Identification and partial purification of ABGP205, an integral membrane glycoprotein from brain that binds ankyrin

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1. A procedure was devised that allows the membrane-skeletal proteins brain spectrin and ankyrin to be extracted selectively from a membrane-skeletal preparation, together with some actin, an M_r -103 000 protein and a population of glycoproteins. 2. Ankyrin-binding activities of the glycoproteins were investigated by affinity chromatography. We detected only one, M_r 205 000, that binds ankyrin and is prevented from binding by the cytoplasmic domain of Band 3, the established erythrocyte-membrane-binding site for ankyrin. The M_r -205 000 glycoprotein, designated ABGP205, may be a candidate for a membrane-binding site for ankyrin.

INTRODUCTION

One of the major requirements of neuronal plasma membranes is that they should have a specific organization of their transmembrane proteins: thus ion channels and receptors must be restricted to points on the cell surface where their activities are to be expressed. How this is achieved is still unclear, but precedents from other cell types suggest that this may occur by association of the cytoskeleton with plasma-membrane proteins. It is therefore of great interest to identify those proteins involved in linking the cytoskeleton to the membrane. One linkage mechanism probably involves the proteins brain spectrin (fodrin) and ankyrin. Both these proteins are found in areas of the neurone where transmembrane proteins are subject to specific organization, such as the Node of Ranvier and the post-synaptic density (see Baines, 1984; Bennett, 1985). The cytoskeleton in both these areas of the cell is rich in filamentous actin (Koenig & Repasky, 1985; Carlin *et al.*, 1983): hence spectrin, which binds actin directly, may link actin to the membrane.

How is this linkage achieved? There is probably more than one linkage mechanism involved, since spectrin has both direct and indirect modes of attachment to the membrane. In areas of cell-cell contact it may bind directly to the neuronal cell-adhesion molecule NCAM 180 (Pollerberg *et al.*, 1986). An alternative is connection through ankyrin, a protein which itself may constitute a membrane binding site for microtubules (Davis & Bennett, 1984; Bennett *et al.*, 1985a). Ankyrin is a peripheral membrane protein, and has two isoforms in neurones apparently related in sequence and functionally identical (M_r 220 000 and 210 000) (Nelson & Lazarides, 1984; Bennett *et al.*, 1985a). The neuronal plasma-membrane binding sites for ankyrin are unidentified, but they seem to be proteinase-sensitive and integral to the membrane (Davis & Bennett, 1986). In cells and tissues requiring high rates of anion exchange, such as erythrocytes and kidney epithelia, the ankyrin-binding site is located in the cytoplasmic domain of an anion channel (Cox *et al.*, 1985; Drenckhahn & Bennett, 1987).

The erythrocyte anion channel, known as Band 3, exists in ankyrin-bound and unbound forms: the rotational and lateral diffusion coefficient for cytoskeleton-linked Band 3 are lower than that for unlinked Band 3 by a factor of 50 (see Bennett, 1985). Thus association with the spectrin/ankyrin cytoskeleton can restrict the mobility of integral membrane proteins. However, no protein closely related to Band 3 is expressed in brain. In the present paper we describe the identification of a brain protein which has the characteristics required of a membrane-binding site for ankyrin, namely it is an integral glycoprotein which binds to ankyrin *in vitro*. The interaction with ankyrin is specific for the membrane-binding domain of ankyrin, since it is blocked by the cytoplasmic fragment of erythrocyte Band 3. We designate the protein that we have identified 'ABGP205' (ankyrin-binding glycoprotein of M_r 205 000), and propose that it is a candidate for a membrane-binding site for brain ankyrin.

MATERIALS AND METHODS

Bovine brain was obtained at the point of slaughter from a local slaughter house. Cerebral cortices were processed for storage at -80°C as described by Bennett *et al.*, (1985b). For preparation of brain membranes, frozen brain (no more than 6 weeks in storage) was thawed rapidly by placing it in a large volume of 0.15 M-NaCl/10 mM-sodium phosphate, pH 7.4, at room temperature. Brains were homogenized with 4 vol. of ice-cold homogenization buffer [0.32 M-sucrose, 10 mM-Hepes/NaOH, pH 7.4, 2 mM-EDTA, 1 mM-dithiothreitol (DTT), 1 mM-phenylmethanesulphonyl fluoride, pepstatin (5 $\mu\text{g}/\text{ml}$), leupeptin (5 $\mu\text{g}/\text{ml}$)]. Demyelinated brain membranes (a synaptosomal/mitochondrial preparation) were prepared from the homogenate as described by Bennett *et al.* (1985b).

