1982), S-100 (Moore, 1965; Means, 1981; Van Eldik et al., 1982), parvalbumin (Celio & Heizmann, 1981), and the M_r 28 000 vitamin D dependent calcium binding protein (calbindin-D_{28k} or CaBP; Jande et al., 1981; Baimbridge & Miller, 1982; Baimbridge et al., 1982; Feldman & Christakos, 1983; Parkes et al., 1984; Christakos et al., 1989; Parmentier et al., 1989). Although calbindin-D_{28k} was first described in avian intestine by Wasserman and Taylor (1966), only recently has calbindin-D_{28k} been described in mammalian tissues that are not regulators of serum calcium, such as brain. Previous studies have indicated an extensive distribution of calbindin-D_{28k} in most but not all neuronal cell groups and fiber tracts (Jande et al., 1981; Baimbridge & Miller, 1982; Feldman & Christakos, 1983) as well as a high concentration of calbindin in the brain (1-2% of the total soluble protein in the cerebellum; Baimbridge et al., 1982; Sonnenberg et al., 1984). These results suggest that calbindin plays a significant role in the regulation of mammalian brain function. A protein of M_r 29 000 which cross-reacts with antibodies raised against chick intestinal calbindin-D_{28k} was reported, by use of Western blot analysis, in rat cerebellum (Pochet et al., 1985) and in the brains of birds, reptiles, and amphibia (Parmentier et al., 1987). Although no biochemical data have been available concerning the M_r 29 000 protein in cerebellum, this protein was immunoprecipitated together with the M_r 28 000 CaBP from cell-free translation products of rat cerebellar mRNA, and it was suggested that it represents another form of the M_r 28 000 calbindin (Pochet et al., 1985). In order to provide an increased understanding of the relationship between these calcium binding proteins, thereby facilitating the elucidation of their function, we report the biochemical characterization of the M_r 29 000 cerebellar calcium binding protein, including sequence analysis by tandem mass spectrometry, and its comparison with rat brain calbindin-D_{28k}. Our findings indicate that these two calcium binding proteins in cerebellum are not two forms of the same protein as previously suggested and that earlier immunological studies using antisera which

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cross-reacted with both proteins detected not only calbindin but also another related but different calcium binding protein.

Materials and Methods

Sephadex G-100 and molecular weight standards were products of Pharmacia (Piscataway, NJ). $^{45}CaCl$, was purchased from ICN Radiochemicals (Irvine, CA) at a specific activity of 15–20 μCi/mg. Chelex 100 resin (200–400 mesh, sodium free) and all electrophoresis reagents were ordered from Bio-Rad (Richmond, CA). Dialysis membranes were purchased from Spectrapor (Los Angeles, CA). Leupeptin was obtained from Calbiochem (San Diego, CA) and phenylmethanesulfonyl fluoride (PMSF) was ordered from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other reagents were of the finest available grade from commercial sources. Antiserum directed against rat renal calbindin-D_{28k} was prepared in rabbits as described previously (Pansini & Christakos, 1984).

Preparation of Tissue Extracts. For purification of brain proteins, rat cerebelli (33 g from 65 rats/preparation) were obtained from 6–8 week old male Sprague-Dawley rats (Taconic Farms, Germantown, NY) fed a routine laboratory chow. Brain tissue was washed with cold saline solution, homogenized at 4 °C in 2 volumes of Tris (0.01 M Tris-HCl and 0.15 M NaCl, pH 7.4, containing 10^{-4} M PMSF and 10^{-5} M leupetin) with a Polytron tissue homogenizer, and centrifuged at 38000*g* for 60 min at 4 °C to obtain a postmitochondrial supernatant. Chick (day-old white Leghorn chicks from Shamrock Poultry and Breeding Farms, North Brunswick, NJ), bovine (from Max Cohen, Livingston, NJ), and human (from the National Disease Research Interchange, Philadelphia, PA) brain tissue as well as rat kidney tissue were similarly extracted.

