Cross-Reaction of Anti-Rat B-50: Characterization and Isolation of a "B-50 Phosphoprotein" from Bovine Brain

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Abstract: Antibodies to the phosphoprotein B-50 of rat brain were used to trace cross-reacting brain proteins of vertebrates. With the SDS-gel-immunoperoxidase method, a cross-reacting protein (CP) of apparent M_r 53,000 was demonstrated in the homogenate and the synaptic plasma membrane fraction of bovine brain. Sequence 1–24 of adrenocorticotropin ($ACTH_{1-24}$) (10^{-5} M and 10^{-4} M) inhibited endogenous phosphorylation of CP in synaptic plasma membranes. The protein was partially characterized and purified to homogeneity from bovine brain by procedures previously described for rat B-50. CP was enriched in ammonium sulfate precipitated protein (ASP) fractions and phosphorylated by an endogenous protein kinase. Two-dimensional gel analysis of bovine and rat ASP showed that the cross-reacting protein had an isoelectric point less acidic than B-50. Limited proteolysis by *Staphylococcus aureus* protease yielded a

"peptide map" analogous to B-50. Two major fragments of M_r 30,000 and 17,000 were produced. In addition, CP exhibited other similarities to rat B-50: phosphorylation by rat brain protein kinase C, microheterogeneity observed after isoelectric focusing, and possibly degradation by endogenous proteolysis. Cross-reaction of proteins in brain homogenates of other mammalian species and of chicken was demonstrated: the M_r of the proteins ranged from 47,000 to 53,000. We conclude that (1) the cross-reacting bovine protein is a "B-50 protein," and (2) the M_r of the "B-50 protein" varies from species to species. **Key Words:** ACTH—Antibodies—Bovine brain—Phosphoproteins—Rat B-50—Synaptic plasma membranes. **Oestreicher A. B. et al.** Cross-reaction of anti-rat B-50: Characterization and isolation of a "B-50 phosphoprotein" from bovine brain. *J. Neurochem.* **43**, 935–943 (1984).

Phosphoproteins are particularly enriched in the brain (Weller, 1979). Changes in their phosphorylation state are supposed to be involved in various functions of the neuronal plasma membrane such as synaptic transmission and ionic conductance (for reviews see Greengard, 1979, 1981; Oestreicher et al., 1982; Rodnight, 1982).

Rat B-50, a membrane-bound phosphoprotein with an apparent M_r of 48,000 and isoelectric point (IEP) of 4.5 (Zwiers et al., 1980), is localized exclusively in nerve tissue (Kristjansson et al., 1982). B-50 was first described in studies of the *in vitro* ef-

fects of behaviorally active adrenocorticotropin (ACTH) peptides on endogenous protein phosphorylation in synaptic plasma membranes (SPM) isolated from rat brain (Zwiers et al., 1976). Zwiers et al. (1976, 1978) showed that the neuropeptide inhibited cyclic-nucleotide-insensitive protein kinase activity of SPM, but it did not influence the activity of protein phosphatases and ATPases. Subsequently, the ACTH-sensitive, Ca^{2+} - and Mg^{2+} -requiring protein kinase and the B-50 protein, one of its substrates, were concomitantly extracted from rat brain membranes (Zwiers et al., 1979) and pu-

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Abbreviations used: ACTH, Adrenocorticotropin; $ACTH_{1-24}$, Sequence 1–24 of adrenocorticotropin; ASP, Ammonium sulfate precipitated proteins; ASP (57–82%), Proteins precipitated between 57 and 82% saturation of ammonium sulfate; BSA, Bovine

serum albumin; CP, Cross-reacting protein; DEAE, Diethylaminoethyl; DTT, Dithiothreitol; IEP, Isoelectric point; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate-buffered saline; P_2 , Mitochondrial-synaptosomal fraction; P_2P , Fraction of P_2 discarded in Po; Po, Pn, Old and new procedures; SAP, *Staphylococcus aureus* protease; SDS, Sodium dodecyl sulfate; SGIP, SDS-gel-immunoperoxidase; SPM, Synaptic plasma membranes.

rified (Zwiers et al., 1980). Further characterization of the B-50 protein demonstrated that it is enriched in a fraction of proteins precipitated between 57 and 82% saturation of ammonium sulfate [ASP (57–82%)] obtained after diethylaminoethyl (DEAE)-cellulose ion-exchange chromatography of a Triton X-100-KCl extract of rat brain membranes (Zwiers et al., 1980). Rat B-50 is degraded by endogenous proteolytic activity and yields a specific fragment, the B-60 protein (M_r 46,000) (Zwiers et al., 1982a).