Preparation of membrane skeletons

Freshly prepared demyelinated brain membranes from 400 g of brain were washed once with 20 mM-Tris/1 mM-

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; ConA, concanavalin A; DTT, dithiothreitol.

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EDTA/1 mM-DTT/1 mM-phenylmethanesulphonyl fluoride, pH 8.0 (buffer A), and resuspended to 1.5 litres with buffer A containing 1% Triton X-100, leupeptin (5 µg/ml), pepstatin (5 µg/ml) and 0.3 M- or 0.6 M-KCl. (We have found that there is no overall difference in the protein pattern of resulting membrane skeletons whether they are prepared with 0.3 M- or 0.6 M-KCl: however, membrane skeletons prepared in 0.3 M-KCl are much less glutinous, so this is the method routinely used.) The resuspended membranes were left on ice for 30 min: the Triton-insoluble residues ('membrane skeletons') were collected by centrifugation at 30000 $g_{av.}$ for 30 min. The membrane skeletons were washed once in buffer A/1% Triton X-100/1 mM-DTT and once more in buffer A/0.1 mM-DTT. The insoluble residue after this treatment is referred to as 'washed membrane skeletons'.

Preparation of Tris extract

The washed membrane skeletons were resuspended with a minimal volume of buffer A to give a freely mobile suspension. An equal volume of 2 M-Tris/HCl (pH 8.0)/0.2% Triton X-100 was added and thoroughly mixed. The mixture was incubated at 35 °C for 20 min with occasional mixing. Insoluble residues were removed by centrifugation at 130000 $g_{av.}$ (40000 rev./min in a Beckman 45Ti rotor) for 40 min. The supernatant routinely contains 0.2–0.3 mg of protein/ml [measured by the Bradford (1976) method] and is referred to as the 'Tris extract'. Approx. 20–40 mg of total protein is obtained per 100 g of brain. The extract is somewhat turbid, and cannot be clarified, since the particles causing turbidity are buoyant in the Tris buffer. Nevertheless, all the membrane-skeletal proteins seem to be fully dissociated.

SDS/polyacrylamide-gel electrophoresis and blotting

SDS/PAGE was done as described by Laemmli (1970). Gels were stained with either Coomassie Blue R250 or the silver reagent of Morrissey (1981). Proteins were transferred to nitrocellulose paper (Schleicher & Schuell; 0.45 µm pore size) (Gershoni & Palade, 1983) for probing with lectins or antibodies. Wheat-germ agglutinin (Sigma) was radiolabelled with Na¹²⁵I and chloramine-T (Hunter & Greenwood, 1962). Blots were probed with ¹²⁵I-wheat-germ agglutinin by the method of Bartles & Hubbard (1984). Blots were probed with concanavalin A (ConA) and horseradish peroxidase by modifications of the method of Faye & Crispeels (1985). Immediately after transfer, blots were fixed and stained for protein with 20% (v/v) propan-2-ol/5% (v/v) acetic acid/0.2% Ponceau S. Background stain was removed by rinsing several times with distilled deionized water. Ponceau stain was removed from the protein bands by washing with 0.5 M-NaCl/40 mM-Tris/HCl, pH 7.4 (TBS), containing 0.3% Tween 20. This solution also served to block protein-binding sites on the nitrocellulose. After 30 min in this solution, blots were transferred to fresh TBS containing 0.05% Tween 20, 1 mM-MnCl₂, 1 mM-CaCl₂ and 100 µg of ConA (Sigma)/ml for 1 h. Unbound ConA was removed by washing five times (2 min each) in TBS/0.05% Tween 20. The blots were incubated with horseradish peroxidase (Sigma, Type II) in TBS/0.05% Tween 20/1 mM-MnCl₂/1 mM-CaCl₂ for 1 h, and unbound peroxidase was removed as above. Colour was developed with 4-chloro-1-naphthol (Faye & Crispeels, 1985).

