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THE PROTEIN KINASE C PHOSPHOSITE(S) IN B-50 (GAP-43) ARE CONFINED TO 15 K PHOSPHOFRAGMENTS PRODUCED BY *STAPHYLOCOCCUS AUREUS* V8 PROTEASE

A. B. Oestreicher,* P. N. E. De Graan, L. H. Schrama,
V. A. F. Lamme, R. J. Bloemen, P. Schotman and W. H. Gispen
Division of Molecular Neurobiology, Rudolf Magnus Institute and Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

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Abstract—The neuron-specific phosphoprotein B-50 (= GAP-43/F1/pp46/P57) is an endogenous substrate of protein kinase C (PKC) in rat brain. To examine the location of the PKC phosphosites, phosphorylated B-50 was digested by *Staphylococcus aureus* V8 protease (SAP). The products migrated in SDS-polyacrylamide gel electrophoresis as two phosphoprotein bands of apparent molecular weight of 15 and 28 kDa (indicated as 15 and 28 K). This study reports further characterization of the 15 and 28 K phosphobands. $ACTH_{1-24}$, a characteristic inhibitor, inhibited equally effective the [^{32}P]phosphate-incorporation into the 15 and 28 K phosphobands formed by SAP from B-50 endogenously phosphorylated in synaptosomal plasma membrane (SPM). Tests using immunoprecipitation or immunoblotting showed that all polyclonal rabbit B-50 antisera recognized the 28 K phosphoband, but only a minor population B-50 antibodies of a recently developed antiserum 8613 reacted with the 15 K phosphoband. The time course of the SAP digestion indicated that B-50 is degraded first to the 28 K band and then to the 15 K band. [^{32}P]phosphate incorporated in B-50 was totally recovered in these phosphobands. Isoelectric focusing resolved the SAP products into one 28 K phosphopeptide of isoelectric point (IEP) 4.8 and the 15 K phosphofragment in at least 4 phosphopeptides, with IEP of 6.1, 6.6, 6.9 and 7.0, respectively. SAP digests of extensively phosphorylated B-50 analysed by isoelectric focusing in narrow pH gradients, showed microheterogeneity in the undigested B-50, the 28 and 15 K phosphofragments. The time course of SAP digestion of B-50 in endogenously phosphorylated SPM and in phosphorylated nerve growth cone membranes, demonstrated that in both types of membranes, 28 and 15 K phosphofragments are consecutively formed, with IEP identical to the phosphofragments derived from isolated B-50. Our findings suggest that the PKC phosphosite(s) in the B-50 protein are restricted to neutral 15 K peptides of the B-50 molecule.

The membrane-bound phosphoprotein B-50, migrating in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight (M_r) of 48 kDa (Zwiers *et al.*, 1976), has been purified from rat brain [isoelectric point (IEP) 4.5, Zwiers *et al.*, 1980]. B-50 has been characterized as a major endogenous substrate of protein kinase C (PKC) in synaptosomal plasma membranes (SPM, Aloyo *et al.*, 1983; Eichberg *et al.*, 1986; De Graan *et al.*, 1989) and in membranes of nerve growth cones isolated from fetal rat brain (De Graan *et al.*, 1985; Van Hooff *et al.*, 1988).

The B-50 protein is neuron-specific (Kristjansson *et al.*, 1982) and identical to the growth-associated protein GAP-43, to F1, and to pp46 (see review of Benowitz and Routtenberg, 1987; Nielander *et al.*, 1987). B-50 phosphorylation in rat brain is influenced by behaviourally active neuropeptides, in particular by adrenocorticotropin (ACTH) peptides (De Graan *et al.*, 1986; Gispen, 1986; Schrama *et al.*, 1986; Van Hooff *et al.*, 1988). B-50 phosphorylation has been implicated in modulation of neuronal membrane signal transduction specifically associated with receptor-mediated hydrolysis of polyphospho-

*To whom correspondence should be addressed.

Abbreviations: ASP, ammonium sulphate precipitate; 1-, 2-D, one- and two-dimensional; GCp, the nerve growth cone particulate fraction; IEF, isoelectric focusing; IEP, apparent isoelectric point; IgGs, immunoglobulins; M_r, apparent molecular weight; PKC, protein kinase C; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SPM, synaptosomal plasma membranes.

inositides (Gispen *et al.*, 1985), in neurotransmission (Dekker *et al.*, 1989), in the molecular mechanism underlying long-term potentiation (Akers *et al.*, 1986; Routtenberg, 1986), in neuronal differentiation (Perrone-Bizzozero *et al.*, 1986), in neurite outgrowth and axon regeneration (Skene and Willard, 1981a, b; Kalil and Skene, 1986; Verhaagen *et al.*, 1986; Zwiers *et al.*, 1987; Gorgels *et al.*, 1987) and rat brain development (Oestreicher and Gispen, 1986).

Studies on the structure and chemical properties of B-50 have revealed that rat and bovine B-50 exhibit anomalous migration behaviour on SDS-electrophoresis in gels of varying concentration of polyacrylamide (Gower and Rodnight, 1982; Oestreicher *et al.*, 1984; Schrama *et al.*, 1987; Benowitz *et al.*, 1987). The B-50 of various species yields on limited digestion by *Staphylococcus aureus* V8 protease (SAP) two phosphoprotein bands, those of rat B-50 have been studied by SDS-electrophoresis on 15% (w/v) polyacrylamide gels, displaying migration rates corresponding to protein entities of M_r of 15 and 28 kDa (Oestreicher *et al.*, 1984; Zwiers *et al.*, 1985). The purified B-50 exhibits microheterogeneity after isoelectric focusing (IEF) in a narrow pH gradient. The isoforms are in part interconvertible by exhaustive phosphorylation and dephosphorylation (Zwiers *et al.*, 1985), indicating that B-50 may contain more than one phosphosite.