Studies of Jolles et al. (1980, 1981) have suggested a modulatory role of the phosphorylation state of B-50 on (poly)phosphoinositide metabolism in rat brain membranes. This concept is supported by observations that affinity-purified anti-rat B-50 antibodies interfere with membrane protein and lipid phosphorylation (Oestreicher et al., 1983a). B-50 is distributed throughout the rat brain, concentrated in regions rich in synaptic contacts (Oestreicher et al., 1981), and is presumably localized in the presynaptic membranes (Sorensen et al., 1981). Recent immunoelectron microscope studies give similar indications (Oestreicher et al., 1983b).

Various specific phosphoproteins first characterized in one species have been found to occur in other species as well, with conservation of many salient properties together with minor distinctions (Greengard, 1981; Goelz et al., 1981; Huang et al., 1982; Martinez-Millan and Rodnight, 1982). The purpose of this paper is to explore the possible occurrence of a "B-50 protein" in bovine brain and in brain fractions of several vertebrate species. Using antibodies to rat B-50 and a comparative biochemical approach, we report the partial characterization and purification to homogeneity of a 53K cross-reacting protein (CP), a "B-50 phosphoprotein" from bovine brain.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. Particular reagents were the same as reported in Oestreicher et al. (1983a). Carrier-free [γ - 32 P]ATP was purchased from New England Nuclear. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories Ltd (England).

Preparation of brain homogenates and membrane fractions

Brains of one-year-old calves obtained from the local slaughterhouse were transported immersed in ice to the laboratory approximately 30 min after sacrifice. All isolation procedures were carried out at 0–4°C.

For the comparative study, laboratory rats (150–200 g) of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). An autopsy sample of frozen human fetal cerebral cortex was obtained from the Academic Hospital of the University of Utrecht. Except for cow and rabbit, laboratory animals (mouse, rat, Russian hamster,

chicken, frog, and fish) were sacrificed by decapitation; rabbits received a heart puncture. One gram wet weight of brain was homogenized in 5 volumes (vol/wt) of 0.32 M sucrose. For SDS-gel-immunoperoxidase (SGIP) analysis, freshly prepared homogenates were solubilized at a protein concentration of 2 μ g/ μ l in sodium dodecyl sulfate (SDS)-electrophoresis sample buffer in final concentrations containing buffer P (10 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM calcium acetate at pH 6.5) and solubilizing solution S [2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (wt/vol) 2-mercaptoethanol, 0.001% (wt/vol) bromophenol blue, and 62.5 mM Tris-HCl, pH 6.8].

The *mitochondrial-synaptosomal fractions* (P_2) of various species and the SPM from rat cerebrum and bovine cerebral cortex were isolated according to the procedure of Kristjansson et al. (1982).

Isolation of rat B-50, ASP(57–82%), and CP from bovine brain

Rat B-50 protein and rat ASP(57–82%) were purified by an old procedure (Po) (Zwiers et al., 1980, Oestreicher et al., 1983a), yielding native proteins, and a new procedure (Pn), including alkaline extraction, as described in detail by Oestreicher et al. (1983a).

Po and Pn were applied to isolate CP from bovine brain. All fractions of the CP isolation were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and checked for specific immunoreactivity to anti-rat B-50 antibodies.

Old procedure. To adapt Po (Zwiers et al., 1980) to bovine brain, fresh bovine cerebral cortex (approximately 240 g wet weight) was homogenized in 0.32 M sucrose (5 ml per gram tissue) in a Waring Blender tissue grinder for 15 s. The homogenate was centrifuged at $900 \times g$ for 10 min and the supernatant subsequently for 20 min at $10,000 \times g$ (Sorvall GSA rotor). The crude mitochondrial pellet was subjected to osmotic shock for 15 min in 320 ml double distilled water, followed by centrifugation for 20 min at $48,000 \times g$ (Sorvall SS-34). The pellet of the light membranes was suspended in 160 ml buffer A [6 mM Tris-HCl, 0.1 mM dithiothreitol (DTT), pH 8.1] and mixed and extracted for 15 min under stirring with an equal volume of 150 mM KCl, 1.0% (vol/vol) Triton X-100 in buffer A. After residual fragments were pelleted at $48,000 \times g$ for 20 min, the extract (310 ml) was diluted with an equal volume of buffer B (10 mM Tris-HCl, 0.1 mM DTT, pH 7.4). The extract was adjusted to pH 7.4 and mixed with 200 ml of a 1:1 (vol/vol) suspension of DEAE-cellulose (DE-32, Whatman) equilibrated in buffer B and stirred for 15 min to allow binding of the proteins. Unbound proteins were removed by suction and rinsing four times with 200 ml buffer B. The DEAE-cellulose containing the bound proteins was then packed in a column (60 $\times$ 2 cm) for ion-exchange chromatography. Proteins were eluted with a linear salt gradient from 0 to 400 mM NaCl in buffer B (400 ml). Collection of fractions (4.5 ml) was monitored by measurement of the absorbance at 280 nm and the conductivity of the effluent. Four pools (1–4) of four consecutive fractions were obtained from the effluent with conductivity equal to 150–250 mM NaCl, the salt concentration at which rat B-50 elutes. The pools were subjected to ammonium sulfate fractionation. The resulting four ASP (57–82%) fractions were dialyzed for 3 h against double distilled water (Zwiers et al., 1980) and analyzed (Fig. 2).