Preparation of erythrocyte proteins and antibodies

Erythrocyte ankyrin and the M_r -40000 N-terminal cytoplasmic domain of Band 3 (containing the ankyrin-binding site) were prepared by the method of Bennett (1983). Affinity columns were prepared by coupling the proteins (1 mg of protein/ml of packed gel) to CNBr-activated Sepharose CL4B. Antibodies were raised against native ankyrin, native brain spectrin or SDS-denatured Band 3 fragment in New Zealand White rabbits. Anti-Band 3 antisera were affinity-purified on a column of Band 3-fragment-Sepharose. A monoclonal antibody against the C-terminal cytoplasmic domain of Band 3 (Bric 130) was generously given by Dr. M. J. Tanner (University of Bristol).

RESULTS

Preparation of Tris extract of brain membrane skeletons

In the process of investigating conditions that would extract membrane-skeletal components from brain, it became apparent that 1 M-Tris/0.1% Triton X-100, pH 8.0, effected an extremely selective solubilization. Tris at high concentration has been used in other systems, e.g. coated vesicles (Zaremba & Keen, 1983) and erythrocyte membrane skeletons (Ohanian & Gratzer, 1984) to render soluble membrane-associated components under conditions that do not irreversibly denature proteins.

The protein components of the Tris extract are shown in Figs. 1 and 2. SDS/PAGE revealed the major components to be brain spectrin and actin. An unidentified protein of M_r 103000 and variable amounts of myelin basic protein (M_r 20000) are other major components (the presence of myelin basic protein reflects residual myelin in the membrane preparation) (Fig. 2). Brain ankyrin and a population of glycoproteins are also present.

The protein composition of this preparation is strikingly simple, and may allow better definition of the brain membrane skeleton. Spectrin, ankyrin and actin are extracted in the 1 M-Tris buffer from both brain and red cells (cf. Ohanian & Gratzer, 1984). Co-extracted with these proteins are Band 4.1 protein (from red cells) and the M_r -103000 protein (from brain). However, Band 4.1 and the M_r -103000 protein do not appear to be closely related (N. Hayes & A. J. Baines, unpublished work), and the nature of the M_r -103000 protein is not yet clear.

The glycoproteins in the extract are recognized by ConA, but not wheat-germ agglutinin (Fig. 1), suggesting they do not correspond to the neuronal cell-adhesion class of molecules (NCAM; Edelman, 1986). They stain very poorly with Coomassie Blue after SDS/PAGE. The three major ConA-binding proteins show a similar pattern of relative mobilities to the synaptic-junction glycoproteins described by Mena & Cotman (1982). No additional sugar-bearing proteins are detected by the sugar-specific stain of Gershoni *et al.* (1985). A minor ConA-binding protein (M_r 110000) has a Ca²⁺-dependent interaction with calmodulin, as judged by affinity chromatography (results not shown). The presence of detergent seems to be essential for stable solubilization of the ConA-binding proteins: the effectiveness of the detergents tested in solubilizing the ConA-binding proteins appeared similar in all cases, although Triton X-

Fig. 1. Protein composition of brain membranes, membrane skeletons and Tris extract

Protein samples were analysed on replicate SDS/polyacrylamide gels (5–15% linear gradient). Lanes: 1, demyelinated brain membranes; 2, membrane skeletons; 3, Tris extract. In all three lanes, protein loadings were normalized for brain spectrin (prominent bands at M_r 240 000). Stains: (a) Coomassie Brilliant Blue R250; (b) ^{125}I -wheat-germ-agglutinin; (c) ConA-peroxidase. Actin and myelin basic protein are the major bands at M_r 42 000 and 20 000 respectively.

100 and Nonidet P40 extracted a broader range of glycoproteins than did Brij, Lubrol PX or Tween 20. Optimal solubilization was achieved at pH 8.0.

All the glycoproteins detected in the Tris extract are operationally defined as integral membrane proteins, since they are retained in whole membranes after extraction with dissociating aqueous solutions (1 M-KI, 0.1 M-NaOH or 0.2 M- Na_2CO_3 , pH 11.0).

Detection of an ankyrin-binding glycoprotein

Since no convenient assay exists for the detection of an ankyrin-binding glycoprotein in crude extracts containing endogenous ankyrin, it was necessary to purify the glycoproteins partially in order to detect an ankyrin-binding activity.

Initially we attempted this by lectin affinity chromatography using ConA-agarose. The extract was diluted to lower the Tris concentration to 0.5 M, and MgCl_2 was added to 1 mM. Under these conditions, all the ConA-binding proteins are adsorbed, but the conditions are not sufficiently dissociating to prevent aggregation of the extract on the column. ConA-binding proteins could not be recovered from the column.

