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Characterization and Location of Divalent Cation Binding Sites in Bovine Glial Fibrillary Acidic Protein†

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ABSTRACT: In our previous work [Yang, Z. W., & Babitch, J. A. (1988) *Biochemistry* (preceding paper in this issue)] divalent cations were found to be more effective promoters of astroglial filament formation than were monovalent cations. To determine if one or more divalent cation binding sites were the basis for this difference, glial fibrillary acidic protein (GFAP) was attached to nitrocellulose membranes and bathed in 1 μM $^{45}CaCl_2$ in 60 mM KCl, 0.5 mM $MgCl_2$, and 10 mM imidazole hydrochloride, pH 7.4. After removal of unbound $^{45}Ca^{2+}$, GFAP was observed to bind calcium. Flow dialysis experiments showed that GFAP, dissolved in 2 mM Tris-HCl, pH 7.5, contained three classes of binding sites and 0.61 ± 0.08 (SD), 1.7 ± 0.4, and 4.6 ± 0.2 sites per GFAP molecule with dissociation constants of 0.66 ± 0.01 μM, 6.6 ± 0.3 μM, and 44 ± 1 μM, respectively. After addition of 0.5 mM $MgSO_4$ to the flow dialysis solution, the high- and low-affinity sites were not observed while the remaining sites (1.95 ± 0.15 per GFAP molecule) had a K_d = 2.16 ± 0.25 μM. This showed that the high- and low-affinity sites are "Ca^{2+}–Mg^{2+}" sites while sites with intermediate affinity are calcium specific. To locate the calcium-binding regions, GFAP peptides were examined for calcium binding by calcium-45 autoradiography. The calcium-specific binding areas were localized in coil I. Computer-assisted analysis of the GFAP sequence revealed several EF-hand-like areas which could be the calcium binding sites. We conclude that divalent cations may play both structural and regulatory roles in astroglial intermediate filaments.

Intermediate filament proteins consist of a predominantly helical rod domain and two flanking end pieces (Geisler et al., 1982, 1983). In low ionic strength solutions, intermediate filaments are soluble, but increases in ionic strength promote filament assembly (Steinert et al., 1976, 1981; Rueger et al., 1979; Huiatt et al., 1980; Yang & Babitch, 1988). Divalent cations may act through different mechanisms from monovalent cations in accelerating filament formation (Fukuyama et al., 1978).

Glial fibrillary acidic protein (GFAP)[1] is an intermediate

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filament protein. Its sequence was determined from a cloned DNA fragment by Lewis et al. (1984). Proteolytic and chemical cleavages have been used to study the possible involvement of its domains in antigenicity and filament assembly (Hong & Davison, 1981; Dahl et al., 1984). Our previous work (Yang & Babitch, 1988) revealed that GFAP polymerization was promoted much more efficiently by divalent than monovalent cations. Here we examined this effect in more detail by characterizing divalent cation binding properties of GFAP by flow dialysis. We located the calcium-specific binding sites by limited proteolysis and chemical cleavages to coil I.

Materials and Methods

Flow dialysis membrane (Spectrapor, M_r 8000 cutoff) was purchased from Scientific Products (Grand Prairie, TX). $^{45}CaCl_2$ (5–8.6 mCi/mg of Ca) was from ICN Biomedicals (Irvine, CA). $MgSO_4$ (ultrapure) came from Alfa Products (Danvers, MA). BNPS-skatole was from Pierce (Rockford, IL). All other chemicals were of the purest grade available. Water was twice deionized and glass distilled. For flow dialysis experiments, additional purification utilized the Millipore Milli Q system.

GFAP Isolation. GFAP was isolated from bovine spinal cords and was purified by two cycles of disassembly–assembly as described in the accompanying paper. It was stored in 8 M urea made up in 2 mM Tris-HCl, pH 7.5. Urea was removed by extensive dialysis at 4 °C against 2 mM Tris-HCl, pH 7.5, prior to proteolysis or flow dialysis experiments.