New procedure. Isolation of CP from fresh bovine brain and from bovine brain stored at -20°C was carried out by Pn according to the method of Oestreicher et al. (1983a), except that steps (a) and (b) were substituted by step (ab), as described below.

(ab) Alkaline extracts were prepared from: (1) 125 g bovine brain homogenized in five volumes of double distilled water in a Teflon-glass Potter-Elvehjem type homogenizer using seven up-and-down strokes at 700 rpm, and (2) the pellet of the osmotically shocked mitochondrial fraction (= P_2P) which is discarded in the Po. The pH of the homogenate or the P_2P was adjusted to 11.5 with 1 M NaOH to a final volume of 10 ml per gram wet weight.

CP was purified to homogeneity from bovine ASP (57–82%) of Po and Pn by means of preparative isoelectric focusing as described by Oestreicher et al. (1983a). Purified CP was desalted on Sephadex G-25, lyophilized, and stored at -20°C .

Production, purification, and detection of antibodies

Antiserum raised against purified rat B-50 in rabbit 8002, as described previously (Oestreicher et al., 1983a), was used in this study. Two new antisera (8206 and 8207) against rat B-50 purified by Po were raised in White New Zealand rabbits (Oestreicher et al. 1981, 1983a). Rat B-50 antibodies were purified by affinity chromatography on a solid immunosorbent prepared from rat B-50 (Oestreicher et al., 1983a). Control immunoglobulins were isolated from preimmune and normal rabbit serum by affinity chromatography on a Protein-A-conjugated Sepharose column (2 ml of swollen gel) by a similar procedure.

Purification and formation of antibodies to B-50 were monitored by an enzyme-linked immunosorbent assay (Engvall, 1980). Immunospecificity was determined by the SGIP method (Van Raamsdonk et al., 1977), with modifications as described by Oestreicher et al. (1983a). Antibody binding was routinely detected by goat anti-rabbit antibodies conjugated to horseradish peroxidase. Immunospecificity was also checked by absorption of the antibody samples for 2 h at 4°C by an excess of purified B-50 protein and of SPM.

Protein phosphorylation

Endogenous protein phosphorylation was accomplished as previously described in detail by Oestreicher et al. (1983a).

Phosphorylation by added protein kinase C was accomplished as follows: Purified B-50, CP, and ASP (57–82%) obtained by Pn from rat and bovine brain were phosphorylated with purified protein kinase C (Aloyo et al., 1983) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (7.5 μM), as described by Aloyo et al. (1982, 1983). One milligram ASP was incubated for 15 min at 30°C with 7.5 μM ATP, containing 50–75 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. act. 3,000 Ci/mmol) and 20 $\mu\text{g/ml}$ phosphatidylserine, to activate 2 μg kinase C in buffer P in a final volume of 150 μl .

Separation of proteins by one- and two-dimensional electrophoresis

One-dimensional SDS-PAGE of proteins solubilized in solution S was conducted as previously described by

Zwiers et al. (1976). For two-dimensional analysis, the protein phosphorylation was stopped by immersion in liquid nitrogen. Separation of proteins was carried out by slab gel isoelectric focusing, followed by SDS-PAGE as described previously (Zwiers et al., 1980; Kristjansson et al., 1982).

Incorporation of ^{32}P was estimated by two procedures:

1. Microdensitometry of autoradiograms with a Zeiss PMQ-II spectrophotometer and linear gel scanner at 550 nm. Peak areas were measured with a planimeter (Zwiers et al., 1978).

2. Quantitative determination by liquid-scintillation counting in excised protein bands and spots of dried gels (Wiegant et al., 1978).