The purpose of the study was to characterize the peptide domains of the B-50 protein which contain the phosphosite(s) for PKC, that is the serine residue(s) phosphorylated by PKC. By analysis by SDS-PAGE and isoelectric focusing of the SAP proteolysis products of B-50 phosphorylated by PKC, we show that the phosphosite(s) is confined to 15 K peptides with IEP ranging from 6.1 to 7.0.*

EXPERIMENTAL PROCEDURES

Materials

Staphylococcus aureus V8 protease from ICN Immuno-Biologicals, Lisle, Ill. (U.S.A.); [γ-^{32}P]ATP (S.A. 3000 Ci/mmol) from Amersham (U.K.); Sephadex IEF and molecular weight markers from Pharmacia Fine Chemicals and Ampholines (pH 3–5, pH 4–6, pH 5–7 and pH 3.5–10) from LKB Produkter (Sweden); goat-anti-rabbit immunoglobulins/gold and silver enhancer, bought as the Auro-Probe BL kit from Janssen Life Sciences Products, Beerse, (Belgium). Formaldehyde-inactivated *Staphylococcus aureus* cell membranes prepared according to Kronvall (1973) were kindly provided by Dr W. H. Jansen (RIVM, Bilthoven, The Netherlands).

*We refer to B-50 and its peptides with M_r as derived from SDS-PAGE.

Isolation of B-50

Alkaline extraction of proteins was applied to rat brains stored at −20°C and B-50 protein was purified as described by Zwiers *et al.* (1985). When occasionally the resulting B-50 preparation contained some of its proteolytic breakdown products (e.g. the 40 K protein), the B-50 protein was refractionated by SDS-PAGE followed by excising the 48 kDa B-50 band and extraction from the gel piece. Protein content of fractions was determined by the method of Lowry *et al.* (1951) and that of B-50 by densitometric scanning as described by Zwiers *et al.* (1985).

Isolation of rat brain membranes

SPM from adult rat brain were prepared as reported by Kristjansson *et al.* (1982). Growth cone membranes, the nerve growth cone particulate fraction (GCp), were isolated from fetal rat brains (De Graan *et al.*, 1985).

Protein phosphorylation

Endogenous phosphorylation of 10–20 μg SPM was assayed as described by Zwiers *et al.* (1976), except for a minor modification in the incubation buffer (IB) that consisted of 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10 mM Tris–HCl at pH 7.4. Phosphorylation by added PKC (0.12 μg/μg B-50, purified according to Kikkawa *et al.*, 1986) of 1–2 μg B-50 protein or 10 μg protein of SPM or of GCp was routinely carried out in buffer IB containing 7.5 μM (2–5 μCi) radioactive [γ-^{32}P]ATP (S.A. 3000 Ci/mmol, Amersham, U.K.) and 1 μg of phosphatidylserine/25 μl, at 30°C for 15 min and in a final incubation volume of 25 μl. The reaction was terminated by addition of 12.5 μl denaturing solution S, containing 6% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) 2-mercaptoethanol, 0.003% (w/v) bromophenol blue and 187 mM Tris–HCl at pH 6.8. Quantitative analysis of phosphorylation proceeded by separation of the proteins by SDS-PAGE, autoradiography, excising the protein bands from the gel and counting radioactivity by liquid scintillation, as reported previously (Oestreicher *et al.*, 1984).

Proteolysis

Two procedures were used: (1) proteolysis during electrophoresis as previously described by Oestreicher *et al.* (1984) and adapted from Cleveland *et al.* (1977); B-50 bands were excised from wet 11 or 15% (w/v) polyacrylamide gels. The gel pieces were soaked in 3-fold diluted denaturing solution S (1/3 S). Thereafter the gel pieces were placed in a slot of a second SDS-PAGE gel, covered with 1/3 S containing 0.65% (w/v) agarose and SAP. The stacking occurred in the presence of SAP during slow electrophoresis in a 5% (w/v) stacking gel (at 5–10 mA for 2 h). When the samples had migrated into the running gel of 15% (w/v) polyacrylamide, the current was increased to 30–40 mA. (2) Excised gel pieces with the protein(s) were homogenized in medium 1/3 S and subjected to digestion *in vitro* with SAP at 30°C at pH 6.8 for various times. The digestion was stopped by heating for 15 min at 100°C or at 55°C. Further analysis was carried out by SDS-PAGE and autoradiography.

Determination of isoelectric point (IEP) and two-dimensional (2-D) electrophoresis

Protein samples were pretreated with (final concentrations) urea (8 M), Triton X-100 (0.5% v/v), glycerol (11% v/v), a mixture of ampholines (4.6% v/v) of pH range 3.5–10.0 and when samples contained SDS, Nonidet P-40 (2.5% v/v) was included. Separation of the proteins was

carried out by 4.7% (w/v) polyacrylamide slab gel isoelectric focusing (IEF). The one-dimensional (1-D) isoelectric focusing gel was analysed by autoradiography and by determination of the pH gradient with a surface micro pH electrode. The IEF fractionated phosphoproteins were identified by the second step of 2-D-electrophoresis. For this purpose, individual tracks were cut out from the IEF gel and mounted on consecutive SDS-polyacrylamide (15% w/v) gel (Zwiers *et al.*, 1985).

Immunochemical analyses

The immunoprecipitation assay was carried out on ^{32}P-labelled purified B-50 or B-50 in SPM as described by De Graan *et al.* (1988). In short, phosphorylated samples were adjusted to 1 μg protein/20 μl with 3 times diluted S. Subsequently 280 μl buffer A (containing 200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4 and 0.5% (v/v) Nonidet P-40) and 100 μl rabbit anti-B-50 serum (8103, 8420, 8502 and 8613; Oestreicher *et al.*, 1983, 1986; Oestreicher and Gispen, 1986); or preimmune serum (diluted in buffer A) were added, resulting in a final SDS concentration of 0.1% (w/v) and a total volume of 400 μl. Incubation took place overnight at 4°C. To immunoprecipitate the antigen–antibody complex, 50 μl of a washed formaldehyde-inactivated *Staphylococcus aureus* cell membrane (4% w/v) suspension in buffer A was added and incubated for 30 min at room temperature. After centrifugation, the immunoprecipitated pellets were analysed by 11% (w/v) SDS-PAGE, followed by autoradiography.

