Immunochemical Characterization of Proteins from Mouse Liver Plasma Membranes

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1. Antiserum was prepared in rabbits against a purified mouse liver plasma-membrane fraction. 2. The antiserum was made to react with an ^{125}I -labelled alkaline-EDTA extract of the plasma membranes, and the immunoprecipitate analysed by polyacrylamide-gel electrophoresis. Seven proteins were immunoprecipitated and a single glycoprotein present in the alkaline-EDTA-soluble fraction was found to be a major component. 3. The alkaline-EDTA-soluble fraction was analysed by two-dimensional immunoelectrophoresis and this procedure indicated the presence of six antigenic components. 4. The plasma membranes were also extracted with 1% deoxycholate-1% Triton X-100; 50% of the protein, 80% of the alkaline phosphodiesterase activity and 30% of the 5'-nucleotidase activity were solubilized. 5. Two-dimensional immunoelectrophoresis of the deoxycholate-Triton X-100 extract indicated the presence of six antigens. 6. The relative distribution of the six antigens among the fractions obtained during the extraction procedure was examined immunoelectrophoretically to provide information on their disposition within the membrane.

Although progress has been made in studying the composition and in separating the proteins and glycoproteins of the liver surface-membrane fraction, relatively little is known about the arrangement of these components in the membrane. Information about the organization of the surface of cells has been obtained by using antibodies; for example the fluid properties of membranes, and the question of the distribution of surface antigens, have been approached by using antibodies (Taylor *et al.*, 1971; Boyse *et al.*, 1968). In the present study, antiserum raised against a mouse liver plasma membrane was used to study the distribution of antigenic specificities in various chemically characterized fractions derived from the liver plasma membrane. It was shown (Gurd *et al.*, 1972*b*) that the antigenic determinants of a mouse liver plasma-membrane fraction were differentially solubilized by sequential extraction with alkaline EDTA and aqueous pyridine solutions, and that additional antigenic sites became exposed on the membranes after the removal by these reagents of certain categories of membrane proteins and glycoproteins. The present work studies more quantitatively the distribution of antigenic determinants present in liver surface membranes by using polyacrylamide-gel electrophoresis to examine labelled immunoprecipitates and two-dimensional immuno-

electrophoresis. Six or seven major antigens were found to be distributed among the various fractions prepared. One of these was shown to correspond to a major liver membrane glycoprotein, which was extracted from the plasma-membrane fraction by an alkaline-EDTA mixture.

Experimental

Preparation of plasma-membrane extracts

Plasma membranes were prepared from the livers of 75 Parkes' white mice (25g) by rate zonal centrifugation followed by discontinuous sucrose-gradient centrifugation (Evans, 1970; Gurd *et al.*, 1972*a*). The purified plasma-membrane fraction was washed by centrifugation with 0.15M-NaCl-5mM-Tris-HCl, pH 7.6, and sequentially extracted with 50mM-NaHCO₃-Na₂CO₃ buffer, pH 10.2, containing 10mM-EDTA and aq. 33% (v/v) pyridine (Gurd *et al.*, 1972*a*). Membrane fractions were stored at -20°C, except for the pyridine-soluble fraction, which was stored at 4°C.

Extraction of washed plasma membranes with detergents was performed by homogenizing the membranes in an equal volume of 2% (w/v) deoxycholate-2% (v/v) Triton X-100-10mM-Tris-HCl, pH 8.9 (final concentration of membrane 5-6mg/ml), and immediately centrifuging the suspension at 108000g for 30min. The supernatant was used for immunoelectrophoretic studies. Fresh deoxycholate-Triton X-100 extracts were used for each experiment.

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Iodination of alkaline-EDTA-soluble proteins

Iodination was performed by the ICl procedure as described by McFarlane (1958). A portion of the alkaline-EDTA-soluble fraction to be iodinated was rapidly added to a solution of ^{125}I containing sufficient ICl to yield 1 atom of I/molecule assuming an average molecular weight of 70000 and 50% incorporation. Free I_2 was removed by exhaustive dialysis against 10mM-EDTA–30mM-NaI–10mM-Tris–HCl, pH8.0, and finally against 10mM-Tris–HCl, pH8.0. After dialysis more than 98% of the ^{125}I could be precipitated with 5% (w/v) trichloroacetic acid. The specific radioactivity of the iodinated fraction was 25×10^6 d.p.m./mg.

Production of antiserum, immunoprecipitation and absorption of antiserum

Rabbit anti-(plasma-membrane) serum was produced as previously described (Gurd *et al.*, 1972b). For immunoprecipitation, portions of the ^{125}I -labelled alkaline-EDTA-soluble fraction were incubated, with sufficient antiserum to give maximum precipitation of protein and radioactivity (determined in preliminary experiments), for 1h at 37°C and then overnight at 4°C, and then centrifuged at 2500g for 40min. The precipitate was washed three times with 0.15M-NaCl–5mM-Tris–HCl, pH7.6, before counting of radioactivity or gel electrophoresis. In the presence of detergent the fraction to be precipitated was incubated with 1% Triton X-100 for 15min before addition of antiserum. When a preliminary precipitation with haemocyanin–anti-haemocyanin was performed, haemocyanin was added to the alkaline-EDTA-soluble fraction, followed by an excess of anti-haemocyanin, and precipitation was allowed to proceed overnight at 4°C. After centrifugation (2500g, 40min) portions of the supernatant were made to react with anti-(plasma-membrane) serum as described above.