Measurement of Calcium-Binding Constants. The calcium-binding constants of GFAP were measured at room temperature by flow dialysis (Colowick & Womack, 1969) with modifications. Calcium was depleted from GFAP by 10 h of dialysis at 4 °C against Chelex beads or 0.5 mM EGTA made up in 2 mM Tris-HCl, pH 7.5, followed by a 16-h dialysis at 4 °C with one change against 2 mM Tris-HCl, pH 7.5. This "calcium-free" GFAP was titrated with $^{45}CaCl_2$ solution in a flow dialysis cell described by Feldmann (1978). $^{45}CaCl_2$ solution was added to the upper chamber containing GFAP in 2 mM Tris-HCl, pH 7.5. The flow rate through the lower chamber was 1.0 mL/min, and six fractions (1.0 mL/fraction) were collected after each addition of less than 5 μL of $^{45}CaCl_2$. In these experiments only plasticware was used, and it was acid washed and rinsed with ultrapure water prior to use. Flow dialysis data were analyzed with spline functions (Wold, 1974). Models were prepared and tested by regression procedures as described by Smith (1979).

Cyanogen Bromide Digestion of GFAP. GFAP in 70% formic acid was cleaved at room temperature for 18 h with 100 equiv (to methionine residues) of BrCN. An equal volume of double-concentrated SDS gel sample buffer (20% glycerol, 10% β-mercaptoethanol, 10 mM Tris, pH 8.0, 6% SDS) was added, and the pH was brought to 8.0 with 1 N NaOH. After heating at 95 °C for 3 min, this sample was subjected to SDS gel electrophoresis and calcium-45 autoradiography.

Tryptic Digestion of GFAP. GFAP in 2 mM Tris-HCl, pH 7.5, was incubated at room temperature for 5 to 10 min at a substrate to enzyme ratio of 20:1. Digestion was terminated by addition of PMSF to 0.2 mM and heating at 95 °C for 3 min immediately after mixing with an equal volume of double-concentrated SDS gel sample buffer. The resulting tryptic digests were subjected to SDS–polyacrylamide gel electrophoresis.

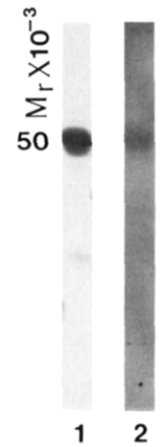

FIGURE 1: SDS–polyacrylamide gel electrophoresis and $^{45}Ca^{2+}$ autoradiography of purified GFAP. Bovine GFAP was isolated from detergent-insoluble fractions by hydroxylapatite chromatography and was purified by two cycles of disassembly–assembly. Electrophoresis was performed on 12–20% linear gradient SDS gels as originally described by Laemmli (1970) and modified by De Blas et al. (1979). GFAP was transferred to nitrocellulose membranes on which ^{45}Ca autoradiography was performed as described by Maruyama et al. (1984) with modifications (Yang & Babitch, 1986). (Lane 1) Coomassie blue stained protein; (lane 2) autoradiogram of $^{45}Ca^{2+}$ binding.

Chymotrypsin and BNPS-skatole Cleavage of GFAP and Electrophoresis of GFAP or GFAP Peptides. Limited proteolysis of GFAP with chymotrypsin or chemical cleavage by BNPS-skatole was conducted as described in the accompanying paper. The resulting GFAP peptides were subjected to electrophoresis on 12.8% or 12–20% linear gradient SDS–polyacrylamide gels as described by Laemmli (1970) and modified by De Blas et al. (1979).

Transblotting and Autoradiography of GFAP or GFAP Peptides. GFAP or gel-separated peptides were transferred to nitrocellulose membranes by overnight electroblotting at 120 mA by the method of Towbin et al. (1979). The calcium-binding ability of the transblotted GFAP fragments was examined by ^{45}Ca autoradiography which was conducted as described by Maruyama et al. (1984) with modifications (Yang & Babitch, 1986); 1 μM $^{45}CaCl_2$, 60 mM KCl, and 0.5 mM $MgCl_2$ were included in the calcium-binding buffer, 10 mM imidazole hydrochloride, pH 7.4.

Results

GFAP, isolated from bovine spinal cords by detergent treatment and hydroxylapatite chromatography and purified by two cycles of disassembly–assembly, contained predominately intact GFAP of M_r 50 000 as shown in Figure 1 (lane 1). ^{45}Ca autoradiography, performed on GFAP which was electrophoretically transferred to nitrocellulose membranes before renaturation, demonstrated that GFAP bound calcium at 1 μM. Inclusion of 0.5 mM $MgCl_2$ and 60 mM KCl in the buffers did not abolish this binding (Figure 1), indicating that $^{45}Ca^{2+}$ bound to calcium-specific sites. Negative controls for all autoradiography experiments included observing that molecular weight marker proteins did not bind $^{45}Ca^{2+}$. Occasionally, additional non-calcium-binding proteins, e.g., hemoglobin, troponin T, and troponin I, also were examined (data not shown).