One-dimensional peptide maps

Partial proteolysis of rat and bovine B-50 protein with *Staphylococcus aureus* V8 protease was carried out according to the methods of Cleveland et al. (1977) and Hames (1981). Two different procedures were used:

1. Proteolysis of proteins during electrophoresis: ^{32}P -phosphorylated ASP obtained from rat and bovine brain by Pn were separated by one-dimensional SDS electrophoresis on a slab gel composed of a 2-cm high stacking gel [3% (wt/vol) acrylamide, 0.2% (wt/vol) bisacrylamide] and a 9-cm high running gel [11% or 15% (wt/vol) acrylamide, 0.2% (wt/vol) bisacrylamide], both of 1-mm thickness. The gel was lightly fixed and stained with Fast Green sufficiently to distinguish B-50 and CP. These bands were excised and soaked in 5 ml 0.125 M Tris-HCl at pH 6.8 containing 0.1% (wt/vol) SDS (= buffer C). Each equilibrated excised band was placed in a slot of another slab gel of similar composition (thickness 1.3 mm) and covered with buffer C containing 20% (vol/vol) glycerol. Next, variable amounts of *Staphylococcus aureus* protease (SAP) were added in buffer C, which also contained, in final concentration, 10% (vol/vol) glycerol and 0.001% (wt/vol) bromophenol blue. The electrophoresis was started at 7–10 mA for 2 h during stacking and run at 30–40 mA.

2. Purified CP and rat B-50 (2.5 μg) were incubated *in vitro* for 30 min at 37°C in the presence of increasing amounts of SAP in buffer C containing 10% (vol/vol) glycerol and 0.001% (wt/vol) bromophenol blue in a final volume of 50 μl . The incubation was terminated by addition of 20 μl solution S and heating for 5 min at 100°C . Analysis was carried out by SDS electrophoresis on 15% (wt/vol) polyacrylamide running gel.

Other analyses

The M_r of proteins and peptide fragments after SDS-PAGE was estimated according to the method of Weber and Osborn (1969). Their electrophoretic mobility was compared with that of standard proteins analyzed in the same gel. The standards were the following proteins (M_r in parentheses): phosphorylase *a* (94K), albumin (67K), catalase (60K), ovalbumin (43K), lactate dehydrogenase (36K), carbonic anhydrase (30K), trypsin inhibitor (20K), and α -lactalbumin (14.4K).

Protein content was measured by the method of Lowry et al. (1951). Purified B-50 was quantified by densitometric scanning at 650 nm of the Fast-Green-stained band in SDS-polyacrylamide gels with bovine serum albumin (BSA) as a standard.

RESULTS

Immunodetection of a cross-reacting protein

Membrane fractions of bovine brain were compared by means of the SGIP method with rat brain SPM for the occurrence of components that cross-react with antibodies to rat B-50 (Fig. 1). Anti-rat B-50 serum immunostained a 53K protein band, designated cross-reacting protein (CP), in fractions of bovine brain (Fig. 1, tracks 2 and 4). CP migrated with an electrophoretic mobility somewhat slower than that of B-50 in rat SPM (track 1). Separation of a mixture of SPM proteins of bovine and rat brain in one slot by SDS-PAGE and incubation with antiserum to B-50 resulted in immunostaining of two distinct bands: B-50 and CP (track 3). The weaker immunostaining of CP in track 4 may suggest that the CP content of the bovine P₂ fraction is less than that of bovine SPM. Similar results were found with affinity-purified antibodies to rat B-50 and anti-B-50 serum of two other rabbits. Immunostaining of CP and of rat B-50 was prevented by absorption of anti-B-50 antibodies by purified rat B-50 or rat SPM (data not shown).

Purification of CP

In order to find out if CP and rat B-50 are similar proteins, isolation of CP was carried out by procedures previously used to purify rat B-50:

Old procedure. A Triton X-100-KCl extract prepared from membranes of fresh bovine brain was fractionated on a DEAE-cellulose column and subjected to two-step ammonium sulfate precipitation as described by Zwiers et al. (1980). Four pools (1–4) of ASP (57–82%) were obtained, incubated with [γ -³²P]ATP to phosphorylate the proteins, and examined by the SGIP method. Most of the protein-

and immunostaining of the 53K CP was found in pools 2 and 3 (Fig. 2A and B). The major phosphorylated band comigrated with CP (Fig. 2C), indicating that CP may be a phosphoprotein (compare Fig. 5). Bovine ammonium sulfate precipitated proteins (ASP) also contained CP protein kinase activity, concentrated in pools 3 and 4 (Fig. 2C). As in the case of rat B-50 and its B-50 kinase (Zwiers et al., 1980), the substrate CP and its protein kinase were enriched in the same ASP fraction (highest yield in fraction 3).

New procedure. As expected (Oestreicher et al., 1983a), the protein composition and content of ASP (Fig. 3, track 2) prepared from an alkaline extract of bovine brain homogenate was very different from the ASP of Po (Fig. 2). The ASP of Pn was devoid of measurable protein kinase, phosphatase, and peptidase activity. CP, one of the major proteins, was about 100-fold enriched in this ASP fraction relative to the homogenate.

