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MICROHETEROGENEITY OF THE GROWTH-ASSOCIATED NEURONAL PROTEIN B-50 (GAP-43)

CONTRIBUTION OF PHOSPHORYLATION BY PROTEIN KINASE C[a]

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SUMMARY

The neuron-specific, growth-associated protein B-50, also known as GAP-43, F1 and neuromodulin, shows a striking heterogeneous behaviour in many chromatographic and electrophoretic systems. A modulatory function has been proposed for the protein in receptor-mediated processes in the presynaptic membrane. Fatty acid acylation, calmodulin binding and phosphorylation appear to be tools in this respect. At least three discrete isoforms were present in separations made by reversed-phase fast protein liquid chromatography (FPLC) of the phosphorylated protein. In anion-exchange FPLC chromatography a conglomerate of eight peaks was eluted, which migrated as eight parallel curves in electrophoretic mobility studies. After dephosphorylation of the protein this number was reduced to two. Under non-reducing conditions, the phosphoprotein was eluted from an FPLC gel filtration column at M_r = 270 kDa, *i.e.* 8–12 times the size of the monomer (m = 23.6 kDa). In sodium dodecyl sulphate polyacrylamide gel electrophoresis all isoforms showed only B-50 at M_r of 48 kDa and its breakdown product (M_r = 40 kDa) in a constant ratio. It was concluded that phosphorylation by protein kinase C of a single serine residue is only one factor in the microheterogeneity of B-50. Multimeric forms may also add to the heterogeneous behaviour of phosphorylated B-50.

INTRODUCTION

The neuron-specific, calmoculin-binding phosphoprotein B-50 is localized in the presynaptic membrane and has been implicated in the modulation of synaptic plasticity and neurite outgrowth[1,2]. During the past 2 years, independently, four groups of investigators have reported the predicted amino acid sequence of the growth-associated protein GAP-43[3,4], the plasticity protein F1[5], the phosphoprotein

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B-50[6] and the calmodulin-binding protein P-57[7], also indicated as neuromodulin[8]. It appeared that these proteins are identical[6].

The protein is one of the major substrates for protein kinase C (PKC) in the synaptic membrane[1]. Its migration in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is strongly dependent on the conditions used, resulting in a apparent molecular mass (M_r) ranging from 35 to 55 kDa[1–7,9,10]. This feature was used for recognition of the protein in different neuronal systems of various species[2]. The protein also showed a heterogeneous behaviour in several separation systems[11–14]. The microheterogeneity in the isoelectric point seemed to be related to its phosphorylation by PKC[12–14]. This led us to the hypothesis of multiple phosphorylation sites for PKC[12].

The primary structure of the protein, obtained from cDNA cloning, revealed that it consists of one highly hydrophilic, elongated polypeptide chain with 1% α-helix, 21% β-sheet and 78% random coil structure and an M_r of 23.6 kDa[6,9]. The mobility of the recombinant protein in SDS-PAGE is similar to that of the protein purified from brain[6,13]. Hence its anomalous migration at M_r 48 kDa in comparison with marker proteins must be explained from its primary structure, as has been described for other very hydrophilic proteins[6,8,10].

Phosphorylation of the *in vitro* translation products of the full-length cRNA and of truncated cRNAs by PKC showed that only serine at position 41 is phosphorylated[15]. Nevertheless, the purified protein shows extensive microheterogeneity with respect to the isoelectric point[12–14]. Recently, a dynamic and reversible interaction of the protein with growth cone membranes via fatty acid acylation has been reported[13,16].

In this study, extensive heterogeneity of the purified protein phosphorylated by PKC was demonstrated both in systems that separate on the basis of hydrophilicity and in systems that use primarily hydrophobic features. The possible contributions of phosphorylation, other known post-translational modifications (thio ester binding and palmitoylation) and multimerization of B-50 will be discussed.