The calcium-binding constants of GFAP were measured by flow dialysis. Calcium-free GFAP (approximately 10 μM) was titrated by stepwise addition of $^{45}CaCl_2$ solution in a flow

[1] Abbreviations: GFAP, glial fibrillary acidic protein; BNPS-skatole, 2-[2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

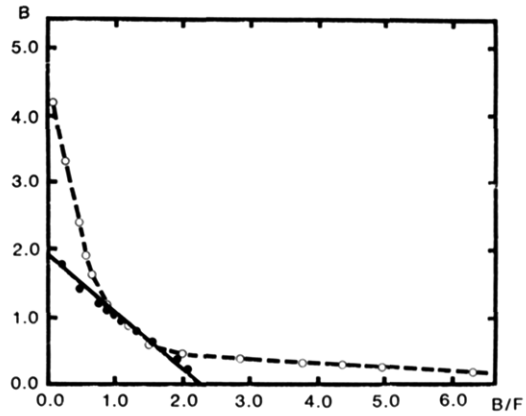

FIGURE 2: Characterization of calcium binding to GFAP in the presence and absence of 0.5 mM $MgSO_4$. Residual calcium was removed as described under Materials and Methods from GFAP made up in 2 mM Tris-HCl, pH 7.5. GFAP (approximately 10 μM) was titrated in a flow dialysis cell by stepwise addition of $^{45}CaCl_2$. The free calcium concentration in the GFAP solution equaled its equilibrium concentration in the diffusate. The total calcium concentration in the upper chamber was measured in the control experiments in which GFAP was absent. Values for the concentration of free (F) and bound (B) calcium were derived and plotted. Dissociation constants could be obtained by application of the equation $K_d = (n - B)F/B$ for a single class of sites or by spline analysis for multiple classes of sites, where n represented the number of calcium binding sites. (○) $^{45}Ca^{2+}$ binding in the absence of Mg^{2+}; (●) $^{45}Ca^{2+}$ binding in the presence of 0.5 mM $MgSO_4$.

dialysis cell. The specific radioactivity in the diffusate was measured in the presence or absence of GFAP. The equilibrium concentration of free calcium in the upper chamber was calculated from the radioactivity in the diffusate collected from the lower chamber. The total calcium concentration in the upper chamber was calculated from a control experiment in which no GFAP was added to the upper chamber. These data were calculated as described by Colowick and Womack (1969) and plotted as shown in Figure 2.

Results obtained in the absence of any Mg^{2+} showed that GFAP contained either two or three classes of calcium binding sites. A model proposing two classes of sites yielded 0.98 ± 0.10 (SD) high-affinity sites with a dissociation constant of (1.48 ± 0.19) × 10^{-6} M and 4.6 ± 0.2 low-affinity sites with a dissociation constant of (4.4 ± 0.1) × 10^{-5} M. The model proposing three classes of binding sites yielded numbers of sites of 0.61 ± 0.08, 1.7 ± 0.4, and 4.6 ± 0.2 per GFAP molecule with dissociation constants of (6.6 ± 0.1) × 10^{-7} M, (6.6 ± 0.3) × 10^{-6} M, and (4.4 ± 0.1) × 10^{-5} M for the high-, intermediate-, and low-affinity sites, respectively.

In the presence of 0.5 mM $MgSO_4$ the high- and low-affinity sites were undetectable. Instead, GFAP was found to have 1.95 ± 0.15 calcium-specific sites with a dissociation constant of (2.16 ± 0.25) × 10^{-6} M (Figure 2). The same results were obtained whether calcium was removed from GFAP by dialysis against EGTA or Chelex beads. This result showed that EGTA did not bind to GFAP so tightly that it was not removed by subsequent dialysis steps.