CP was further purified to homogeneity from ASP of Pn and Po (pool 2 of Fig. 2) by preparative isoelectric focusing on a flat bed of Sephadex-IEF containing a pH gradient of 4–6 (Oestreicher et al., 1983a). In both cases, one of the fractions obtained contained only the 53K CP, as visualized by SDS-PAGE (Fig. 3, fraction 12). A more acidic protein of 49K was also separated in another fraction (11).

The recovery of CP by Po was much lower than by Pn, since the P₂P fraction, also a source of CP, is discarded in Po. The yield of Pn was 35–45 μ g CP per gram wet weight brain tissue.

Comparison of properties of CP and rat B-50

Two-dimensional analysis: IEP. The preparative isoelectric focusing purification had indicated that CP was an acidic protein. Bovine ASP isolated by Pn was phosphorylated by added protein kinase C as described by Aloyo et al. (1982, 1983). Under these conditions, CP is strongly labeled and the major phosphorylated protein of the ASP fraction. Phosphorylated bovine and rat proteins were separated by analytical two-dimensional electrophoresis (Kristjansson et al., 1982) and the IEP of CP was compared with that of rat B-50. The findings (Fig. 4) indicate the following:

1. The IEP of the 53K CP was approximately 4.6, and that of the other immunoreactive 49K protein was approximately 4.5 (Fig. 4, panel AI). The 53K CP exhibited more immunostaining and ³²P-incorporation than the 49K protein (Fig. 4, panel I).
2. CP had an IEP less acidic than rat B-50 (IEP = 4.5, Zwiers et al., 1980), which, as expected, reacted more strongly with the antibodies and was highly phosphorylated by protein kinase C (Aloyo et al., 1982).
3. The minor cross-reacting 49K protein of the bovine ASP fraction had an IEP close to that of rat B-50 (overlap in Fig. 4, panel II). As in the case of

FIG. 1. Immunodetection of a 53K protein band in bovine membrane fractions using antiserum to rat B-50 and the SGIP-method. SPM proteins from rat (track 1: 50 μ g) and bovine brain (track 2: 60 μ g), a mixture of both (track 3), and a bovine membrane fraction (track 4: 70 μ g of P₂ protein) were separated by electrophoresis on an SDS-polyacrylamide gel of 3 mm thickness as described by Oestreicher et al. (1981, 1983a). After electrophoresis, pieces of 20 cm² were excised, frozen, and sectioned to 50- μ m thick sections in a cryostat microtome. **A:** The remaining gel piece was stained with Fast Green to reveal the pattern of separated protein bands in the gel. **B:** Immunostaining after incubation of the fixed gel sections with 1:40 diluted antiserum to rat B-50 (rabbit 8002).

FIG. 2. Ammonium sulfate precipitate (57–82%) pools: intermediates in the purification of CP from bovine brain. 2–4 µg ASP (57–82%) of pools (1–4) were endogenously phosphorylated *in vitro* at 30°C for 15 s in an incubation medium (25 µl) containing 7.5 µM ATP (2–3 µCi [γ - 32 P]ATP, 3,000 Ci/mmol), 10 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM calcium acetate, pH 6.5. The reaction was terminated by addition of 12.5 µl solubilizing solution S, concentrated three times (see Materials and Methods). The labeled proteins were separated by electrophoresis on SDS-polyacrylamide [11% (wt/vol)] running gel of 1.3 mm thickness. 32 P-labeled rat B-50 (track 5) was used as a marker. An autoradiogram obtained from the frozen gel was used to localize the region of interest (M_r 45–67K) (not shown). This region was excised, analyzed by the SGIP method, and subjected to autoradiography. The protein staining pattern (A) of the original gel, the immunostaining pattern (B) by anti-rat B-50, and the autoradiogram (C) of the 50-µm thick gel sections are shown.

rat B-60 (IEP = 4.3, panel BII), the 49K CP (CP-60, Table 1) had incorporated less labeled phosphate than the 53K CP and rat B-50 (panel BI of Fig. 4).

Equivalent results were obtained with ASP purified by Po from bovine and rat brain (data not shown). It was notable that in endogenously phosphorylated ASP of Po the 53K CP incorporated 5–10-fold more radioactive phosphate than the 49K CP. This phosphorylation behavior of the 53K and 49K CP is quite comparable to that of rat B-50 and rat B-60.

Microheterogeneity of purified CP and rat B-50 was observed after isoelectrofocusing of the purified proteins using a narrow pH gradient of 3.5–5.0 in the first dimension and SDS-PAGE for the second

dimension (Zwiers et al., 1984). Both proteins were resolved in four adjacent spots (equal M_r) of different IEP (data not shown).