EXPERIMENTAL

Isolation and purification of B-50 from rat brain

Frozen (−20°C) brain material from Wistar rats (150–200 g body weight) was used to prepare a crude B-50 preparation (ammonium sulphate precipitate, ASP, 57–82% saturation[17,18]. B-50 was dialysed and further purified by anion-exchange chromatography [Pharmacia–LKB mono-Q HR 5/5 fast protein liquid chromatographic (FPLC) column)] at pH 7.4[15] as a final step. B-50-containing fractions (eluting between 190 and 260 mM potassium chloride) were pooled, dialysed and lyophilized. B-50 was identified by SDS-PAGE and immuno-blotting[18].

Phosphorylation and dephosphorylation

PKC was isolated from rat brain as described by Aloyo *et al.*[19]. The conditions for phosphorylation of purified B-50 by PKC and dephosphorylation by alkaline phosphatase were as described by Zwiers *et al.*[12]. Excess of [γ-^{32}P]ATP was removed by dialysis. In some experiments B-50 was separated from the enzymes using another run on the mono-Q FPLC column. Alkaline phosphatase elutes from mono-Q at about 70 m*M* potassium chloride.

Electrophoretic mobility variation curves

About 25 μg of B-50, purified as a last step by anion-exchange chromatogrpahy on a mono-Q FPLC column, was applied and electrophoresed on a flat bed of 5% polyacrylamide isoelectric focusing gel, prefocused in the second dimension, according to Rosengren *et al.*[20]. Ampholines of a broad (4.5–9.5) or a narrow pH range (3.8–5.2) (Pharmacia–LKB) were used.

Anion-exchange chromatography

Anion-exchange chromatography of B-50 was performed using a mono-Q FPLC column (50 × 5 mm I.D.). The mobile phase contained 20 m*M* Tris–HCl (pH 7.4). 1 m*M* ethylene glycol tetraacetate (EGTA), 1 m*M* dithiotreitol (DTT) and 40 m*M* sodium fluoride to inhibit phosphatases and proteases. A linear gradient of 0–0.4 *M* potassium chloride was run at a flow-rate of 1 ml/min in 40 min with UV detection at 214 nm. Fractions of maximally 0.5 ml were collected by peak detection and analysed by SDS-PAGE.

Reversed-phase chromatography

Reversed-phase chromatography of B-50 was performed using a ProRPC FPLC column (50 × 5 mm I.D.) (Pharmacia–LKB). The mobile phase was 0.1% trifluoroacetic acid (TFA), pH 2.1. A 0–20% linear gradient of acetonitrile–0.1% TFA (9:1, v/v) was run at a flow-rate of 1.0 ml/min in 20 min. In some runs 10 m*M* tetrapropylammonium bromide (TPA) was added as an ion-pairing reagent for negatively charged groups.

Gel filtration

Gel filtration of ^{32}P-phosphorylated B-50 (in the presence of PKC) was performed using a Superose 6 FPLC column (300 × 10 mm I.D.) (Pharmacia–LKB). The mobile phase contained 0.1 *M* ammonium acetate buffer (pH 6.5) (flow-rate 0.5 ml/min, fraction size 0.5 ml). The column was calibrated using dextran blue (void volume), thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (67 kDa) and cytidine (<1 kDa) (Pharmacia–LKB) as molecular weight markers.

SDS-PAGE

Proteins were analysed by SDS-PAGE using 11% acrylamide gels[17] and molecular weight marker proteins. The position of B-50 was just above the 46 kDa ovalbumin marker.