Localization of the Calcium-Specific Cation Binding Sites. GFAP was treated by a variety of chemical and proteolytic cleavage methods as described in the accompanying paper. GFAP fragments were separated on polyacrylamide gels and transferred to nitrocellulose sheets for $^{45}Ca^{2+}$ binding as described under Materials and Methods. Chymotrypsin treatment for 10 min at 20:1 GFAP:chymotrypsin digested the GFAP N-terminal and C-terminal regions (Figure 3A, lanes 1 and 2) yielding a M_r 38 000 core domain which bound $^{45}Ca^{2+}$ (Figure 3A, lane 3). BNPS-skatole treatment cleaved GFAP

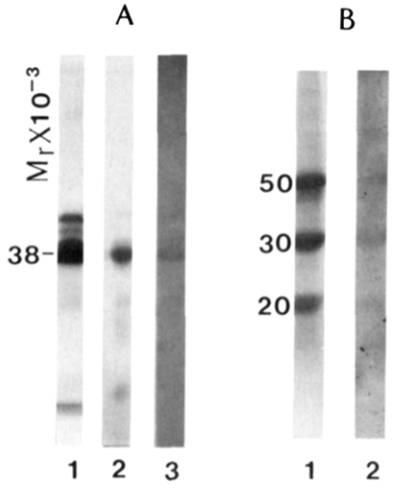

FIGURE 3: Calcium binding to chymotryptic (A) and BNPS-skatole (B) digests of GFAP. (A) GFAP, dissolved in 2 mM Tris-HCl, pH 7.5, was incubated at room temperature for 10 min with α-chymotrypsin at a substrate to enzyme ratio of 200:1 (lane 1) or 20:1 (lane 2). Digestion was terminated by addition of PMSF to 0.2 mM. ^{45}Ca autoradiography was performed on nitrocellulose membranes after transfer of polypeptides from the SDS–polyacrylamide gel as described in Figure 1. Lanes 1 and 2 were chymotryptic digests of GFAP. Lane 3 was the ^{45}Ca autoradiogram of lane 2. (B) GFAP was treated with BNPS-skatole in 50% acetic acid at room temperature for 2 days. The digests were collected by trichloroacetic acid precipitation. The resulting pellet was subjected to electrophoresis on a 12–20% linear gradient SDS–polyacrylamide gel (lane 1), and the polypeptides were transferred to a nitrocellulose membrane on which ^{45}Ca autoradiography was performed as described in Figure 1. Lane 2 was the resulting autoradiogram of lane 1.

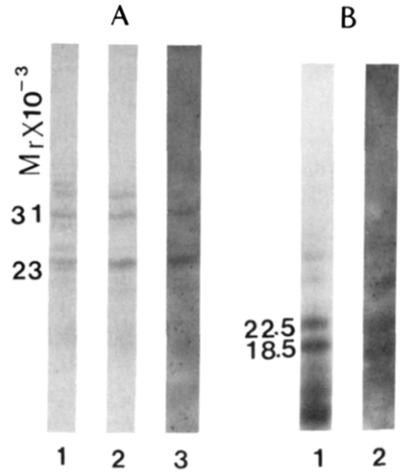

FIGURE 4: Calcium binding to tryptic (A) and BrCN (B) digests of GFAP. (A) GFAP, dissolved in 2 mM Tris-HCl, pH 7.5, was digested at room temperature with trypsin at a substrate to enzyme ratio of 20:1. Digestion was terminated by addition of PMSF to 0.2 mM. The peptides, separated on a 12–20% linear gradient SDS–polyacrylamide gel, were transferred to a nitrocellulose membrane and were examined for calcium binding as described in Figure 1. Lanes 1 and 2 show the resulting fragments for 5- and 10-min digestions, respectively. Lane 3 was the ^{45}Ca autoradiogram of lane 2. (B) One hundred equivalents (to methionine residues) of BrCN was added to a GFAP solution, made up in 70% formic acid. Digestion was carried out overnight at room temperature. The resulting peptides were transferred to a nitrocellulose membrane after separation on a 12.8% SDS–polyacrylamide gel. ^{45}Ca autoradiography was performed as described in Figure 1. (Lane 1) BrCN-digested GFAP; (lane 2) ^{45}Ca autoradiogram of lane 1.

at the sole tryptophan, yielding some residual intact GFAP and some M_r 30 000 amino-terminal peptide (Figure 3B, lane 1) both of which bound $^{45}Ca^{2+}$. The M_r 20 000 carboxyl-terminal peptide did not bind $^{45}Ca^{2+}$ (Figure 3B, lane 2). When GFAP was extensively digested with trypsin, the major