Effect of ACTH_{1–24} on phosphorylation. SPM was isolated from fresh bovine brain to study the possibility of inhibition by sequence 1–24 of adrenocorticotropin (ACTH_{1–24}) of the endogenous phosphorylation of CP, and to test for this "B-50" property. As in the case of rat SPM (Zwiers et al., 1980), a dose-dependent inhibition by ACTH_{1–24} of endogenous phosphorylation of bovine SPM proteins was observed. Inhibition affected the 53K band and several low and medium molecular weight protein bands. The extent of inhibition was quantified by densitometric scanning of the autoradiogram (not

FIG. 3. Protein profiles of fractions obtained during the purification of CP from bovine ASP by isoelectric focusing. ASP (57–82%) prepared according to Pn from bovine brain was separated by isoelectric focusing. Samples (0.5–2% of the fractions) were analyzed by SDS-PAGE. The protein staining profiles of the fractions are shown. Tracks: 1, 0.5 µg rat B-50; 2, 25 µg bovine ASP (57–82%); 3, molecular weight markers and fractions 12 and 13 contained purified CP (approximately 2 and 4 µg protein, respectively), while fraction 11 consisted of a bovine protein comparable to rat B-60 and a trace CP.

FIG. 4. Two-dimensional analysis of ³²P-labeled ASP (57–82%) fractions from bovine and rat brain. Bovine ASP and rat ASP prepared according to Pn and Po, respectively, were phosphorylated as described in Materials and Methods. The ³²P-labeled proteins were separated by slab gel isoelectric focusing followed by SDS-PAGE. Longitudinal sections of two-dimensional gels were analyzed by the SGIP method (**A**) and subjected to autoradiography (**B**). **IA** and **B**: IEP of CP and of a 49K protein separated from 50 μg bovine ASP (57–82%) of Pn. **IIA** and **B**: IEP of the proteins separated from a mixture of bovine (25 μg) and rat (6 μg) ASP.

shown) and amounted to 16% and 37% for 10^{−5} M and 10^{−4} M ACTH_{1–24}, respectively. Although it was shown that ACTH_{1–24} affects bovine brain protein phosphorylation, it was still uncertain whether CP was indeed one of the inhibited proteins of the bovine 53K band, since one-dimensional gel electrophoresis is often not sufficient to resolve proteins with small M_r differences. The answer to this question was obtained by two-dimensional analysis. The exact position of CP in the two-dimensional

TABLE 1. Comparison of apparent molecular weights of CP, CP-60, rat B-50, and the two major SAP fragments

Polypeptide	Apparent molecular weight (× 10 ^{−3})			
	11% PAGE	(n)	15% PAGE	(n)
Rat B-50	49.2 ± 0.2	(8)	42.3 ± 0.7	(4)
CP	53.4 ± 0.2	(8)	45.2 ± 0.5	(5)
CP-60	49.8 ± 0.2	(3)		
Rat fragment I			28.2 ± 0.4	(4)
Rat fragment II			15.7 ± 0.4	(4)
Bovine fragment I			30.2 ± 0.3	(5)
Bovine fragment II			16.9 ± 0.2	(5)

The results (mean ± SEM) were determined by SDS-PAGE as described in Materials and Methods. n, Number of determinations.

autographic map of bovine SPM was located with the aid of [³²P]CP of bovine ASP (Fig. 4, BI). The relevant part of the autographic map of bovine SPM phosphorylated in the presence and absence of ACTH_{1–24} is presented in Fig. 5A and B, respectively. In the absence of ACTH_{1–24}, at least two radioactive spots were found at the 53K position in the IEP range of 4–5 (Fig. 5B). In the presence of ACTH_{1–24} (Fig. 5A), the labeling of the more acidic protein, CP, was greatly decreased (see arrow, Fig. 5B). The inhibition was quantified by counting the excised labeled CP spots. The inhibition amounted to 30% and 60% for 10^{−5} M and 10^{−4} M ACTH_{1–24}, respectively. The increase was due to separation of the uninhibited SPM proteins of the 53K band and their removal.

One-dimensional peptide map. One-dimensional peptide maps were made to compare the primary structures of CP and rat B-50. Excised bands of phosphorylated CP and rat B-50 were subjected to limited proteolysis with *Staphylococcus aureus* V8 protease during electrophoresis in an SDS 15% (wt/vol) polyacrylamide running gel. The map of CP yielded a peptide pattern rather similar to that of rat B-50 (Fig. 6). Two major phosphopeptides, I and II, were produced. These fragments differed in electrophoretic mobility from those of rat B-50 in the same manner as the parent proteins (Fig. 6A and B). *In vitro* incubation of purified CP or rat B-50 with the protease for 30 min at 37°C, followed by analysis by SDS-PAGE, yielded equivalent results (data not shown). Table 1 presents a comparison of M_rs of CP, CP-60, rat B-50, and the major fragments.