Purification of Calcium Binding Proteins. The brain calcium binding proteins were further purified from the postmitochondrial supernatant fraction obtained from rat cerebelli by heat treatment at 65 °C for 15 min followed by Sephadex G-100 column chromatography as previously described for the purification of rat kidney calbindin-D_{28k} (Pansini & Christakos, 1984; Christakos et al., 1987b). After gel filtration on Sephadex G-100 the fractions containing the calcium binding activity, as determined by the Chelex resin assay (Briggs & Fleishman, 1965), were pooled and concentrated by lyophilization or Amicon ultrafiltration with a PM 10 filter and further purified by preparative SDS gel electrophoresis. The M_r 28 000 and 29 000 calcium binding proteins were identified by the ^{45}Ca blot assay described below. The proteins from the respective gel slices were eluted by overnight incubation in 0.01 M Tris and 0.15 M NaCl, pH 7.4, buffer. SDS was removed by using an Affinity-pak, Extracti-gel D column (Pierce, Rockford, IL). The final step in the purification process was reverse-phase HPLC performed on an Applied Biosystems Model 130A separations system. Sample (50 μg in 45 μL of 0.1% aqueous trifluoroacetic acid) was injected into a narrow-bore RP300 Aquapore column (2.1 mm × 3 cm) and eluted with a 40-min linear gradient of 0–60% acetonitrile (0.085% trifluoroacetic acid) in 0.1% trifluoroacetic acid. Column effluent was monitored at 214 nm, and fractions were collected.

^{45}Ca Blot Technique. Calcium binding proteins were detected by ^{45}Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate (SDS) gel electrophoresis essentially as described by Maruyama et al. (1984). Briefly, after electrophoretic transfer of proteins to the nitrocellulose membrane the membrane is washed in 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole hydrochloride, pH 6.8, buffer and then incubated in the same buffer containing 1 mCi/L ^{45}Ca for 10 min. The membrane is rinsed in distilled water for 5 min, air-dried, and exposed to Kodak XAR-5 film for 12–24 h.

Chelex Assay. The calcium binding activity was measured by the ^{45}Ca/Chelex resin assay of Briggs and Fleishman (1965) modified as previously described (Pansini & Christakos, 1984; Christakos et al., 1987a,b).

Immunoblot Technique (Western Blot). Proteins were separated by SDS–polyacrylamide gel electrophoresis (12.5% acrylamide) as described by Laemmli (1970), and the binding of rat calbindin-D_{28k} antibodies (Pansini & Christakos, 1984; Christakos et al., 1987b) to the electrophoretically separated proteins was assayed by the immunoblot technique essentially as described by Burnette (1981) with minor modifications (Gona et al., 1986).

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis of partially purified rat cerebellar proteins was performed according to the method of O'Farrell and O'Farrell (1977).

Proteolytic Cleavage Reactions. Proteolytic cleavage with either trypsin, chymotrypsin, endoproteinase Lys-C, or endoproteinase Asp-N (approximately 1 μg of each) and 30–150 μg of S-carboxymethylated or native protein was performed in degassed 100 mM Tris-HCl buffer (1 M in guanidine hydrochloride, pH 8.6) at 37 °C for 12–24 h. Reactions were terminated by lyophilization of the reaction mixture. Peptides were then fractionated by reverse-phase HPLC as described above. For subdigestion of peptides so isolated, elastase, endoproteinase Asp-N, or endoproteinase Glu-C (1 μg of each) was added to the peptide in 50 mM ammonium bicarbonate, pH 8.6. Reaction was allowed to proceed at 37 °C for 1–3 h and was then terminated by lyophilization of the solution. Products were fractionated by reverse-phase HPLC.

Mass Spectrometry. Mass spectra were recorded on either a TSQ-70 triple quadrupole (Finnigan-MAT, San Jose, CA) or a quadrupole Fourier transform instrument constructed at the University of Virginia. Operation of the latter instrument has been described previously (Hunt et al., 1987b). Sample ionization and volatilization in the TSQ-70 instrument were induced by particle bombardment from a cesium ion gun (Antek, Palo Alto, CA) operated at 6 keV. For ion detection, the conversion dynode of this instrument was operated at 15 keV. Collision-activated dissociation spectra were recorded on the TSQ-70 instrument with argon at a pressure of 4 mTorr as the collision gas (Hunt et al., 1986). Laser photodissociation spectra were recorded on the Fourier transform instrument as described previously (Hunt et al, 1987a; Michel et al., 1988). Samples for analysis on either of the above instruments were prepared by adding 0.5 μL of 0.1% trifluoroacetic acid solution, containing peptide at the 10–100-pmol level, to 0.5 μL of monothioglycerol matrix on a gold-plated stainless steel probe.

Other Methods. The protein concentration was measured by the method of Lowry et al. (1951) or by the method of Bradford (1976) with fraction V defatted bovine albumin (Sigma) as the protein standard.