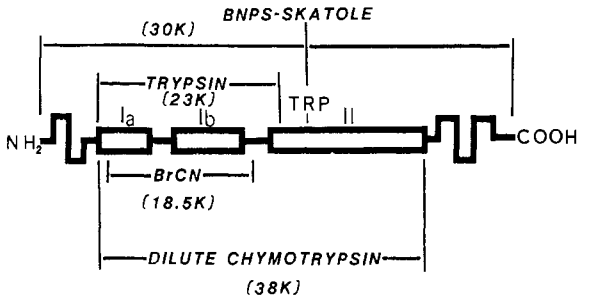

FIGURE 5: Schematic structure of GFAP and the locations of specific calcium-binding peptides obtained from enzymatic or chemical digestion. This scheme was based on the structural model of intermediate filaments proposed by Geisler et al. (1982) and the mouse GFAP sequence reported by Lewis et al. (1984).

product was a M_r 23 000 polypeptide (Figure 4A, lanes 1 and 2) which we determined previously to have originated from the coli I region. This fragment bound $^{45}Ca^{2+}$. Contained within this region is a somewhat smaller (M_r 18 500) fragment which can be liberated from GFAP by cyanogen bromide treatment (Figure 4B). This M_r 18 500 fragment was the smallest we could obtain from GFAP which still retained the capacity to bind $^{45}Ca^{2+}$ in the presence of 0.5 mM Mg^{2+} (Figure 4B, lane 2).

The results of the cleavage, blotting, and $^{45}Ca^{2+}$ binding experiments are summarized in Figure 5. The calcium-specific binding sites are localized in coil I because all of the labeled peptides (except for the M_r 20 000 BNPS-skatole peptide) bound $^{45}Ca^{2+}$ in the presence of 0.5 mM Mg^{2+}.

Discussion

GFAP was found to bind $^{45}Ca^{2+}$ when incubated with 1 μM $^{45}CaCl_2$, approximately 0.1 mCi/L, one-tenth as much as was used in the method of Maruyama et al. (1984). Using a similar method, Serrano et al. (1986) localized the high-affinity calcium binding sites on α- and β-tubulin. This again demonstrated that the ^{45}Ca overlay technique was capable of detecting calcium-binding proteins or peptides.

In the highly helical rod domain of intermediate filaments most of the aromatic amino acid residues probably are buried inside, leading to their extraordinary resistance to chymotryptic digestion which is known to cleave peptide bonds involving aromatic amino acid residues. Rueger et al. (1981) reported a chymotrypsin-resistant M_r 37 000 polypeptide after GFAP cleavage. A desmin rod domain also was obtained in the same way (Geisler et al., 1982). Our experimental data suggested that the divalent cation binding sites were localized on this rod section.

GFAP contains a unique tryptophan residue in the rod domain, at position 226 (Lewis et al., 1984), which can be cleaved by BNPS-skatole treatment. Autoradiography demonstrated that only the M_r 30 000 N-terminal polypeptide bound calcium in the presence of 0.5 mM Mg^{2+}, suggesting that calcium-specific binding sites were localized in the section between amino acid residues 1 and 226 (Figures 3 and 5).

The polypeptide gel pattern which resulted from trypsin-digested GFAP (Figure 4A, lanes 1 and 2) was comparable with that reported by Rueger et al. (1981), who demonstrated that GFAP was initially cleaved through several intermediates to a polypeptide of about M_r 37 000, and this polypeptide was further cleaved into a M_r 21 000 polypeptide. This trypsin-resistant polypeptide was found to bind calcium (Figure 4A, lane 3).

Hong and Davison (1981) treated GFAP with cyanogen bromide and separated a prominent, immunoactive peptide of M_r 16 000 from the digests. Rueger et al. (1981) also detected this M_r 18 000 polypeptide in cyanogen bromide digests of GFAP, and later Dahl et al. (1984) demonstrated that the proteolysis-resistant M_r 21 000 polypeptide from the tryptic digestion of GFAP contained this M_r 18 000 polypeptide. This was the smallest calcium-binding fragment we obtained (Figure 4B, lane 2). The sequence from mouse GFAP (Lewis et al., 1984) suggested that this peptide probably consisted of amino acids 45–193.