Table 2 summarizes similarities of rat B-50 and CP.

Cross-reaction of antibodies to B-50 with brain proteins of various species

The SGIP method was applied to examine the proteins of brain membrane fractions and homogenates of various species for the presence of antigen determinants of rat B-50. Figure 7 shows that an-

FIG. 5. Two-dimensional analysis of bovine SPM phosphorylated in the presence and absence of ACTH_{1–24}. 140 μg bovine SPM protein was incubated at 30°C for 15 s with 25 μCi [³²P]ATP at a concentration of 7.5 μM. Fifteen seconds before zero time ACTH_{1–24} was added, while the control received only the solvent (buffer P). Phosphorylation of SPM and two-dimensional analysis were performed as described in Materials and Methods. Autoradiograms: (**A**) 10^{−4} M ACTH_{1–24}; (**B**) control. Arrow indicates position of CP.

FIG. 6. Comparison of peptide maps of bovine CP and rat B-50. Peptide maps were obtained as described in Materials and Methods. Gel pieces containing 1–2 μ g CP and rat B-50, both isolated from 32 P-labeled ASP fractions, were placed with various amounts of SAP in the slots of the stacking gel. Slots: 1, control; 2, 0.5 ng; 3, 5 ng; tracks with *, purified CP and rat B-50; track M_r , molecular weight marker proteins. Protein staining pattern (A) and autoradiogram (B) are shown. Two 32 P-labeled polypeptides, I and II, were produced from CP and rat B-50 (see also Table 1).

tibodies to rat B-50 immunostained a cross-reacting protein band in the molecular weight range of 47K to 53K (Table 3) in all vertebrate species tested, except the fishes and one amphibian. Similar results were obtained with affinity-purified antibodies and the anti-rat B-50 sera of two other rabbits.

DISCUSSION

Comparison of "bovine B-50" with rat B-50

Until recently, studies on the B-50 protein were limited to rat tissues (Zwiers et al., 1976, 1982b). We report here the use of antibodies against rat B-50 to screen for the presence of B-50-like proteins in brains of cow and several other species. We demonstrated the occurrence of a cross-reacting phosphoprotein (CP) in bovine brain membranes. Po and Pn developed for rat B-50, with minor modifications, turned out to be suitable to purify CP to homogeneity. The purified 53K protein cross-reacted with anti-rat B-50 antibodies purified from antisera of three rabbits (results similar to Fig. 1B, track 2). From this evidence, we conclude that CP is a B-50-like protein.

Protein phosphorylation in plasma membranes of bovine brain has been studied mainly to identify substrate proteins for the cyclic-AMP-dependent kinase (Dunkley et al., 1977; Lohmann et al., 1980). Recently, Martinez-Millan and Rodnight (1982) made a comparison of intrinsic protein phosphorylation in SPM preparations from cerebral cortex of several mammalian species, including rat and ox.

We found that CP is phosphorylated in bovine SPM and is inhibited dose-dependently by $ACTH_{1-24}$ in the same manner as rat B-50 (Zwiers et al., 1976).

CP is distinct from rat B-50 in IEP and M_r . This difference is also reflected in the sizes of its proteolytic fragments. As shown in Table 1, the M_r s of B-50 and CP were found to depend on the gel system (e.g., concentration of acrylamide). The migration rates of B-50 and CP in the 11% (wt/vol) polyacrylamide gel were slower than that of ovalbumin, whereas in the 15% (wt/vol) polyacrylamide gel the mobilities were close to that of ovalbumin (Fig. 6). Such anomalous migration behavior in SDS-PAGE has been reported for the γ_5 (possibly identical to B-50) of rat SPM by Gower and Rodnight (1982) and for polypeptides containing carbohydrate or membrane lipids (Maddy, 1976). Since the content of carbohydrate and lipid of B-50 was below the detection level of sensitive standard procedures (Zwiers et al., 1984), the reason for this behavior is unknown. However, this behavior appears to be another common feature.