Gel-filtration chromatography can be conveniently performed on samples in high concentrations of Tris (Zaremba & Keen, 1983; Ohanian & Gratzner, 1984).

Chromatography on Sepharose CL4B [2.5 cm $\times$ 90 cm column, equilibrated in 1 M-Tris/HCl (pH 8.0)/0.1% Triton X-100/0.02% NaN_3] resolved the ConA-binding proteins from ankyrin, spectrin (eluted in fractions 16–22 in Fig. 3) and actin (fractions 30–36). The partially purified glycoproteins can be analysed by affinity chromatography on a column of ankyrin-Sepharose. In all affinity-chromatography experiments, we have used erythrocyte ankyrin. Despite numerous attempts we have been unable to obtain sufficient brain ankyrin for preparation of an affinity column. However, erythrocyte ankyrin is a good model for brain ankyrin, since (a) brain and erythrocyte ankyrins compete for the same site(s) on both brain and erythrocyte membranes in rebinding studies, and (b) the erythrocyte-membrane binding site for ankyrin will displace both brain and erythrocyte ankyrin from brain membranes (Bennett *et al.*, 1985a; Davis & Bennett, 1986).

Before affinity chromatography, the pooled peak of ConA-binding proteins from the gel-filtration column was dialysed against buffer B (50 mM-NaCl, 10 mM-sodium phosphate, 1 mM-EGTA, 0.2 mM-DTT, 0.1% Triton X-100, 1 mM- NaN_3 , pH 7.4), which allows ankyrin to rebind to brain membranes. Dialysed extract (50 ml) was run on to a 4 ml column of ankyrin-Sepharose (equilibrated in buffer B) at 10 ml/h, and residual un-

Fig. 2. Brain spectrin and ankyrin are components of Tris extract

Replicate SDS/5–15%-polyacrylamide gels of the Tris extract were stained with Coomassie Blue (CB) or transferred to nitrocellulose and stained with anti-(brain spectrin) serum (aSp) or anti-(erythrocyte ankyrin) serum (aA). The cross-reactive bands co-migrate on the gels with authentic purified standards. Control blots showed no reaction.

bound material was removed by washing with 10 column vol. of buffer B. Material bound to the column was recovered by elution with a dissociating buffer (0.8 M-NaBr, 50 mM-NaCl, 10 mM-sodium phosphate, 1 mM-EGTA, 0.1% Triton X-100, 0.2 mM-DTT, pH 7.4). Bound proteins were eluted slowly, in approx. 3 column volumes, and were too dilute for convenient analysis. They were concentrated 20-fold by dialysis against poly(ethylene glycol) flakes and analysed by SDS/PAGE. Fig. 4 shows the results of such an experiment. Several polypeptides were retained by the ankyrin column, but only two were recognized by ConA (M_r 220 000 and 205 000).

Fig. 3. Separation of ConA-binding proteins from spectrin and ankyrin by gel filtration

Tris extract (30 ml) was chromatographed on a 2.5 cm $\times$ 90 cm column of Sepharose CL4B. The column was equilibrated with 1 M-Tris/HCl (pH 8.0)/0.1% Triton X-100/1 mM- NaN_3 , and developed at 30 ml/h; 13 ml fractions were collected, of which 100 μ l samples were analysed by SDS/PAGE. Stains: (a) Coomassie Blue; (b) ConA-peroxidase. Spectrin and ankyrin are eluted principally in fractions 16–22. ConA-binding proteins eluted in fractions 24–30 were pooled.

Since it is known that the M_r -40 000 cytoplasmic (ankyrin-binding) domain of erythrocyte Band-3 protein will compete with brain membranes for rebinding of brain ankyrin (Bennett *et al.*, 1985a) exogenous Band-3 fragment was used in some experiments to block the membrane-binding site of ankyrin. For this, 4 μ M-Band-3 fragment was added to the pooled glycoproteins before the ankyrin column. Of the two ankyrin- and ConA-binding glycoproteins, only one, of M_r 205 000 was displaced (Fig. 5). The M_r -205 000 species therefore specifically recognizes the membrane-binding domain of ankyrin. We designate the M_r -205 000 species ABGP205 (ankyrin-binding glycoprotein, M_r 205 000). ABGP205 is a candidate for a membrane-binding site for ankyrin.