Flow dialysis experiments characterized the divalent cation binding sites. Statistically the model proposing three classes of sites fits the data slightly better than a model proposing two classes of sites. That there are three classes rather than two also fits better with the data obtained in the presence of 0.5 mM Mg^{2+} where the high- and low-affinity sites could not be detected by $^{45}CaCl_2$ additions. The remaining sites are rather similar in number and affinity to the intermediate sites in the three-site model. The probability that GFAP contains three classes of sites is supported also by our previous experience with flow dialysis. We detected two classes of calcium binding sites in tubulin (Kong et al., 1988). When tubulin was examined, the binding data generated by the flow dialysis technique were nearly straight lines with clear intercepts which is indicative of the accuracy and precision which can be obtained by this method (Haiech et al., 1979; Bryant & Andrews, 1983). This is to be contrasted to the GFAP flow dialysis data with its substantial curvature, suggesting the existence of a third class of sites with intermediate affinity for calcium.

Both the high- and low-affinity binding sites probably were calcium–magnesium sites, on the basis of the fact that these sites were saturated by 0.5 mM Mg^{2+} and were no longer detectable. The closeness in the dissociation constants of these three classes of binding sites made the analysis of the intermediate-affinity sites rather difficult. Inclusion of 0.5 mM Mg^{2+} in the GFAP solution gave a quite clear result: only one class of binding sites with a dissociation constant of $(2.16 \pm 0.25) \times 10^{-6}$ M was detected. This class of binding sites appeared to be regulatory calcium binding sites in vivo where the magnesium concentration is approximately 0.1–0.3 mM (Heinonen & Åkerman, 1987).

The above results suggest the (probably) two calcium-specific binding sites are localized in coil I between residues 45–193. The Ca–Mg sites may be in other regions. In an attempt to locate the calcium-binding regions, we prepared computer programs to detect EF-hand- (Kretsinger, 1980), elbow- (Stuart et al., 1986), and lock-washer- (Vyas et al., 1987) type calcium binding sites. These programs included provisions for inserting or deleting amino acids from traditional calcium-binding sequences because several calcium-binding proteins have been shown to utilize such modified sequences, e.g., S100 PAPIb (Isobe & Okuyama, 1978), intestinal calcium-binding protein (Szebenyi et al., 1981), and α-lactalbumin (Stuart et al., 1986). This analysis is limited by the necessity of studying the amino acid sequence deduced from a mouse cDNA sequence cloned by Lewis et al. (1984), because the bovine sequence is unavailable. However, the reports of Geisler and Weber (1983) and Zang and Nilaver (1986) suggest that the interspecies variability of amino acid sequences of intermediate filament proteins is relatively rare, particularly in the rod domain. With this in mind, several putative calcium-binding sequences were detected with the computer programs (Table I).

The sequence most like a classical EF-hand (Kretsinger, 1980) may be residues 290–319. It contains three acidic ligands and is the only sequence with a preferred glycine near the center of the putative calcium-binding loop. This loop has

Table I: Putative Calcium-Binding Amino Acid Sequences in GFAP[a]

			Anionic
VERTICES:	X Y Z -Y -X -Z	SCORES (of 16)	ligands in loop
T-K TEST:	E n n n n * * * G I * E n n n n		
89	E L R E L R L R L D Q T A N S A R L E V E R D N F A Q D L 118	10(12)	2 + 1
96	R L D Q L A N S A R L E V E R D N F A Q D L G T L R Q K L 125	7(10)	3 + 1
117	D L G T L R Q K L Q D E T N L R L E A E N N L A A Y R Q E A 146	8(12)	2 + 2
125	L Q D E T N L R L E A E N N L A A Y R Q E A D E A L A R V 154	7(11)	3 + 0
127	D E T N L R L E A E N N L A A Y R Q E A D E A T L A R V D L 156	6(11)	3 + 1
134	E A E N N L A A Y R Q E A D E A T L A R V D L E R K V E S L 163	7(13)	3 + 1
144	Q E A D E A T L A R V D L E R K V E S L E E E I Q F L R K I 173	7(10)	4 + 2
145	E A D E A L A R V D L E R K V E S L E E E I Q F L R K I Y 174	8(11)	3 + 3
148	E A T L A R V D L E R K V E S L E E E I Q F L R K I Y E E E 177	7(11)	3 + 2
165	E E I Q F L R K I E E E V R D L R E Q L A Q Q Q V H V E M 194	9(9)	3 + 2
290	E S A S Y Q E A L A R L E E E G Q S L K E E M A R H L Q E Y 319	8(10)	3 + 2