RESULTS

Purification of B-50

B-50 protein was purified by anion-exchange chromatography using a mono-Q FPLC column as a final step[14]. Fractions eluting between 190 and 260 m*M* potassium chloride were pooled. On SDS-PAGE, a major 48 kDa band and a minor 40 kDa band were found (see Fig. 2A, lane S). Both were identified as B-50 products by ACTH-sensitive phosphorylation by PKC[14] and with affinity-purified anit-B-50 IgGs[18]. The 48-kDa band represents full-length B-50 and the 40-kDa band an N-

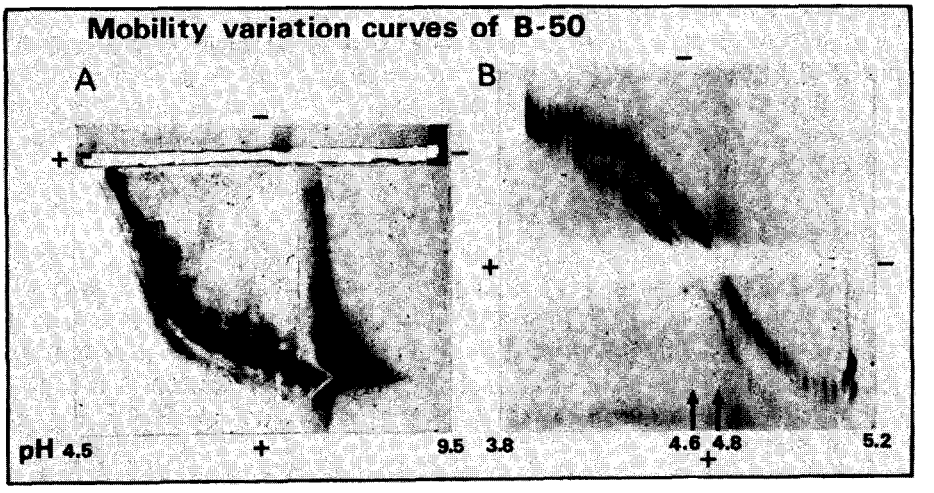

Fig. 1. Results for 25 μg of purified B-50 applied to prefocused 5% polyacrylamide isoelectric focusing gels. (A) Ampholines with pH range 4.5–9.5; (B) Ampholines with pH range 3.8–5.2.