Results

Immunological Studies. Western blot analysis was done by using rat renal calbindin-D_{28k} antisera (Pansini & Christakos, 1984; Sonnenberg et al., 1984; Christakos et al., 1987b) and rat cerebellar postmitochondrial supernatant proteins. When increasing concentrations of cerebellar proteins were analyzed, unlike previous findings with chick antiserum (Pochet et al., 1985; Parmentier et al., 1987), only the M_r 28 000

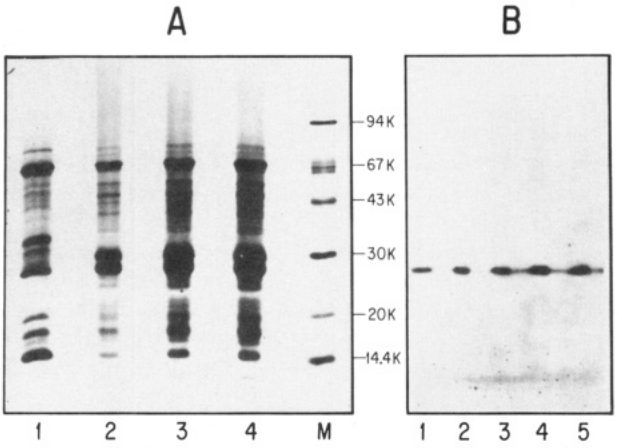

FIGURE 1: Immunoblot analysis of proteins in rat kidney (lane 1) and rat cerebellum (lanes 2–5) using rabbit antiserum to rat renal calbindin-D_{28k}. Proteins were separated by SDS–polyacrylamide slab gel electrophoresis and transferred to nitrocellulose sheets. Proteins were either stained with Coomassie Brilliant Blue in the gel slabs (A) or visualized by binding of radioiodinated *Staphylococcus* protein A to the immune complex, following incubation with antiserum (B). Increasing cerebellar protein concentrations are as follows: 100 μg, lane 2; 200 μg, lane 3; 250 μg, lane 4; 300 μg, lane 5 (not shown in A). The concentration of kidney protein in lane 1 is 100 μg. The migration pattern for the calibration markers is shown in the center (M).

protein cross-reacted with rat renal calbindin-D_{28k} antibodies (Figure 1) even when high cerebellar protein concentrations were used (Figure 1, lanes 3–5). Previous studies have indicated biochemical identity between rat brain and rat kidney calbindin-D_{28k} (Intrator et al., 1985). Additional evidence that our antiserum cross-reacted only with calbindin-D_{28k} in brain was obtained by Ouchterlony immunodiffusion and immunoelectrophoresis experiments. Ouchterlony immunodiffusion, using postmitochondrial rat renal and rat brain supernatant from cerebellum or cerebrum and rat renal calbindin-D_{28k} antiserum, resulted in immunoprecipitin lines joining with total coalescence. After immunoelectrophoresis, the precipitin arcs observed with rat renal and rat brain supernatants were equidistant from the origin and no other arcs were formed, indicating that the antiserum reacted only with calbindin-D_{28k} (data not shown).

^{45}Ca Blot Analyses. Although rat renal calbindin-D_{28k} antisera did not cross-react with a higher molecular weight brain protein, in order to examine further the possibility that an additional calcium binding protein, similar in molecular weight to calbindin-D_{28k}, was present in brain, ^{45}Ca blot analyses were done. Postmitochondrial supernatants of rat renal and cerebellar homogenates were fractionated on a Sephadex G-100 column, and fractions with calcium binding activity in the M_r 28 000 region, as measured by the Chelex resin assay (Briggs & Fleishman, 1965; Pansini & Christakos, 1984), were pooled and concentrated. Equivalent aliquots of protein from the rat renal or rat cerebellar preparations were separated by SDS gel electrophoresis. The separated proteins were transferred to nitrocellulose paper, which was then incubated with ^{45}Ca to detect calcium binding proteins by autoradiography (see Materials and Methods). The result obtained is shown in Figure 2A. The band in lane 3 (rat kidney) is equivalent in molecular weight to calbindin-D_{28k} (see lane 1, Figure 1B). Lane 4 (rat cerebellum) contains, in addition to the M_r 28 000 protein, a second calcium binding protein of higher molecular weight. ^{45}Ca blot analysis of rat cerebellar samples indicated the presence of both calcium binding proteins even after chelation of calcium (data not shown), suggesting that the two different molecular weight proteins are