Pre-absorption of antiserum was performed by incubating 0.2ml of antiserum for 1h at 25°C in 0.15M-NaCl–5mM-Tris–HCl, pH7.6, with the indicated amounts of membrane protein in a total volume of 0.5ml. The mixture was then centrifuged at 2500g for 40min and the supernatant used for analysis. The pyridine-soluble proteins were freeze-dried and dissolved in 5mM-Tris–HCl, pH8.0, for pre-absorption experiments.

Gel electrophoresis and two-dimensional immunoelectrophoresis

Membrane samples used for polyacrylamide-gel electrophoresis were heated at 90°C for 3–5min in 4M-urea–1% sodium dodecyl sulphate–1% 2-mercaptoethanol solution and then dialysed overnight against 0.1% sodium dodecyl sulphate–10mM-

mercaptoethanol–30mM-sodium phosphate buffer, pH7.2. Washed immunoprecipitates from the ^{125}I -labelled alkaline-EDTA-soluble fraction were dissolved in 4M-urea–1% sodium dodecyl sulphate–1% mercaptoethanol by heating at 90°C for 3–5min and 125mg of unlabelled alkaline-EDTA-soluble proteins was added to aid in the detection of protein bands. Polyacrylamide gels [7.5% (w/v) polyacrylamide, 10cm $\times$ 8mm] were prepared as described by Weber & Osborne (1969) and samples were run at 4mA/tube for 16h. After electrophoresis, gels were stained either for protein with Amido Black or for carbohydrate by the periodate–Schiff procedure (Zacharias *et al.*, 1969). For radioautography gels were sliced longitudinally into four sections: the two centre flat slices were removed and stained for protein or carbohydrate. The stained gel slices were dried under vacuum on cellophan sheets and mounted for radioautography on Ilford Ilfex X-ray film. Radioautograms were developed for 24h. Densitometric traces of the radioautograms and stained gels were made by using a Joyce–Loebl Chromoscan.

Two-dimensional immunoelectrophoresis of the alkaline-EDTA-soluble fraction (35–40 μg) and of the deoxycholate–Triton X-100 membrane extract (40–50 μg) was performed by the method of Laurell (1965). The agarose used for electrophoresis in the second dimension contained 0.2ml of whole or pre-absorbed antiserum. After electrophoresis, the plates were dried, washed thoroughly with 0.15M-NaCl–5mM-Tris–HCl, pH7.6, and stained for protein with Amido Black.

Other methods

Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard. 5'-Nucleotidase activity was measured spectrophotometrically in the presence of excess of adenosine deaminase (Ipata, 1967), and alkaline phosphodiesterase I activity was measured spectrophotometrically as described by Razzel (1963). ^{125}I radioactivity was counted in a Panax Nuclear sodium iodide crystal γ -radiation counter. The efficiency was 40%.

Materials

All chemicals and reagents were of analytical grade and were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., or from British Drug Houses, Poole, Dorset, U.K. Agarose was purchased from Paines and Byrne Ltd., Greenford, Middx., U.K. Glass-distilled water was used throughout. Reoviral proteins were obtained from Dr. J. J. Skehel of this Institute. Haemocyanin and rabbit anti-haemocyanin serum were a gift of Dr. R. M. E. Parkhouse of this Institute.

Results

Isolated mouse liver plasma membranes were injected into rabbits and antiserum demonstrable by Ouchterlony double diffusion and by precipitation reaction was obtained. The antiserum was specific for the surface-membrane fraction, and showed a low degree of cross-reactivity with subcellular fractions containing intracellular membranes or organelles (Gurd *et al.*, 1972b). This antiserum, which reacted with each of the fractions obtained by sequential extraction of the surface membranes with alkaline EDTA and aqueous pyridine, was used in the following experiments.

Fig. 1. Polyacrylamide-gel electrophoresis densitometer tracings of ^{125}I -labelled alkaline-EDTA-soluble mouse liver plasma-membrane proteins

Tracings are of gels of the alkaline-EDTA-soluble fraction stained for protein (a) or of radioautograms of the ^{125}I -labelled alkaline-EDTA-soluble fraction (b), the immunoprecipitate of the alkaline-EDTA-soluble fraction (c) and the immunoprecipitate of the alkaline-EDTA-soluble fraction obtained in the presence of 1% Triton X-100 after a preliminary precipitation with haemocyanin-anti-haemocyanin (d). The hatched areas represent the position of glycoproteins stained by the periodate-Schiff procedure. The direction of electrophoresis was from left to right.