Immunoblotting was performed as described by Van Hooff *et al.* (1988). The separated proteins on the nitrocellulose blots were incubated with antibodies [1:2000 and 1:4000 diluted anti-B-50 IgGs, affinity-purified from antiserum 8420 or 8613 (Oestreicher *et al.*, 1983, 1986), and 1:400 and 1:16000 diluted anti-B-50 serum 8613]. The bound immunocomplex was visualized with goat anti-rabbit IgGs adsorbed to gold particles and enhanced by silver with IntenSE according to the protocol provided by Janssen Life Sciences Products.

RESULTS

Effect of ACTH on the phosphofragments of B-50 in SPM

The phosphorylation of B-50 in SPM is inhibited dose-dependently by ACTH peptides (Zwiers *et al.*, 1976). It has been suggested that B-50 has more than one phosphorylation site for PKC (Zwiers *et al.*, 1985). We examined if there was a differential effect of the ACTH inhibition on the SAP phosphofragments of B-50 to distinguish the possible PKC phosphosites. Digestion by SAP of SPM endogenously phosphorylated in the absence and presence of 10 μM $ACTH_{1-24}$ resulted in the production of two, 15 and 28 K, phosphoprotein bands [Fig. 1(B)]. ACTH inhibited ^{32}P-labelling of B-50 [Fig. 1(A), lanes +] and that of the 15 K and 28 K phosphobands [Fig. 1(B), lanes +]. Table 1 shows that the phosphorylation of both fragment bands are equally affected by $ACTH_{1-24}$. Thus, the test with the peptide does not allow to differentiate phosphosites of the two fragment bands.

Immunoreactivity of the phosphofragments of B-50

By immunoprecipitation and immunoblotting we examined if the 15 and 28 K fragments formed from

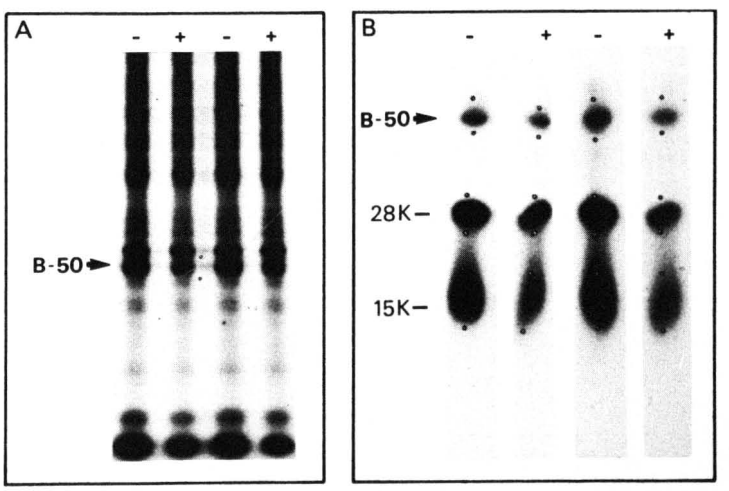

Fig. 1. The effect of $ACTH_{1-24}$ on endogenous B-50 phosphorylation in SPM (A) and on ^{32}P-incorporation of the two phosphobands obtained by SAP digestion (B). 20 μg SPM proteins endogenously phosphorylated in the absence (−) and presence (+) of 10 μM $ACTH_{1-24}$ were separated by SDS-PAGE, subjected to autoradiography (A). The radiolabelled B-50 protein was excised from the gel, and digested with SAP during SDS electrophoresis on a second 15% polyacrylamide gel [autoradiogram shown in (B)]. $ACTH_{1-24}$ inhibited equally the phosphorylation of B-50, 28 K and 15 K phosphofragments (see Table 1).

Table 1. Effect of 10 μm $ACTH_{1-24}$ on ^{32}P-incorporation into the B-50 phosphobands obtained by SAP digestion

Addition	Incorporation (% inhibition) 28 K	15 K	Ratio 15 K/28 K
−ACTH	9055 ± 54 (0%)	13416 + 56 (0%)	1.48 + 0.09
+ACTH	6225 ± 648 (32%)	9055 + 274 (33%)	1.49 + 0.13

Radioactivity determined in excised bands as cpm ± SD, ratio not significantly different [$n = 5$).

isolated B-50 or from B-50 of rat brain membranes were able to bind anti-B-50 antibodies raised in various rabbits. SPM was phosphorylated in the presence of added PKC, digested with various amounts of SAP for 30 and 60 min. The digestion products were analysed either directly (Fig. 2, panel C) by SDS-PAGE (15% running gel) or priorly immunoprecipitated and then subjected to SDS-PAGE. Figure 2 (left panel) shows that various SPM proteins, including B-50, had been highly phosphorylated, and that with increasing amounts of SAP (lane 3 vs 5) and time (lane 4 vs 5) B-50 decreased and 15 and 28 K phosphobands increased. From the numerous phosphorylated proteins, the B-50 antiserum (8502) immunoprecipitated specifically B-50 and the 28 K phosphoband, but not the 15 K band (Fig. 2, right panel, lanes 3, 4, 5). The result suggests that the polyclonal B-50 antibodies are predominantly directed to epitopes present in B-50 and 28 K phosphofragment. We also examined by immunoprecipitation the reactivity of other anti-B-50 antisera raised in several rabbits (8103, 8420 and 8502; Oestreicher *et al.*, 1983) on SAP digests of purified phosphorylated B-50 (amount approximately equivalent to that in SPM of the experiment of Fig. 2) obtained after 15 and 90 min. The results were comparable to Fig. 2. Antibodies of these B-50 antisera tested at increasing dilution (1:25 and more) reacted with the phosphorylated B-50 and with the 28 K phosphoband, but not with the 15 K phosphoband. The antisera were not effective, even after prolonged digestion, when most of 28 K band was converted to 15 K band (see below). However, when in contrast to the phosphorylated preparations of low specific radioactivity incorporation, a SAP digest of B-50 tracer (S.A. 20 μCi/μg, Oestreicher *et al.*, 1986) was used, affinity-purified antibodies of antiserum 8613 also immunoprecipitated the 15 K phosphoband (Fig. 3). Higher antibody concentrations were required to immunoprecipitate the 15 K phosphofragment than for the B-50 and the 28 K fragment, indicating that the population of antibodies in the serum reacting with epitopes of the 15 K fragment was present in smaller quantity than those recog-