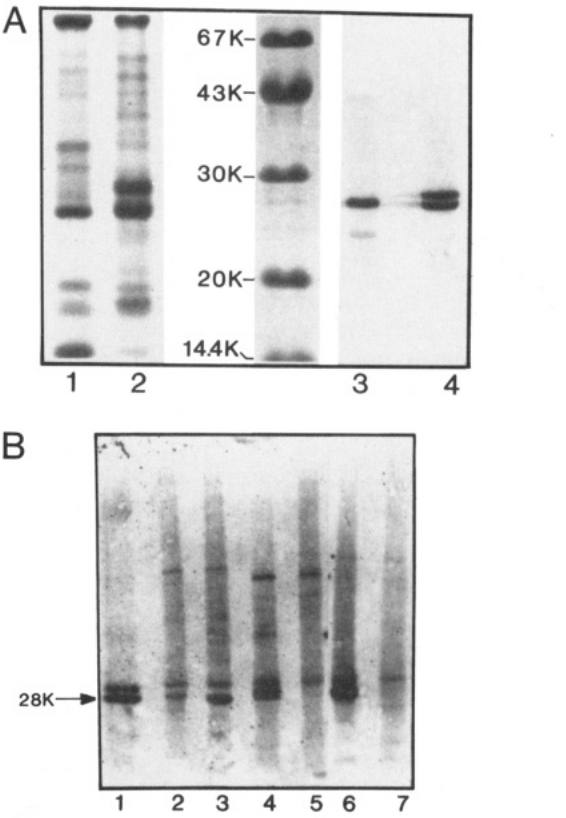

FIGURE 2: ^{45}Ca blot analysis of proteins in rat kidney and brain. (A) Rat kidney proteins (lanes 1 and 3) and rat cerebellar proteins (lanes 2 and 4) were either stained with Coomassie Blue in slab gels (first two lanes) or analyzed for calcium binding (last two lanes). (B) ^{45}Ca blot analysis of brain samples from various species. Lane 1, rat cerebellum; lane 2, rat cerebrum; lane 3, human cerebellum; lane 4, chick cerebellum; lane 5, chick cerebrum; lane 6, bovine cerebellum; lane 7, bovine cerebrum.

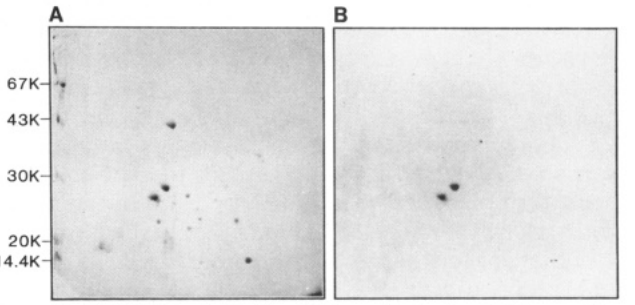

FIGURE 3: ^{45}Ca blot analysis of partially purified rat cerebellar proteins separated by two-dimensional gel electrophoresis. Proteins were either stained with Coomassie Brilliant Blue (A) or visualized by ^{45}Ca blot analysis (B).

not due to differences in mobility in SDS gels which occur in the presence and absence of calcium (Grab et al., 1979; Klee et al., 1979; Burgess et al., 1980; Van Eldik et al., 1980).

In order to determine the distribution in brain and species specificity, additional ^{45}Ca blot analyses were done. Figure 2B represents a ^{45}Ca blot analysis of cerebellar and cerebral extracts from various species after fractionation on Sephadex G-100. Our findings indicate that M_r 28 000 and 29 000 calcium binding proteins do not appear with the same intensity in all species and that an M_r 29 000 calcium binding protein can be detected in the cerebrum as well as the cerebellum of all species examined.

^{45}Ca blot analysis of two-dimensional gels of rat cerebellar proteins (Figure 3) indicated that the proteins have different isoelectric points (M_r 28 000 protein, pI = 4.9; M_r 29 000 protein, pI = 5.1).