To explore the specificity of ABGP205-ankyrin interaction further, glycoproteins from the gel-filtration column were chromatographed on other affinity columns. ABGP205 did not bind to the other columns tested (brain spectrin-Sepharose, ovalbumin-Sepharose or calmodulin-Sepharose), whereas the M_r -220 000 species bound to all the columns tested (Fig. 4). Although we cannot rule out the possibility of the M_r -220 000 glycoprotein having high-affinity, saturable, interactions with proteins bound to these affinity columns, since it

Fig. 4. Affinity chromatography on erythrocyte ankyrin-agarose of pooled fractions from the gel-filtration column

Lanes 1 and 2 show SDS/PAGE (9% acrylamide) of proteins in the dialysed pool from the gel-filtration column: 1, silver stain; 2, ConA. The pool was chromatographed on an ankyrin affinity column, and bound proteins (lanes 3 and 4; also SDS/9% PAGE), were recovered with a dissociating buffer (see the text): 3, silver stain; 4, ConA. Two of the ConA-binding proteins were retained on the column, M_r 220 000 and M_r 205 000. The M_r -205 000 band represents ABGP205.

does not interact appreciably with unconjugated Sepharose, it seems unlikely that the M_r -220 000 species constitutes a membrane-binding site for ankyrin.

Cross-reactivity of ABGP205 with Band 3

Since ABGP205 and Band 3 bind at or close to the same site on ankyrin, it was important to investigate antigenic cross-reactivity between ABGP205 and Band 3. Using immunoblotting and/or immunoprecipitation with affinity-purified anti-Band 3 antibodies or Bric130 monoclonal antibody, we have been unable to detect any cross-reactivity with ABGP205 in whole membranes, cytoskeletons, Tris extract, gel-filtered glycoprotein fractions or affinity-column eluates (results not shown).

DISCUSSION

In this paper we describe the identification and partial purification of an integral glycoprotein which binds ankyrin. ABGP205 is the first glycoprotein from brain to be identified on the basis that it binds ankyrin. It is our conclusion, based on the activity of ABGP205 and its selective co-extraction with ankyrin and other membrane-

Fig. 5. Affinity chromatography of pooled fractions from the gel-filtration column on erythrocyte ankyrin in the presence of the cytoplasmic fragment of erythrocyte band 3 and on ovalbumin-Sepharose

Lane 1: ConA-binding proteins retained by the ankyrin column. Lane 2: as lane 1, but the column was run in the presence of 4 μ M-Band-3 cytoplasmic fragment. Lane 3: ConA-binding proteins retained by ovalbumin-Sepharose.

skeletal proteins, that it may constitute a membrane-binding site for ankyrin. However, until we are able to show co-localization of ankyrin and ABGP205 in whole brain tissue, this must remain conjecture. Moreover, the heterogeneity of the preparations used in our affinity-chromatography experiments does not allow us to rule out the possibility of an indirect interaction between ankyrin and ABGP205 mediated by another protein. However, since ankyrin will rebind *in vitro* to integral sites on brain membranes, we consider this unlikely. We are therefore concentrating on developing a method for obtaining ABGP205 in sufficient quantities for the preparation of antibodies. The methods described in this paper yield only microgram quantities. The small amounts that we have been able to detect suggest that there may be other membrane-binding sites for ankyrin

that are not detected in our procedure. In this context, it is to be noted that our procedure for preparing membrane skeletons involves rigorous washing under conditions that will only allow preservation of high-affinity interactions between ankyrin and membrane proteins. Lower-affinity interactions are likely to be missed by our method. We have been unable to obtain an estimate of the stoichiometry of ankyrin to ABGP205 in brain membranes. The SDS/PAGE pattern of brain membranes is too complex to allow densitometry: estimates of stoichiometry must await the development of an immunological assay.

The relationship between ABGP205 and the known erythrocyte ankyrin-binding protein band 3 cannot be close, since we have been unable to detect any immunological cross-reactivity. Nevertheless, they must bind at or near the same site on ankyrin, since added Band 3 can prevent binding of ABGP205.

This fact also raises the question of whether ankyrin can be considered a likely membrane-binding site for microtubules: tubulin must bind close to this site, since its binding to ankyrin is also blocked by Band 3 (Davis & Bennett, 1984). It seems unlikely that ankyrin can bind to the plasma membrane and microtubules at the same time.

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