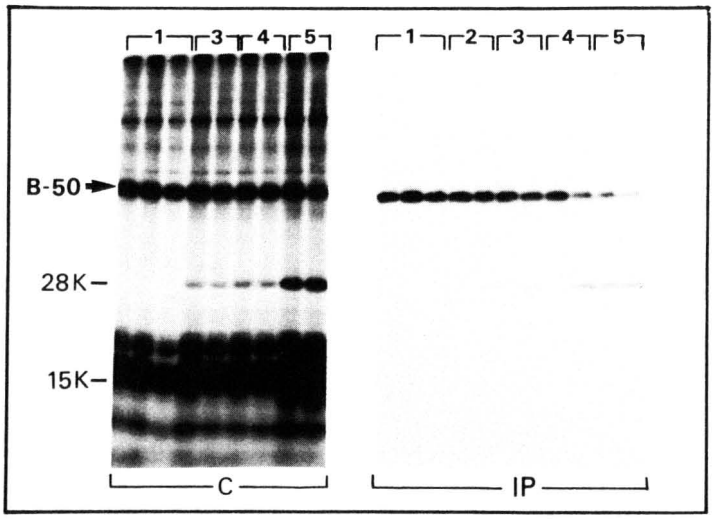

Fig. 2. Immunoprecipitation by anti-B-50 antibodies of the phosphofragments produced by SAP proteolysis from SPM which were prephosphorylated by added PKC for 5 min at 30°C. Samples of 1 μg SPM (containing about 0.05 μg B-50) were either immunoprecipitated with 1:200 diluted B-50 antiserum 8502 followed by analysis by SDS-PAGE (IP) or as controls (C) directly analysed. Thereafter autoradiography was performed of which the results are shown. The samples contained: lane 1, phosphorylated SPM, no SAP added; lanes 2, 3 and 4, 5, respectively, 1 and 5 ng/μl SAP added; lanes 2, 4 and 3, 5, respectively, incubated for 30 and 60 min. Panel (C) shows that with increasing dose of SAP and time B-50 was progressively degraded and 28 K and 15 K fragments were formed.

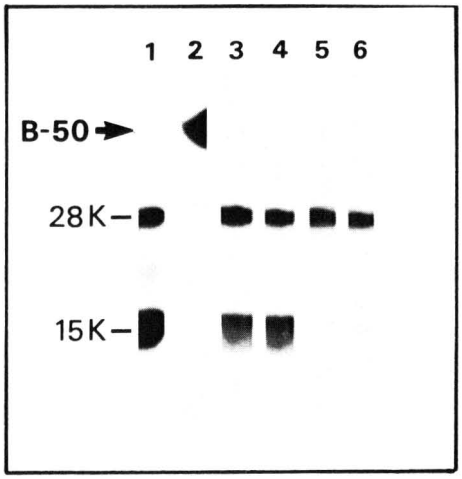

Fig. 3. Autoradiogram of immunoprecipitation by anti-B-50 IgGs of antiserum 8613. Samples of a time course of SAP digestion of as tracer labelled B-50 (approximately 5 ng) were either directly analysed by SDS-PAGE (15% gel) or first immunoprecipitated and then subjected to SDS-PAGE. Results are shown for the following samples: lane 2, B-50; lanes 1, 3 and 5, 40 min digests and lanes 4 and 6, 60 min digests containing the 28 and 15 K phosphofragments. Samples of lanes 2, 5 and 6 were immunoprecipitated with the 1:200 diluted antiserum 8613 and those of lanes 3 and 4 with 1:160 diluted affinity-purified antibodies of 8613.

nizing epitopes of B-50 and 28 K fragment. Analysis of the various antisera on Western blots prepared from SAP digests of purified B-50 or of total SPM, gave similar results. The formation of the fragments was verified by autoradiography. The antibodies in all sera reacted with B-50 and the 28 K band, whereas those of antiserum 8613 also reacted with the 15 K band. Small changes in the phosphorylation state brought about by *in vitro* phosphorylation of B-50 and SPM, appeared not to influence the immunoreactivity of the SAP digested proteins on the blot.

Time course of SAP digestion of B-50

Zwiers *et al.* (1985) reported that on limited SAP digestion of B-50, total incorporated phosphate of B-50 was completely recovered in the two fragments. On basis of the kinetics of the production of the fragments these authors concluded that one cleavage site in B-50 produced simultaneously the 15 and 28 K fragments.

We examined this possibility and an alternative interpretation, by following the time course of B-50 digestion more extensively till the 28 K phosphoband was also degraded. We started with a preparation of B-50, containing a specific 40 K degradation product characterized by IEP 4.5 and crossreacting with antibodies to B-50. These proteins were phosphorylated by purified PKC and then digested by SAP during increasing time periods. The kinetics show that ^{32}P-labelled B-50 and the 40 K protein decreased, while the 28 K phosphoband was formed first and then the 15 K band [Fig. 4(A)]. After longer incubation times (60 min) the 28 K band began to decrease. Finally, the 15K band became the end product [Fig. 4(A), (B)]. Summing up the counted radioactivity in the excised protein bands, shows that the total radioactivity was virtually constant over the examined period [Fig. 4(B)]. Thus, degradation in smaller phosphofragments appeared not to occur. This implies that B-50 and the 40 K protein are both degraded in 28 and 15 K fragments and all phosphosites are conserved. Figure 4(C) corroborates this by demonstrating, that SAP digestion of the excised labelled B-50, 40 and 28 K phosphobands yielded the same products, and finally all the 15 K phosphoband [Fig. 4(C)]. To characterize further the 28 and 15 K polypeptides, samples were fractionated by IEF with various pH gradient, the identity and the isoelectric point of the phosphopeptides was determined by 2-D analysis (Zwiers *et al.*, 1985, see below in section IEP). In Fig. 4(D) we present the results of 1-D IEF on a wide 3.5–8.0 pH gradient, indicating on the left the identity of the detected phosphospots as resolved by consecutive SDS-PAGE analysis. After 30 min SAP digestion, ^{32}P-labelled purified B-50 [Fig. 4(D), lanes 1], 40 K [Fig. 4(D), lanes 2] and 28 K [Fig. 4(D), lanes 3] yielded the same spots: one polypeptide with IEP 4.8, identified as the 28 K fragment, and four polypeptides with IEP varying from 6.1 to 7.0, identified as originating from the 15 K fragment [left panel of Fig. 4(D)]. Digestion for 90 min to "end products" resulted in the same four 15 K peptides [right panel of Fig. 4(D)].