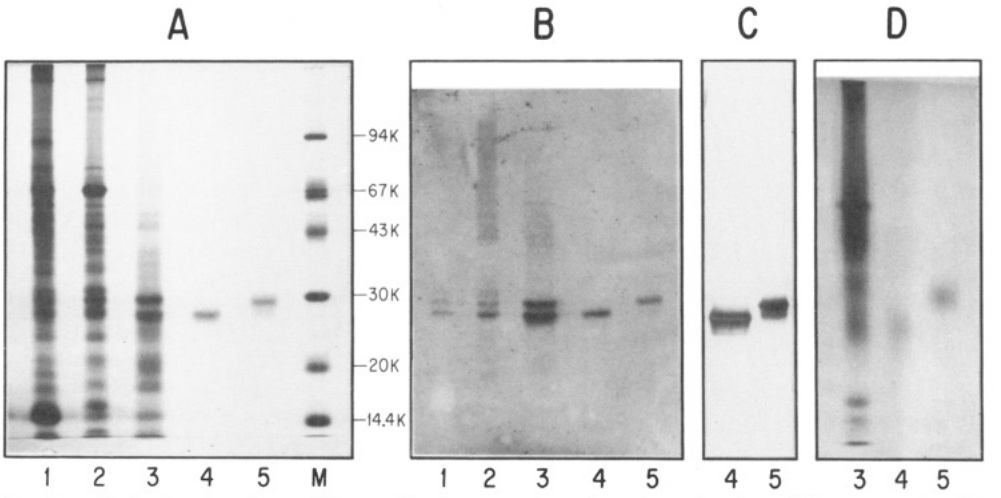

FIGURE 4: Electrophoretic analysis at various stages of the purification procedure. Lane 1, crude rat cerebellar supernatant; lane 2, rat cerebellar supernatant after heat treatment; lane 3, rat cerebellar proteins after gel filtration on Sephadex G-100; lane 4, purified rat cerebellar calbindin-D_{28k} after preparative gel electrophoresis; lane 5, purified rat cerebellar M_r 29 000 protein after preparative gel electrophoresis. (A) Coomassie Blue stain (SDS gel); (B) ^{45}Ca blot analysis; (C) silver stain (5 μg of each purified protein); (D) analytical polyacrylamide gel electrophoresis (25 μg of each purified protein, lanes 4 and 5).

Purification and Sequence Analysis. In order to determine whether the higher molecular weight protein was another form of calbindin-D_{28k} with a different isoelectric point which did not cross-react with rat calbindin-D_{28k} antisera or whether the M_r 28 000 and 29 000 proteins were two different calcium binding proteins, it was necessary to purify the proteins to homogeneity and to obtain sequence information. The M_r 28 000 and 29 000 calcium binding proteins were purified to homogeneity from rat cerebellum as described (see Materials and Methods). Approximately 2.4 mg of the M_r 29 000 protein and 1.9 mg of calbindin-D_{28k} were obtained from 1160 mg of crude cytosol from rat cerebellum. The electrophoretic patterns at the various stages of the purification procedure are shown in Figure 4. Coomassie Blue staining of the SDS gel is represented in Figure 4A, and ^{45}Ca blot analysis of rat cerebellar proteins at various stages of the purification procedure is shown in Figure 4B. Note in Figure 4C that after SDS gel electrophoresis of the purified proteins and silver stain only one band is observed for each calcium binding protein, indicating the homogeneity of the preparations. Similarly, a single band was observed after nondenaturing gel electrophoresis (Figure 4D; $R_f = 0.54$, M_r 28 000 protein, lane 4, and $R_f = 0.40$, M_r 29 000 protein, lane 5; R_f values represent the mean of three determinations). The molecular weights of the purified proteins as determined by SDS gel electrophoresis using protein standards were 27 680 and 29 450.

Exposure of the M_r 28 000 or 29 000 calcium binding proteins to several cycles of Edman degradation on a gas-phase sequenator failed to remove a phenylthiohydantoin derivative, suggesting that both proteins were blocked at the NH_2 terminus. A blocked amino terminus has previously been reported for calbindin (Christakos et al., 1989). In order to determine the primary structure in spite of a blocked amino terminus, the purified proteins were digested in separate experiments with elastase, endopeptidase Glu-C, trypsin, and dilute acid. Peptides produced in these digests were then fractionated by reverse-phase HPLC and either sequenced directly by tandem mass spectrometry or subdigested to produce smaller fragments and then sequenced by mass spectrometric techniques as described (see Materials and Methods).

Shown on line 2 of Figure 5 is the amino acid sequence data obtained on rat brain M_r 28 000 calbindin. Line 1 displays the cDNA-derived sequence of rat spot 35 protein, a protein spot from a two-dimensional gel that was later identified as rat calbindin-D_{28k} (Yamakuni et al., 1986; Wood et al., 1988). This latter sequence was employed to align the peptide fragments sequenced by tandem mass spectrometry. Sequences determined by collision-activated dissociation on the triple quadrupole instrument (Hunt et al., 1986) are labeled by enzyme and the monoisotopic mass of the $(M + H)^+$ ion. Peptides labeled with the prefix "FT-" were characterized by enzyme specificity and the average mass of the corresponding $(M + H)^+$ ion. Fragments labeled with the prefix "PD-FT-" were characterized by laser photodissociation on the Fourier transform instrument (Hunt et al., 1987a; Michel et al., 1988). Note that the amino acids Leu and Ile have identical masses and are not differentiated by tandem mass spectrometry under the conditions employed in the present sequence analysis. The letter X in the sequence indicates that these residues could be either Leu or Ile. Data obtained by tandem mass spectrometry indicate that the blocked N-terminus of the M_r 28 000 protein is *N*-acetylalanine and provide strong evidence that rat spot 35 protein and rat brain M_r 28 000 calbindin are identical proteins.