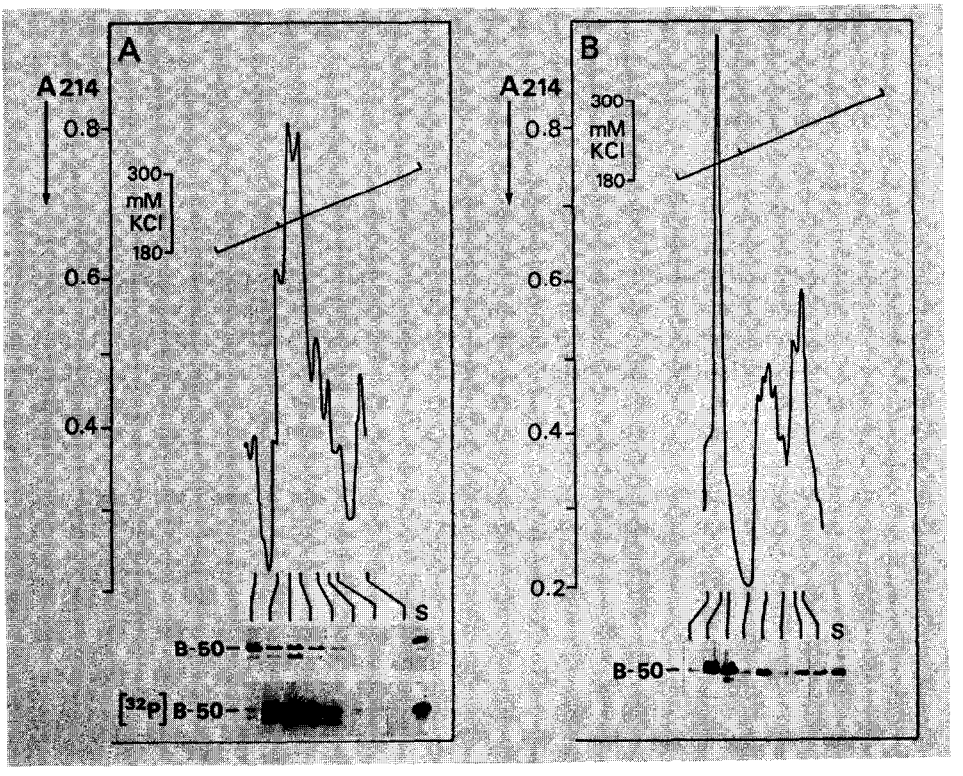

Fig. 2. Mono-Q anion-exchange FPLC. The mobile phase contained 20 m*M* Tris–HCl (pH 7.4), 1 m*M* EGTA, 1 m*M* DTT and 40 m*M* NaF. Flow-rate, 1 ml/min; gradient, 0–0.4 KCl; run rime, 40 min; fraction size, 0.5 ml. The additional mark on the lines of the salt gradient indicates the concentration of 220 m*M* KCl. (A) Purified B-50 was phosphorylated by PKC and [γ-^{32}P]ATP; (B) purified B-50 was phosphorylated by PKC as indicated in Fig. 2A, and subsequently dephosphorylated with alkaline phosphatase.

terminal product formed by proteolysis in brain tissue during storage[15,21–23]. The 40-kDa product showed the same pI and was present in all chromatographic procedures and resulting fractions in a constant ratio to B-50.

Mobility variation curves and mono-Q profiles of B-50 protein after phosphorylation and dephosphorylation

Purified B-50 (about 25 μg) was run on a flat bed, prerun isoelectric focusing gel[20]. Electrophoretic mobility curves were obtained using ampholines with a broad (4.5–9.5; Fig. 1A) and with a narow pH range (3.8–5.2; Fig. 1B). After staining of the protein, a set of parallel curves became apparent. At pH 8.0 the regular pattern was reproducibly distorted (Fig. 1A). At this isoelectric point a series of eight parallel curves are visible (Fig. 1B).

In order to gain more insight into the formation of the isomeric forms of the protein, the purified protein was phosphorylated extensively with PKC and high concentrations of [γ-^{32}P]ATP and run on the mono-Q column in the presence of a phosphatase inhibitor to prevent dephosphorylation (Fig. 2A). The UV profile shows a conglomerate of at least eight peaks. SDS-PAGE revealed that B-50 in all fractions was phosphorylated to about the same extent. This was confirmed by liquid scintillation counting of the fractions. For the 40-kDa product the same was found. Fractions were collected, pooled and in part used to obtain electrophoretic mobility curves in the narrow pH range (3.8–5.2) of the stained (Fig. 3A) and ^{32}P-labelled B-50 protein (Fig. 3B), respectively. A similar number of parallel curves were visible, as shown before (Fig. 1B), although the autoradiogram of the ^{32}P-labelled phosphoprotein (Fig. 3B) did not give the same resolution as obtained with staining of the protein (Fig. 3A) owing to the relatively high energy radiation of ^{32}P.