Occasionally, in some B-50 preparations, we observed another B-60 protein, well known as a degradation product formed from B-50, especially in "native" ASP at low concentrations of Ca^{2+} (Zwiers *et al.*, 1982) as a minor phosphoband migrating just below the B-50 in the 15% gels. In those instances, there appeared on SAP digestion two additional minor phosphobands migrating below the 28 and 15 K phosphobands, respectively. In other experiments, we examined the SAP products of B-50 and the 40 K protein by Coomassie blue and silver staining. In the 15% gels, two prominent staining bands migrating as proteins of 28 and 15 kDa were revealed. For the B-60 protein, the major staining products were 26 and 12 K protein bands.

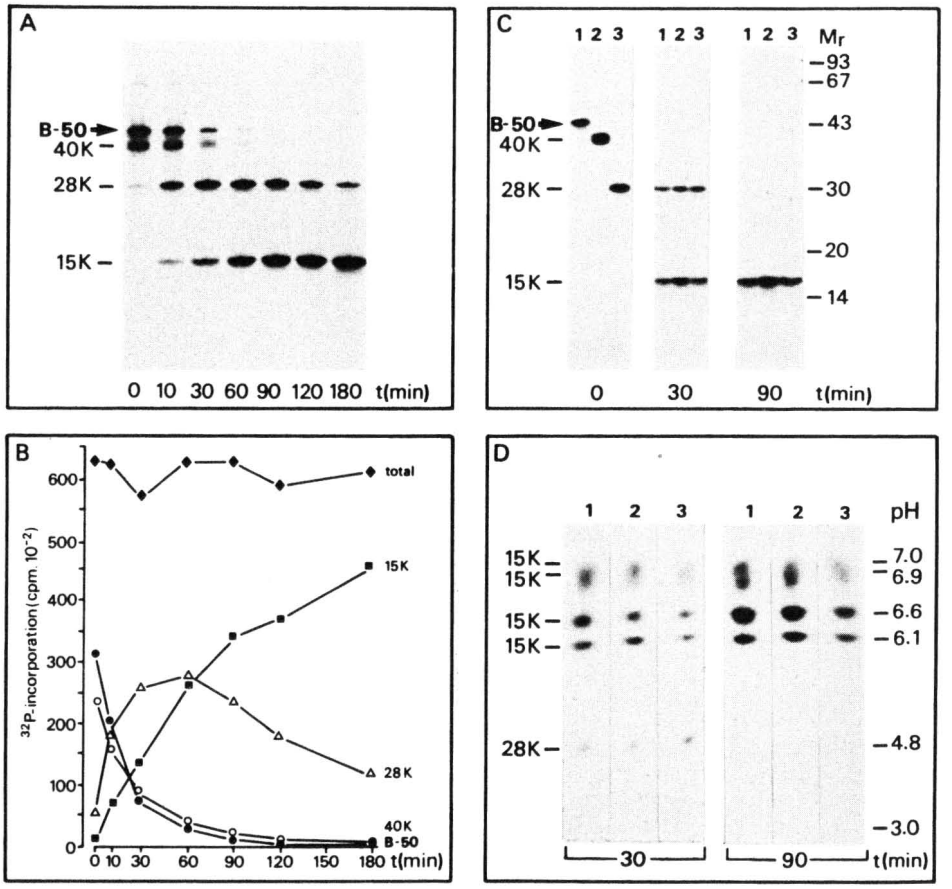

Fig. 4. Time course of SAP digestion of radiolabelled phosphorylated B-50 and characterization of the phosphofragments by IEF. After phosphorylation of B-50 (containing a 40 K proteolytic breakdown product) for 15 min, in the presence of 7.5 μM [^{32}P]ATP and PKC, proteolysis by SAP (10 ng/μl) proceeded for various reaction times in medium S (see Experimental Procedures). Equal amounts of digests (originally containing about 0.5 μg of B-50 and 40 K) were separated by SDS-PAGE, the gels were processed for autoradiography [panel (A)]. Panel (B) presents measurements of the radioactivity incorporated in the bands of B-50, 40, 28 and 15 K phosphobands and total incorporation measured as function of the digestion time. Panel (C) autoradiograms of a second SAP (10 ng/μl) digestion for 0, 10 and 60 min of homogenized protein bands (B-50, 40 and 28 K in lanes 1, 2 and 3, respectively) cut out from the 10 min digestion lane of the gel of panel (A). Panel (D) autoradiograms of IEF analysis on a wide 3.5–8.0 pH gradient of parallel samples of homogenized band B-50, 40 and 28 K (lanes 1, 2 and 3, respectively), digested by SAP for 30 and 90 min. Identification of phosphospots after IEF fractionation was carried out by a consecutively second analysis by 15% SDS-PAGE (see Experimental Procedures).