Shown on line 3 of Figure 6 is the amino acid sequence data obtained on rat cerebellar M_r 29 000 protein. Lines 1 and 2 contain the cDNA-derived partial sequence for rat spot 35 protein, calbindin-D_{28k} (Yamakuni et al., 1986; Wood et al., 1988) and a cDNA-derived partial sequence for chick calretinin (Rogers, 1987), respectively. Both of these sequences can be employed to align the peptide fragments generated from the rat brain M_r 29 000 protein. Note, however, that there is only 52% identity between the sequence for rat calbindin-D_{28k} and the rat brain M_r 29 000 protein. The identity between the sequences for the rat brain M_r 29 000 protein and chick calretinin (Rogers, 1987) is higher, 89%. We conclude that rat cerebellar M_r 28 000 and 29 000 proteins are distinct proteins and not different forms of the same protein as previously suggested. The available evidence suggests that the rat cerebellar M_r 29 000 protein is rat calretinin.

Discussion

Previous studies using anti-chick intestinal calbindin-D_{28k} detected two immunoreactive proteins (molecular weights approximately 28 000 and 29 000; Pochet et al., 1985; Parmentier et al., 1987) in brain. Both proteins were detected by Western blotting and as immunoprecipitates of translation products (Pochet et al., 1985; Parmentier et al., 1987). The