[a] Sequences were examined with the Tufty–Kretsinger EF-hand test (Tufty & Kretsinger, 1975) and are depicted by the single-letter amino acid code. In the test sequences E, G, and I are specified where indicated. n is a hydrophobic residue (L, I, V, F, M), and * is an oxygen-containing amino acid (D, N, E, Q, S, T). Ligand vertices for calcium coordination (X, Y, Z, -Y, -X, -Z) as described by Tufty and Kretsinger are indicated. Amino acids satisfying the tests are underlined. Possible modifications to the tests are double underlined, with the increased scores listed in parentheses. These modifications have been observed in other calcium-binding proteins. Additional acidic groups which may contribute to metal binding are overlined. Regions where one amino acid should be deleted for maximal homology are elevated. The first number under anionic ligands indicates acidic residues in correct positions to be EF-hand vertices; the second number indicates additional acidic residues in the loop.

two additional glutamic acid residues in the loop region (for a total of five) which could give the area sufficient flexibility and charge density to be the highest affinity Ca^{2+}–Mg^{2+} binding site. As such, it may provide structural support for the carboxyl half of the protein. Ca^{2+}–Mg^{2+} sites should not be detected in our autoradiograms because the 0.5 mM $MgCl_2$ we included in the buffer should block binding of $^{45}Ca^{2+}$. However, the high affinity of this site for Ca^{2+} [K_d = (6.6 ± 0.1) × 10^{-7} M] may have allowed small levels of ^{45}Ca binding even in the presence of $MgCl_2$ (Figure 3B, lane 2). This high affinity also may explain why we detected only 0.61 ± 0.08 of these sites per GFAP molecule: endogenous Ca^{2+} which we could not remove probably accounted for the remaining 0.4 site per molecule. This sequence also bears a superficial resemblance to a calcium-binding elbow (Stuart et al., 1986), but the spacing here is not the same as that in the calcium-binding region of α-lactalbumin.

Another probable calcium-binding region containing the highest concentration of acidic amino acids in the molecule (residues 155–166) occurs in three putative EF-hand sequences: 144–173, 145–174, and 148–177. One of these overlapping sequences could be an intermediate-affinity, calcium-specific site.

The sequences in Table I which seem least likely to bind divalent cations are residues 89–118 and 125–154. This is because these sequences have clusters of only three acidic amino acids in the loop regions.

The other sequences in Table I contain at least three acidic groups in vertex positions of the Tufty–Kretsinger (Tufty & Kretsinger, 1975) calcium-binding loop. This was considered to be the minimum necessary for cation binding. Therefore, any could be calcium-binding sequences of relatively high affinity. These assignments agree with the biochemical data which suggest that the calcium-specific sites are in coil I. In summary, the computer analyses suggest that one calcium-specific site occurs in the area of residues 144–177. The high-affinity Ca^{2+}–Mg^{2+} site probably occurs at amino acids 290–319. Lower affinity sites may occur between amino acids 89–154 and 165–194, and it may be these sites which enhance filament formation, as shown in the previous paper.

GFAP has a central amino acid sequence (excluding the head and tail regions) which is highly conserved when compared to those of other intermediate filament types. Our previous observations indicated that the polymerization characteristics of GFAP are similar to those of other intermediate filaments. Therefore, similar divalent cation binding sites are likely to occur on other intermediate filament proteins. That is supported by our preliminary work and the work of Lefebvre and Mushynski (1987) on divalent cation binding to neurofilaments.

A number of cytoskeletal proteins bind calcium, such as tubulin and actin. Binding of calcium to tubulin shows a significant, inhibitory effect on its polymerization (Kircher et al., 1974; Solomon, 1977; Serrano et al., 1986). Calcium

binding to actin inhibits polymerization at the nucleation step (Frieden, 1982; Cooper et al., 1983; Tobacman & Korn, 1983; Gershman et al., 1984). For GFAP the effects of calcium binding are not clear yet, but because the protein contains both Ca^{2+}–Mg^{2+}- and Ca^{2+}-specific sites, calcium is likely to play both structural and regulatory roles.

Added in Proof

Krinks et al. (1988) have shown that squid neurofilament polypeptides bind calcium.

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