IEP and microheterogeneity

As mentioned above, the IEP and M_r of the SAP fragments were estimated by conventional 2-D electrophoresis, first IEF fractionation followed by SDS-PAGE on 15% gel (Fig. 5). We found that the 28 K phosphopeptide had an IEP of 4.8 [Fig. 5(A)] more basic than B-50 (IEP 4.5) and, the 15 K phosphopeptides were resolved, depending on the conditions, in 3–4 peptides, with IEP of 6.1, 6.6, 6.9 and 7.0 [Figs 4(D) and 5(C)]. Occasionally, a 12 K phosphopeptide with an IEP of 7.0 was observed. Analysis of phosphorylated B-50, 40 K and the 28 K phosphofragment on IEF in a narrow pH gradient of 3–5 followed by SDS-PAGE revealed several isoforms of each of these proteins [Fig. 5(A)]. We examined further whether SAP digestion of the four B-50 isoforms would yield fragments with differences in IEP. When microheterogenous phosphorylated B-50 band of a 2-D gel was fractionated by excising

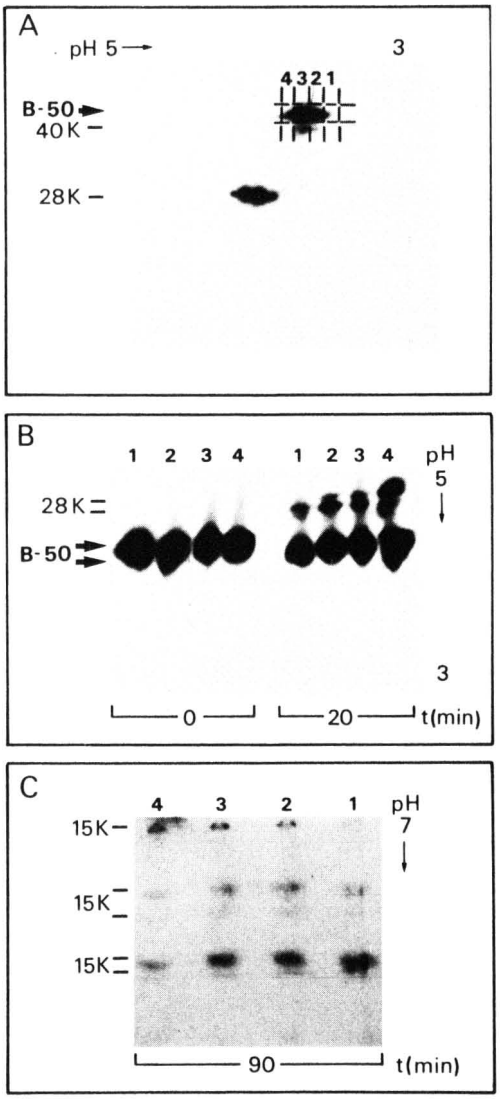

Fig. 5. Microheterogeneity of B-50 (40 K) and its SAP phosphofragments. Autoradiograms are shown. 1–2 μg B-50 was phosphorylated, digested by SAP (5 ng/μl for 5 min). Digests were analysed by 2-D gel electrophoresis, IEF gel with narrow 3–5 pH or 4–7 pH gradient. Panel (A) shows the results of the second part of the 2-D analysis, in 15% SDS-PAGE. As shown, 28, 40 K and B-50 are microheterogeneous. In addition is indicated how the B-50 isoforms (1–4) were excised. Panel (B) shows the first part of the 2-D analysis, fractionation by IEF on a narrow 3–5 pH gradient of the undigested (left) and 20 min digested fractions 1–4 (right). The four B-50 fractions reveal a small difference in IEP of the isoforms and so do those of the 28 K fragment, fractions 1 being more acidic. Panel (C) presents the IEF on a 4–7 pH gradient of the 90 min digests (1–4) yielding more of the acidic 15 K fragments in the sample derived from the more acidic B-50 isoform 1. Phosphoproteins were identified, as described, by 2-D analysis (Zwiers *et al.*, 1985).

four B-50 components according to the quadruplo autoradiographic spot [Fig. 5(A)] from the gel, SAP digestion of these four B-50 spots yielded similar 28 and 15 K phosphofragments on 1-D SDS-PAGE. However, after IEF on narrow pH gradients the more acidic isoforms of B-50 tended to yield more acidic forms of 28 K [Fig. 5(B)] and 15 K fragments [Fig. 5(C)]. The identity of the various forms was assessed by extending the 1-D IEF to a 2-D SDS-PAGE analysis. The results suggest that the microheterogeneity of the 28 and 15 K fragments was a reflection of the microheterogeneity original present in B-50. To see if the extent of phosphorylation of B-50 affected the IEP of the SAP products, we phosphorylated B-50 with 0.1 mM ATP for various times (30 s, 5 and 45 min), purified the phosphorylated B-50, subjected it to SAP digestion for various times (6, 24 and 120 min in the presence of 5 ng SAP/μl) and analysed by IEF. We observed that increased incorporation of phosphate up to 5 min correlated with increasing microheterogeneity of B-50 and its phosphofragments; the proportion of the quantity of the isoforms changed and more of highly labelled acidic isoforms appeared. In another experiment using pulse chase conditions (radioactive ATP pulse of 15 s and chase in the presence of 0.1 mM cold ATP up to 5 min, during which the labelling of the control increased 5-fold), the distribution of label over the isoforms of B-50 and its phosphofragments did not change.

Comparison of the SAP phosphofragments derived from B-50 from growth cone membranes, SPM and from isolated B-50

In order to find out if the phosphorylation of B-50 in growth cone membranes and SPM occurs at similar sites as the isolated B-50, we studied the IEPs of the SAP phosphofragments of B-50 from nerve growth cone particulate fraction (GCp) and SPM (Fig. 6). Samples of B-50, GCp and SPM were phosphorylated in the presence of added purified PKC and for comparison, SPM was also endogenously phosphorylated and analysed by SDS-PAGE [Fig. 6(A)]. In all samples, a strongly ^{32}P-labelled B-50 band was detected. In the isolated B-50 [Fig. 6(A), lane 1] and the GCp preparation [Fig. 6(A), lane 2], an additional 40 K band was labelled. In parallel, the phosphorylated samples were subjected to immunoprecipitation with antibodies to B-50 of serum 8502. This was followed by SAP digestion of the immunoprecipitates for 0, 10 and 60 min and SDS-PAGE analysis. As Fig. 6(B) shows, at zero time B-50 was specifically immuno-