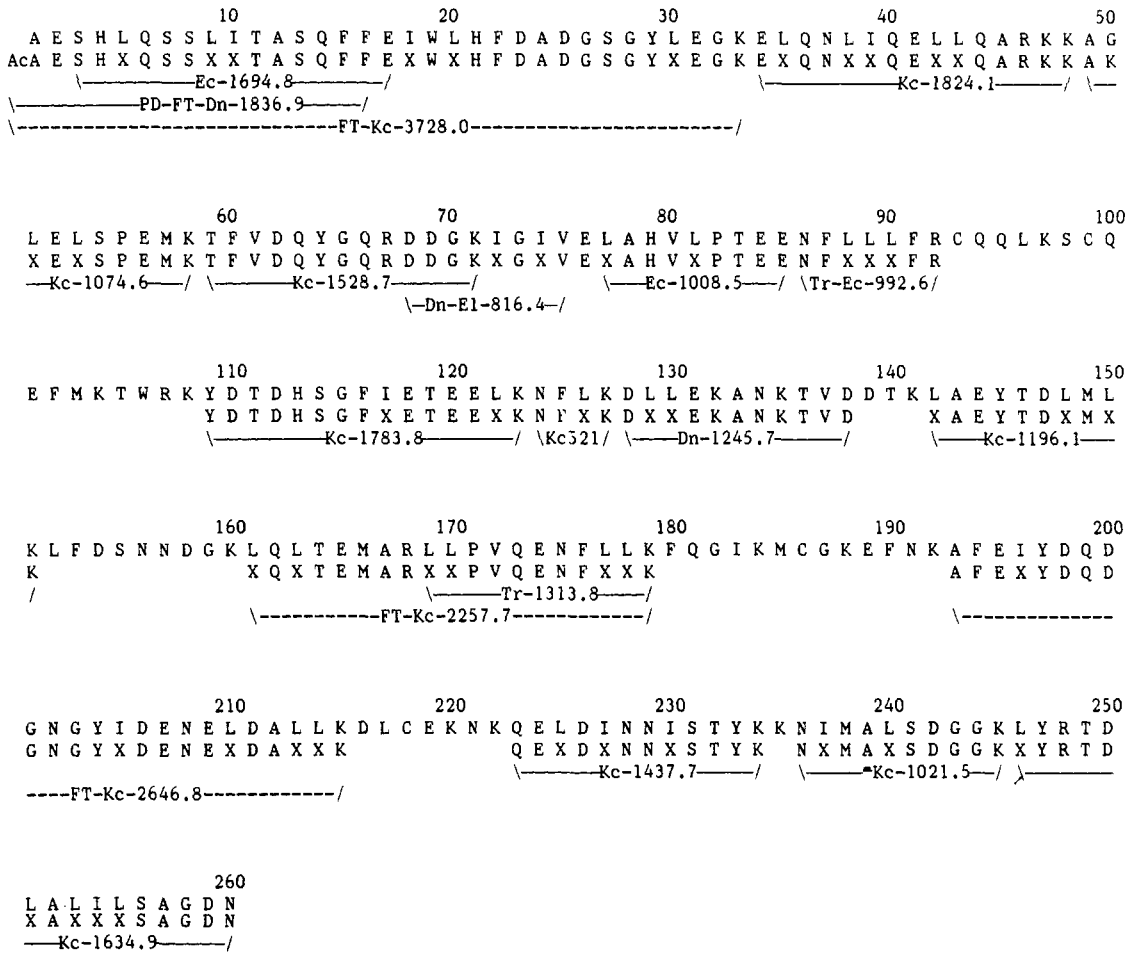

FIGURE 5: Amino acid sequence data obtained on calbindin-D_{28k} isolated from rat brain. Published data (Yamakuni et al., 1986; Wood et al., 1983) obtained from a cDNA sequence analysis of rat spot 35 protein (subsequently identified as rat calbindin-D_{28k}) is shown on line 1. Data obtained from sequence analysis of rat calbindin-D_{28k} by tandem mass spectrometry are shown on line 2. Note that the amino acids Leu and Ile have identical masses and are not differentiated by tandem mass spectrometry under the conditions employed in the present sequence analysis. The letter X in the sequence indicates that these residues could be either Leu or Ile. Fragments sequenced by collision-activated dissociation on the triple quadrupole instrument are labeled by enzyme and the monoisotopic mass of the $(M + H)^+$ ion. Enzymes are designated as follows: Kc, endoproteinase Lys-C; Ec, endoproteinase Glu-C; Dn, endoproteinase Asp-N; Tr, trypsin; Ch, chymotrypsin; and El, elastase. Fragments labeled with the prefix "FT-" were characterized by enzyme specificity and the average mass of the corresponding $(M + H)^+$ ion. Fragments labeled with the prefix "PD-FT-" were characterized by laser photodissociation on the Fourier transform instrument. A superscript "a" indicates that the methionine residue in this fragment is in the form of the corresponding sulfoxide.

proteins were detected in rat cerebellum (Pochet et al., 1985) and in whole brain of rats, birds, reptiles, and amphibians (Parmentier et al., 1987). The rat renal vitamin D dependent calbindin-D_{28k} antiserum cross-reacts only with the M_r 28 000 protein in rat (Feldman & Christakos, 1983), chick (unpublished observation, S. Christakos), and amphibian (Gona et al., 1986) brain. These differences suggest different immunoreactive sites for these antisera and that previous immunocytochemical mapping of calbindin in brain using antisera which crossreacted with both proteins detected not only calbindin but also another related but different calcium binding protein. Indeed, differences in the immunohistochemical mapping of rat brain calbindin have been reported with different calbindin-D_{28k} antisera (Jande et al., 1981; Feldman & Christakos, 1983; Garcia-Segura et al., 1984). The use of antisera with different antigenic sites which are specific for each calcium binding protein will serve as an important tool in distinguishing among these calcium binding proteins in future immunocytochemical mapping studies and in studies designed to measure these proteins in the same sample (such as radioimmunoassay).

Use of tandem mass spectrometry allowed us to obtain extensive sequence information over the whole length of both N-terminally blocked proteins and yet avoid extensive purification of proteolytic fragments. Only in this way were we able to determine that the M_r 28 000 and 29 000 proteins were not two forms of the same protein. Tandem mass spectrometry was recently used to determine that there are two isoforms of the M_r 9000 vitamin D dependent calcium binding protein (calbindin-D_{9k}), which is present primarily in mammalian intestine (Hunt et al., 1989). Unlike our findings for calbindin-D_{28k}, the two forms of calbindin-D_{9k} were found to be identical except for the insertion of a glutamine in the minor form at the site of an intron in the rat calbindin-D_{9k} gene, suggesting that alternative splicing produced the two forms of calbindin-D_{9k}. Thus the regulation of calbindin-D_{9k} and calbindin-D_{28k} may indeed be different. Alternative splicing may be involved in regulating, both developmentally and by hormone, the expression of calbindin-D_{9k} but not the expression of calbindin-D_{28k}.