During purification, CP was enriched about 100-fold in the ASP fraction (relative to the bovine brain homogenate). However, in this fraction, a 49K cross-reacting protein (CP-60) was also detected as

FIG. 7. Immunodetection of cross-reacting brain proteins from various species. 100 μ g protein of membrane fractions (P_2) and of 20% (wt/vol) homogenates of the brain of various species were analyzed by the SGIP method as described in Materials and Methods. The antiserum to rat B-50 of rabbit 8002 was used: dilution 1:20 for tracks 1–5 and 1:40 for tracks 6–11. P_2 fractions were used in tracks 1–5, 7, and 8; homogenates in track 6 and 9; and ASP (50 μ g of Pn) in tracks 10 and 11. Tracks: 1, mouse; 2 and 11, rat; 3, rabbit; 4 and 10, cow; 5 and 7, chicken; 6, *Xenopus laevis*; 8, Russian hamster; 9, human.

TABLE 2. Summary of similarities of CP and rat B-50

1. Immunoreactivity with antibodies to rat B-50.
2. Apparent molecular weight in similar range.
3. Purified by similar procedures.
4. Acidic isoelectric point.
5. Microheterogeneity observed after isoelectric focusing.
6. Phosphorylated endogenously in SPM: inhibition by $ACTH_{1-24}$.
7. Phosphorylated by rat kinase C.
8. Peptide map.
9. Possibly endogenous proteolysis to CP-60, a "B-60-like" protein.

TABLE 3. Immunostaining of B-50-like proteins in brain homogenates of various vertebrate species

Species	Immunostaining	Apparent molecular weight ^a
Human	+	51K
Rat	+	48K
Mouse	+	48K
Russian hamster	+	47K
Rabbit	+	52K
Cow	+	53K
Chicken	+	53K
<i>Xenopus laevis</i>	—	
Goldfish	—	
Trout	—	

The cross-reactivity with antibodies and antiserum to rat B-50 was qualitatively assessed by the SGIP method. +, Positive immunostaining. —, No immunostaining observed.

^aM_r of the immunostained bands was estimated relative to rat B-50 (M_r 48K) and CP (M_r 53K).

a minor component, with an IEP close to that of rat B-50. We have continued the comparisons of CP with rat B-50 and of CP-60 with rat B-60 because of the following arguments: (1) In bovine SPM the only cross-reacting protein is the 53K band, as in the case of rat SPM, in which only B-50 is observed (Kristjansson et al., 1982). (2) In the respective SPM, ACTH₁₋₂₄ inhibits endogenous phosphorylation of CP and B-50, whereas phosphorylation of CP-60 and B-60 has not been observed. (3) During endogenous phosphorylation of ASP (of Po), CP and rat B-50 incorporate much more phosphate than CP-60 and B-60. (4) CP and rat B-50 are major proteins in the ASP fraction, whereas CP-60 and B-60 are minor components. (5) CP-60 and B-60 are alike in having a more acidic IEP than CP and B-50, respectively. Zwiers et al. (1982a) demonstrated that, on incubation of rat ASP isolated by Po at 30°C, B-50 is slowly degraded to B-60 in the absence of calcium ions, whereas an increase in calcium ion concentration will lead to further proteolysis. Preliminary evidence suggests that CP-60 may be formed by proteolysis from CP.

The fact that CP is an "ACTH-sensitive" phosphoprotein with the properties here described (Table 2) leads us to conclude that CP can be classified as a B-50 protein. The availability of bovine brain as an alternative source of the "B-50 protein" creates new possibilities for study of the structure, phosphorylation sites, and biological function of B-50.

Cross-reactivity of anti-rat B-50 antibodies with brain proteins of various species

Many studies on other specific proteins from nervous tissue have demonstrated an interspecies conservation of amino acid sequences and antigenic determinants. Conservation is explained as a decisive determinant for protein function during phylogenetic evolution (Benson, 1982). Antibodies directed

against a particular brain protein of one species have been used to trace and characterize (Moore, 1972; Zomzely-Neurath and Keller, 1977; Jorgensen and Mikoshiba, 1978; Basset et al., 1982; Nieto-Sampedro et al., 1982), to isolate and purify (Bock, 1978), and to determine quantitatively (Goelz et al., 1981) homologous proteins in brain of other species.

The polyspecific antisera to rat B-50 and the affinity-purified antibodies (Oestreicher et al., 1983a) exhibited cross-reaction with a protein of variable molecular weight (Table 3) in brain membrane fractions of various mammals and chicken, but not in brain homogenates of amphibian and fish. This finding raises new questions. As yet, it is speculative to suppose that the B-50 protein is absent in brain tissue of fish and species developed earlier during evolution. Proteins with antigenic determinants different from B-50, but with similar biological properties, may well be present in the lower vertebrates. Such proteins will not be detected by the immunoassay used.

From our interspecies distribution study we conclude that a class of B-50 proteins exists in the brain of mammals and birds. The consistent differences between species in M_r of B-50 might indicate adaptation of this protein to functional requirements of the species.

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