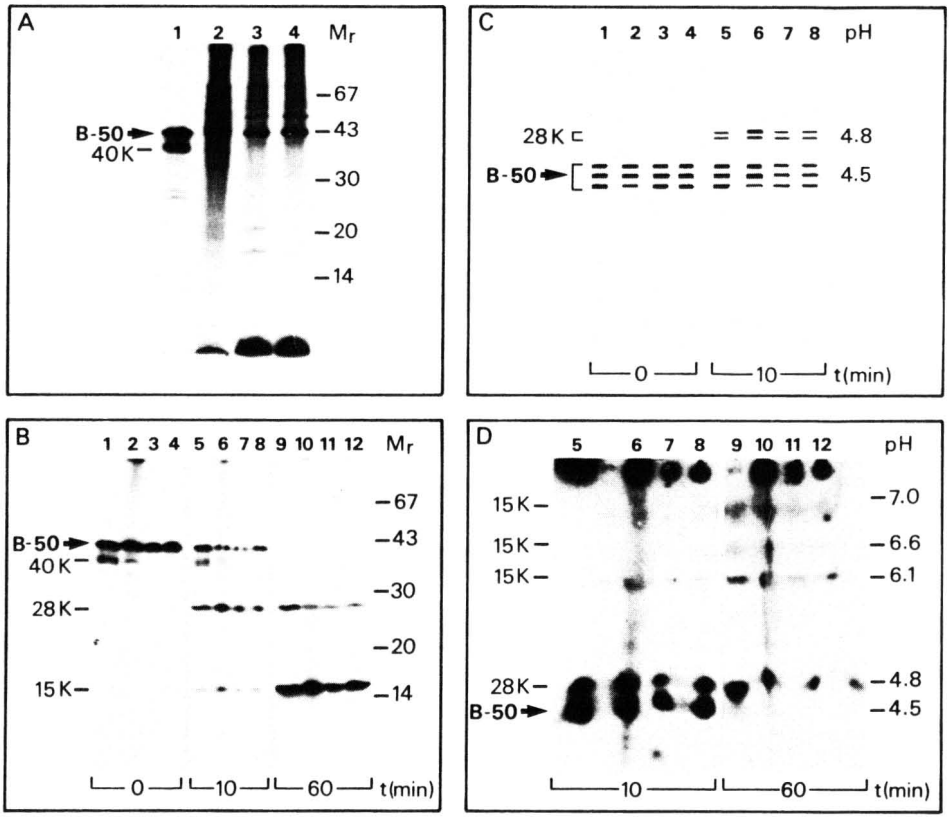

Fig. 6. Comparison of IEP of phosphofragments formed by SAP digestion from isolated B-50, nerve growth cone particulate fraction (GCP) and SPM phosphorylated *in vitro* endogenously and by added PKC. Autoradiograms are shown [except in (C)]. Panel (A) lane 1, B-50 and 40 K; lane 2, GCp; lane 3, SPM; phosphorylated in the presence of PKC and lane 4, endogenously phosphorylated SPM, analysed by 11% SDS-PAGE. Panel (B) directly after the phosphorylation, parallel samples were immunoprecipitated by B-50 antibodies (of antiserum 8502) and treated with SAP for 0, 10 (10 ng/μl) and 60 min (20 ng/μl). Panel (C) IEF analyses of these digests on a narrow pH gradient of 3–5 (camera lucida drawing) and, on a wide pH gradient of 3.5–8.0 in panel (D). The samples were: lanes 1, 5, 9, B-50; lanes 2, 6, 10, GCp; lanes 3, 7, 11 and 4, 8, 12, SPM phosphorylated in the presence of PKC and endogenously, respectively. Products were identified by 2-D analysis. Shown is that SAP digestion of B-50 phosphorylated endogenously in brain membranes or in purified form (phosphorylated by PKC) produces similar phosphofragments.

precipitated from all 4 samples (lanes 1–4), and in addition the 40 K band from the isolated B-50 and the GCp (lanes 1, 2). On SAP digestion immunoprecipitated B-50 (and 40 K) decreased, the four sources yielded mainly 28 K phosphobands [Fig. 6(B), lanes 5–12] and progressively more 15 K phosphobands [Fig. 6(B), lanes 9–12]. Analysis of the same digests by IEF on a narrow pH 3–5 gradient revealed that B-50 from the four sources [Fig. 6(C), lanes 1–4] displayed similar microheterogeneity (3 isoforms) and IEPs. On the 1-DIEF gel, the 40 K protein was not resolved from the isoforms of B-50, having an equal IEP, but on 2-D analysis it can be separated from B-50 [cf. Fig. 5(A)].

The 10 min digest shows a similar pattern of fractionated proteins for the four sources [Fig. 6(C), lanes 5–8] and a difference in IEP of the isoforms of B-50 and 28 K. The 15 K phosphopeptides of the four samples were screened on a wide 3.5–8.0 pH gradient and resolved in three similar 15 K phosphopeptides with identical IEPs (Fig. 6(D)].

DISCUSSION

We report here characterization of the 28 and 15 K phosphofragments, the prominent products of limited SAP digestion of phosphorylated B-50 protein. In agreement with Zwiers *et al.* (1985) all phosphate

incorporated in B-50 was recovered after SAP digestion in the observed phosphofragments of 15 and 28 K. We add evidence that the phosphosites of the 15 and 28 K are equally affected by ACTH inhibiting the endogenous phosphorylation of B-50 in SPM. This is explained by the finding that the phosphopeptides are consecutively formed from B-50 (see below). Further characterization of the 28 and 15 K phosphopolypeptides by their IEPs allowed us to demonstrate that SAP digestion of B-50 phosphorylated in SPM and GCp (Van Hooff *et al.*, 1986, 1988) yielded identical phosphofragments to those obtained from purified phosphorylated B-50. This indicates that the peptide domain phosphorylated in isolated B-50 by PKC is similar to the domain in B-50 endogenously phosphorylated in adult SPM as well by added PKC and that of B-50 in fetal GCp phosphorylated by exogenous PKC. On prolonged SAP digestion, isolated B-50 and B-50 present in the membranes were degraded to the small (15 K) fragments. This also has been reported in recent studies on the molecular properties and the identity of B-50 to GAP43 and protein F1 (Benowitz *et al.*, 1987; Chan *et al.*, 1986; Rosenthal *et al.*, 1987; Snipes *et al.*, 1987).