In our studies, the sequence of the rat cerebellar M_r 29 000 protein was found to be 89% identical with the reported sequence of chick calretinin, also an M_r 29 000 calcium binding protein (Rogers, 1987) whose mRNA was cloned and sequenced from chick retina. Rogers (1987) reported 58% identity between chick calbindin-D_{28k} and chick calretinin and found by in situ hybridization studies that chick calretinin and calbindin are expressed in different sets of neurons throughout

FIGURE 6: Amino acid sequence data obtained on the M_r 29 000 calcium binding protein isolated from rat brain. Published data (Yamakuni et al., 1986; Wood et al., 1988) obtained from a cDNA sequence analysis of rat spot 35 protein (subsequently identified as rat calbindin-D_{28k}) is shown on line 1. The published partial sequence derived from cDNA sequence analysis of chick calretinin (Rogers, 1987) is shown on line 2 (the dots refer to regions of the sequence that were not completed in the published study). Data obtained from sequence analysis of the protein by tandem mass spectrometry are shown on line 3. The letter X in the sequence indicates that these residues could be either Leu or Ile. Lower case letters indicate that the residue assignment requires confirmation. See the legend to Figure 5 for an explanation of the labels attached to each peptide fragment.

the brain. The sequence data presented suggest that the M_r 29 000 calcium binding protein in cerebellum is calretinin with some variation due to species differences or to the presence of an isoform of calretinin. It should be noted however that calretinin RNAs, as determined by both in situ hybridization and Northern blot analysis, were reported to be comparatively rare in chick cerebellum (Rogers, 1987), while calbindin RNAs are most abundant in cerebellum (Rogers, 1987; Varghese et al., 1988). Calbindin is very abundant in Purkinje cells (Christakos et al., 1989) while calretinin is totally absent in these cells in chick (Rogers, 1987) and rat (Rogers, 1989). In rat only weak calretinin immunoreactivity was observed in cerebellum (Rogers, 1989). Antibodies against calretinin stain only the Lugaro cells in rat cerebellum and some granule cells in lobe X, and they stain granule cells in other lobes very weakly. In a recent study by Winsky et al. (1989) the purification of an M_r 29 000 calcium binding protein from guinea pig cerebrum, which appears to be identical with calretinin, was reported. By use of antisera specific to the guinea pig M_r 29 000 protein and immunofluorescence, weak immunoreactivity has been observed in rat and guinea pig cerebellum (L. Winsky, personal communication). However, radioimmunoassay, a more sensitive immunological determination, has indicated between 1–3.5 μg of calretinin/mg of soluble protein in rat cerebellum (Winsky and Jacobowitz, unpublished results). In our studies, Coomassie Blue staining of partially purified preparations containing both calbindin-D_{28k} and the M_r 29 000 protein (see Figure 4A, lane 3, for example) suggested equivalent concentrations of both proteins in rat cerebellum. In addition, Pochet et al. (1985), using chick intestinal calbindin-D_{28k} antiserum following translation of rat cerebellar mRNA in the rabbit reticulocyte lysate system, observed that the M_r 28 000 and 29 000 proteins which were immunoprecipitated were approximately equivalent in intensity. Therefore, one can conclude from our studies and those of Pochet et al. (1985) that there is an M_r 29 000 calcium binding protein in cerebellum which is not a rare protein, and thus one would not expect weak immunoreactivity and barely detectable levels of mRNA, as previously reported for calretinin in cerebellum (Rogers, 1987, 1989). Although the staining in granule cells is weak (Rogers, 1989), it is also widespread. Thus the possibility exists that the overall amount may be high (J. Rogers, personal communication). Barely detectable levels of calretinin mRNA in cerebellum may suggest posttranscriptional regulation. It is also possible that the limits of sensitivity of in situ hybridization did not result in an accurate representation of the abundance of calretinin RNA in cerebellum or that there are species differences (calretinin cerebellar mRNA levels may

be high in rat and barely detectable in chick). Further studies using a cDNA and antisera specific for the M_r 29000 calcium binding protein in mammalian cerebellum are needed before definitive conclusions can be made.

Little is known concerning the exact physiological role of calbindin-D_{28k} in brain. Calbindin-D_{28k} has been hypothesized to act as an intracellular calcium buffer (Baimbridge et al., 1982). The localization of calbindin in nerve terminals (DiFiglia et al., 1989) suggests that the protein might also affect processes associated with synaptic transmission. A neuroprotective role of calbindin was suggested by studies of calbindin in neurodegenerative diseases (Iacopino & Christakos, 1990) and by studies by Scharfman and Schwartzkroin (1989), who showed that an activity-induced increase in intraneuronal calcium can initiate processes resulting in cell deterioration. However, those cells that contained calbindin were less vulnerable to damage. Whether the function of the M_r 29000 protein is similar or different than that of calbindin remains to be determined. Future experiments concerning cellular/subcellular localization, enzyme activation studies, and studies concerning the regulation of these calcium binding proteins will be needed in order to determine the exact role each protein plays in calcium-dependent phenomena critical to the functioning of nervous tissue.

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