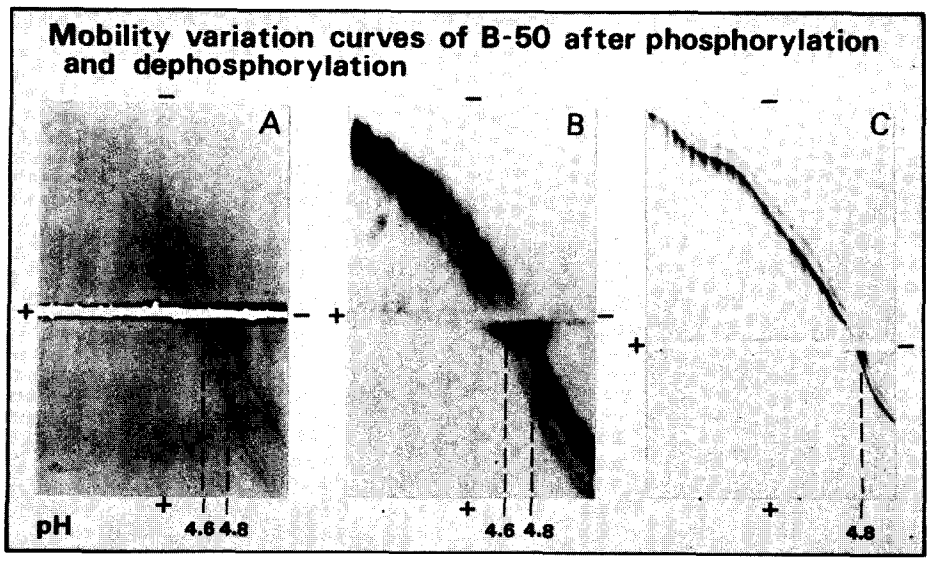

Fig. 3. Fractions pooled from FPLC mono-Q, as indicated in Fig. 2A, run on a flat-bed isoelectric focusing gel (see also Fig. 1B). (A) Staining pattern; (B) autoradiogram, (C) staining pattern after dephosphorylation (see Fig. 2B).

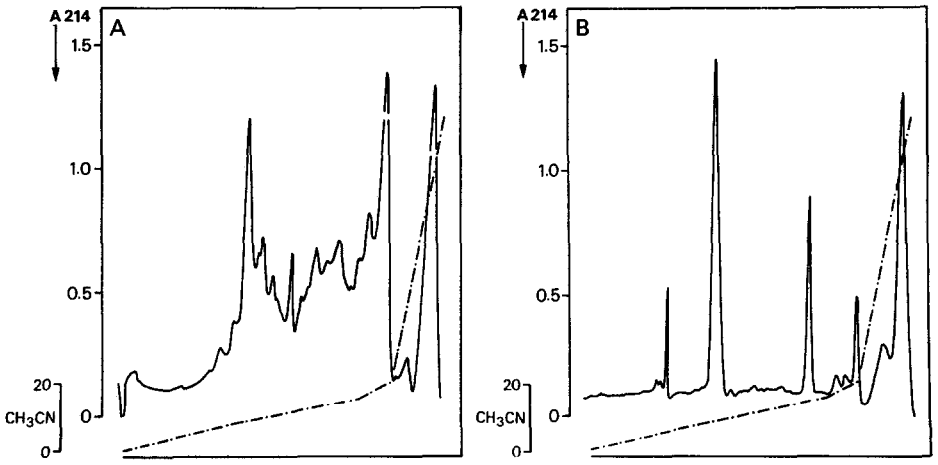

Fig. 4. Purified B-50 phosphorylated by PKC and run on a ProRPC reversed-phase FPLC column. Mobile phase, 0.1% TFA; flow-rate, 1.0 ml/min; gradient, 0–20% acetonitrile (90%); run time, 30 min. (a) Without TPA; (B) with TPA as ion-pairing reagent.

The other part of the ^{32}P-labelled B-50 was incubated with alkaline phosphatase to dephosphorylate the protein, run on the mono-Q column (Fig. 2B) and used to obtain mobility variation curves (Fig. 3C). The UV profile showed a similar number of about eight peaks, but was also shifted with one major peak eluting at the lowest salt concentration. The change in the distribution of B-50 over the peaks was confirmed by SDS-PAGE. The autoradiogram of the gel (not shown) was blank, indicating the effectiveness of the dephosphorylation reaction. The number of mobility curves was reduced to two at the highest pH (Fig. 3C).

Heterogeneity on reversed-phase chromatography

Purified B-50 protein was phosphorylated by PKC as described above, dialysed and run using a ProRPC reversed-phase FPLC column (with C_1/C_8 alkanes as functional groups; Fig. 4A). The UV profile showed extensive heterogeneity ranging from about 5% to more than 20% acetonitrile. Analysis of the amount of B-50 in the fractions by SDS-PAGE combined with protein staining and autoradiography (data not shown) confirmed this UV profile, *i.e.*, ^{32}P-labelled and stained B-50 was present in all fractions with a UV absorption above 0.3 at 214 nm.