Immunoreactivity of the 15 and 28 K phosphofragments

All polyclonal antibodies to B-50 raised formerly in various rabbits reacted with B-50 and the 28 K fragment, but not with the 15 K fragments. Only one recently developed B-50 antiserum 8613 bound to the 15 K fragments. This suggests that just a small population of the B-50 antibodies is directed against epitopes restricted to the 15 K fragments. In principle, it has now become feasible to select antibodies specific for the 15 K polypeptides by affinity-purification.

B-50 is degraded by SAP, first to the 28 K and then to the 15 K phosphofragments

Time course studies of the SAP digestion and analysis by IEF demonstrated that phosphorylated B-50 and the 40 K protein from the various sources were first digested to 28 K phosphofragments, from which subsequently the 15 K polypeptides were formed as end products (Figs 4 and 5). The 40 K protein, a breakdown product of B-50, is found in frozen stored brain preparations, such as GCp and the rat brains used to purify B-50.

IEP and microheterogeneity

SAP digestion of phosphorylated B-50, with IEP 4.5, yielded the somewhat less acidic 28 K phosphofragment of IEP 4.8 and several neutral 15 K phosphopeptides with IEP of 6.0, 6.6, 6.9 and 7.0. In agreement with Zwiers *et al.* (1985) microheterogeneity of phosphorylated B-50 was observed after IEF in a narrow pH gradient of 3–5 and 2-D gel analysis. Microheterogeneity was also noticed for the 28 and 15 K phosphofragments (Figs 5 and 6). Experiments in which the phosphorylation state of B-50 was changed by incorporating additional phosphate altered the microheterogeneity in B-50 (data not shown) and that of its SAP fragments; increasing phosphate incorporation led to more of the acidic isoforms. In our experiments, these isoforms appeared to be produced rather fast, namely, at initial stages (within 20 s) of the phosphorylation, since pulse chase labelling did not affect this. Prolonged digestion seemed to increase the formation of the more basic forms of the 15 K fragments (unpublished data). This may be due to cleavage by SAP of secondary peptide bonds closely located in the B-50 sequence thus producing 15 K peptides differing only in a few amino acid residues (see below). Our evidence suggests that the microheterogeneity found in the fragments is a consequence of that in B-50, probably partly due to its original state as isolated.

Phosphosites are confined to the 15 K polypeptides

During the course of the SAP digestion of B-50 (and of 40 K) the total radioactivity in the reactants was constant (Fig. 4). This indicates that the sites of phosphorylation were finally recovered in the end products, the 15 K peptides. Thus, these contain the PKC phosphosite(s). Zwiers *et al.* (1985) have suggested that B-50 contains multiple phosphosites based on their findings of interconversion of the B-50 isoforms by phosphorylation and dephosphorylation. Our experiments have been carried out under different conditions (e.g. pH 6.8 vs 7.4). With our results the number of phosphosites can not be assessed, however, our data do not exclude one phosphosite. Regardless the number of phosphosites, the phosphosite(s) in B-50 are retained in the breakdown product 40 K, and conserved in 28 and 15 K phosphofragments.

B-60, a B-50 breakdown product (Zwiers *et al.*, 1982), yields on SAP digestion two fragments both smaller in size than the B-50 or 40 K SAP fragments. Sequence analysis of B-60 and B-50 by McMaster *et al.* (1989) indicates that B-60 is formed by the exclusive loss of 40 amino acid residues at the *N*-terminal of B-50. Taking into account this structural relation, it is suggested that the SAP 28 and 15 K fragments

are derived from the N-terminal sequence of B-50. SAP cleaves mainly peptide bonds on the carboxyl terminal side of aspartic and glutamic acid (Drapeau *et al.*, 1972). As shown by Houmard and Drapeau (1972), the amino group of the peptide linkage is not critical, but substrate specificity can be rendered more restrictive to only hydrolysis of glutamoyl bonds in either ammonium bicarbonate buffer at pH 7.8 or in ammonium acetate buffer at pH 4.0. For reasons of comparison with our previous studies (Oestreicher *et al.*, 1984; Van Hooff *et al.*, 1986, 1988), this study was carried out at pH 6.8 in 2% SDS. It is as yet unknown which peptide sequences of B-50 are broken by SAP under the conditions used in this study.

While this study was being completed, the cloning of complementary DNA for B-50/GAP43/F1 and its primary structure was reported (Basi *et al.*, 1987; Cimler *et al.*, 1987; Karns *et al.*, 1987; Neve *et al.*, 1987; Nielander *et al.*, 1987; Rosenthal *et al.*, 1987). These studies indicated that those proteins are identical. B-50 is very likely also identical to P57, a neurospecific aberrant calmodulin binding protein, purified from bovine brain (Cimler *et al.*, 1985, 1987; Alexander *et al.*, 1987, Masure *et al.*, 1986). B-50 is shown to be a very hydrophilic protein, consisting of 226 amino acids corresponding to a molecular mass of 23.6 kDa, which is much smaller than evaluated from its mobility in SDS-PAGE. Phosphorylation of B-50 occurs only at the serine residues (14 residues present). Three potential phosphorylation sites for PKC have been predicted at positions 41, 110 and 122 (Nielander *et al.*, 1987). We have shown that all incorporated phosphate was recovered in the 15 K phosphopeptides of IEP ranging from 6.1 to 7.0. This type of heterogeneity may indicate that the peptides have been formed by cleavage of neighbouring sensitive bonds. Since B-60 yields smaller fragments than B-50, the 15 K fragments must be derived from the N-terminal part and may contain at least the potential site 41.

Further analysis of the 15 K fragments needs to be done to pinpoint the precise location of the PKC phosphosite(s). Alternatively, we hope to resolve this question by production of truncated B-50 mRNAs, translation *in vitro*, phosphorylation by PKC of the synthetic B-50 sequences, SAP digestion of these, in parallel with purified B-50 tracer (Schrama *et al.*, 1988). In summary, our SAP proteolysis studies of B-50 phosphorylated by PKC suggest that, in the purified protein as well as in the protein attached to the membrane, the phosphosite(s) are restricted to peptide domains of small neutral (15 K) phosphopeptides of B-50 (Oestreicher *et al.*, 1988).

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