A second run was performed with TPA as a reagent for ion pairing of negative charges of the protein present in the mobile phase (Fig. 4b). The UV profile clearly showed four peaks. The first three peaks contained about 90% of the pure B-50 and the fourth also showed some impurities. SDS-PAGE (data not shown) showed a similar distribution of the staining and radioactivity of B-50.

Gel filtration

B-50 purified by anion-exchange chromatography on a mono-Q FPLC column as a final step was phosphorylated by PKC. The reaction was stopped and the reaction mixture was run on a Superose 6 FPLC gel filtration column that was calibrated

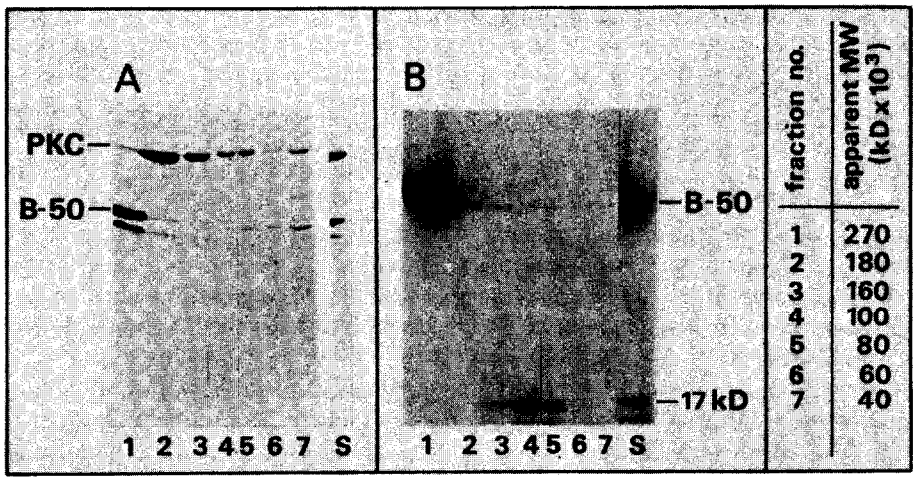

Fig. 5. Purified B-50 phosphorylated by PKC and run on a Superose 6 FPLC gel filtration column. Mobile phase, 0.1 *M* ammonium acetate buffer (pH 6.5); flow-rate, 0.5 ml/min; fraction size, 0.5 ml. Fractions were collected as indicated and subjected to SDS-PAGE. (A) Staining pattern; (B) autoradiogram. Fractions containing protein are shown. Numbers refer to M_r values calculated from a semi-logarithmic plot of marker proteins (see Experimental).

with molecular weight markers in the range 67–670 kDa. Fractions of 1 ml were collected and analysed by SDS-PAGE for protein staining and radioactivity (Fig. 5). Under the conditions used (no reducing agent present), more than 95% of the B-50 (staining and radioactivity) eluted with an apparent M_r of about 270 kDa. Part of the PKC eluted with some of the radioactive B-50. Moreover, a number of minor bands, partly the 40-kDa proteolytic product of B-50 and partly impurities of the PKC preparation, are present. The phospho band at M_r = 17 kDa represents another acylated substrate protein of PKC[11].

DISCUSSION

For many years, the growth-associated, neuron-specific protein B-50 was identified in various neuronal systems of different species by two-dimensional microheterogeneity and anomalous mobility in SDS-PAGE with M_r ranging from 35 to 57 kDa depending on the percentage of acrylamide and cross-linker[1–7,9,10,12]. The molecular weight calculated from the primary structure is 23.6 kDa[3–6,8] and the M_r of 46 kDa seems to be related to its elongated form with very hydrophilic stretches[9]. Strong surface charges of the protein might also result in additional interactions of the protein with matrices for chromatographic and electrophoretic separations used in this study. For example, the extensive heterogeneity in the elution profile of pure B-50 shown in RPC (Fig. 4A) was reduced to four discrete peaks on addition of a second ion-pairing reagent for negative charges (Fig. 4B).

The heterogeneity shown in isoelectric focusing (Figs. 1 and 3) is influenced by phosphorylation and dephosphorylation of the protein[12–14]. Similar changes were observed in the the elution profile from anion-exchange chromatography (Fig. 2).

However, the existence of a phosphorylated and a dephosphorylated form of B-50 cannot explain the large number of isoforms shown. Even after extensive dephosphorylation with alkaline phosphatase resulting in complete removal of all radioactive phosphate incorporated by PKC, two spots in two-dimensional isoelectric focusing–SDS-PAGE[12] or two parallel curves with different IEP remained (Fig. 3B). Only part of the heterogeneity on a mono-Q column disappeared with this treatment (Fig. 2B). A phosphorylation site specific for casein kinase II has been reported in the C-terminal part at Ser^{192} of the molecule[24]; maybe this site is not susceptible to alkaline phosphatase. Recent studies, using various techniques, revealed only one site in B-50 for phosphorylation by PKC[15,23,25]. Nevertheless, the three peaks on reversed-phase chromatography (Fig. 4B) all contained ^{32}P-labelled B-50 after incubation with PKC and [γ-^{32}P]ATP.

The presence of a 40-kDa N-terminal proteolytic fragment of B-50 cannot explain the extra isoforms of B-50. The sequence of the 40-kDa product was recently derived as 1–203 by indirect evidence from the cDNA sequence[15,26]. Its pI and behaviour in chromatography appeared to be identical with that for B-50 and is phosphorylated to the same extent by PKC *in vitro* (see, for instance, the SDS-PAGE lanes in Fig. 2A). Moreover, in all fractions analysed by SDS-PAGE the ratio between the 40-kDa product and B-50 was constant and the same as that in the starting material.

Recently, the existence of isoforms of B-50 with a different hydrophobicity has been reported. Cys^3 and Cys^4 of B-50 appeared to be acylated to fatty acids in embryonal or neonatal rat brain[13,16]. This might explain, in principle, the existence of four isoforms[12,16], which are (i) non-acylated and non-phosphorylated; (ii) acylated and non-phosphorylated; (iii) non-acylated and phosphorylated; and (iv) acylated and phosphorylated B-50. Of the theoretical isoforms, only two are phosphorylated, thereby not explaining the three different phosphorylated forms in Fig. 4B and the additional phosphorylated curves in Fig. 3B. There are no indications for other post-translational modifications such as (de)amination that might contribute to the different isoforms of B-50, although they have not yet been excluded.

The existence of single and double acylated forms of B-50 or the formation of multimers via the second Cys residue might increase the number of possible, phosphorylated isoforms. The latter possibility is supported by the observation of a molecular weight of 270 kDa of phosphorylated B-50 in gel filtration (Fig. 5). Another explanation of this phenomenon might be the formation of micelles of acylated B-50. At physiological salt concentrations, the protein partitions predominantly into the detergent phase, whereas at low ionic strength partitioning is approximately equal between the detergent and aqueous phases[13]. The reproducible disturbance in the mobility variation profile at pH 8 (Fig. 1A) might be related to a phase transition.

In conclusion, the results support the notion that isoforms of B-50 exist on the basis of acylation[13,16], phosphorylation[12,14,15] and multimer formation. The post-translational modifications together with exposure of highly charged stretches of the molecule may result in the formation of a number of aggregation and isoforms of the protein. This, in combination with binding of calmodulin[8,9] and other PKC substrate proteins[11], may play an important role in regulatory aspects of B-50 as a growth-associated protein[1,2] and in the regulation of synaptic plasticity[1,2].

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