Analysis of immunoprecipitates of plasma-membrane fractions by polyacrylamide-gel electrophoresis

Extraction of salt-washed mouse liver plasma membranes with an alkaline-EDTA solution, pH 9.7, removed approx. 20% by weight of the membrane proteins (Gurd *et al.*, 1972a). Antigenic components present in the extract accounted for 60–70% of the antigenic activity of the intact membrane (Gurd *et al.*, 1972b). To identify the solubilized antigens, the alkaline-EDTA-soluble proteins were iodinated and the immunoprecipitate obtained with anti-(plasma-membrane) serum was analysed by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. The distribution of ^{125}I in the gels is shown in Fig. 1. Comparison of the protein stain with the radioautogram indicated that most of the proteins were labelled. Staining the gel by the periodate-Schiff procedure indicated the presence in the alkaline-EDTA extract of only one glycoprotein. When the iodinated alkaline-EDTA-soluble proteins were made to react with sufficient antiserum to give maximum precipitation of radioactivity, seven components were precipitated and the relative concentration of the alkaline-EDTA-extracted glycoprotein (approx. mol. wt. 120000) was increased in the precipitate (Fig. 1c). To examine the possibility of contamination of the immunoprecipitate by non-specifically precipitated proteins owing to the presence of protein aggregates in the extracted fraction, an initial precipitation of the alkaline-EDTA plasma-membrane extract with haemocyanin-anti-haemocyanin was performed and the supernatant was then treated with an excess of anti-(plasma-membrane) serum in the presence of 1% Triton X-100. This concentration of Triton X-100 has been shown not to interfere with the immunoprecipitation reaction (Crumpton & Parkhouse, 1972) but would be expected to diminish the effects of protein aggregation (Steck & Fox, 1972). Although this procedure resulted in a 50–60% decrease in the total amount of radioactivity precipitated, the pattern of labelled proteins was qualitatively unchanged. Analysis of the precipitate showed a further increase in the relative concentration of the alkaline-EDTA-soluble glycoprotein (Fig. 1d) confirming that this liver plasma-membrane glycoprotein represents one of the major membrane antigens extracted by alkaline EDTA.

Analysis of membrane antigens by immunoelectrophoresis

Further characterization of the antigenic determinants present in the plasma membrane, and in membrane fractions obtained by extraction with alkaline EDTA and aqueous pyridine, was obtained by two-dimensional immunoelectrophoresis.

In this technique the height of the precipitation line is inversely proportional to the concentration of antibody present in the agarose used for electrophoresis in the second dimension (Clarke & Freeman, 1966). In the present experiments the concentration of antibody was in turn determined by the activity of the corresponding antigen in the membrane fractions used for pre-absorption of the antiserum.

Analysis of the alkaline-EDTA-soluble fraction by immunoelectrophoresis against whole antiserum showed the presence of four precipitation lines (Plates 1*a* and 1*a'*). A further two precipitation lines were observed when the direction of electrophoresis was towards the cathode, and although these were not studied further, it is relevant that both immunoelectrophoresis and gel electrophoresis indicated the presence of a similar number of antigens in the alkaline-EDTA extract. These results, in conjunction with previous studies that showed that pre-absorption of ^{125}I -labelled anti-(plasma-membrane) serum with the EDTA-soluble fraction decreased binding to liver plasma membranes by 60–70% (Gurd *et al.*, 1972*b*), now indicate that the six antigens extracted by alkaline-EDTA account for approx. two-thirds of the antigenic activity of intact mouse liver plasma membranes.

Pre-absorption of the antiserum with 400 μg of either the whole membrane or the alkaline-EDTA-insoluble residue resulted in the complete removal of the antibody responsible for precipitation line 1 but had only a slight effect on any of the other antibodies (Plates 1*b* and 1*c*). Increasing the amount of membrane used for pre-absorption to 800 μg resulted in a total or possibly a partial removal of all four precipitation lines (Plates 1*b'* and 1*c'*) as indicated by the loss or increased height of the precipitation lines, thus suggesting incomplete extraction of these antigens by alkaline EDTA. In addition these results show that the previously described increase in total antigenic activity after extraction of plasma membranes with alkaline EDTA can be partially attributed to increases in the activity of the alkaline-EDTA-soluble antigens 3 and 4. A partial segregation of antigenic components after pyridine extraction was demonstrated when the effects of pre-absorption of the antiserum with either the pyridine-insoluble or -soluble fraction on the precipitation pattern were compared (Plates 1*d* and 1*e*). Antigen 1 appeared to be fully active in the pyridine-insoluble residue but was considerably decreased in activity in the pyridine extract. Conversely, the activity of antigen 3 was increased in the pyridine extract relative to the intact membrane, whereas it was virtually absent from the pyridine-insoluble residue. Antigens 2 and 4 were inactive in both the pyridine-soluble and -insoluble fractions, accounting in part for the previously observed loss of total antigenicity after pyridine extraction (Gurd *et al.*, 1972*b*).

Extraction of membrane antigens by detergents

Extraction of the plasma membranes with 1% deoxycholate–1% Triton X-100 solubilized 50% of the membrane protein. Although polyacrylamide-gel electrophoresis showed that the protein composition of the detergent extract was generally similar to that of the whole membrane (Fig. 2), some selectivity in the extraction of enzymes was indicated by the different distribution of 5'-nucleotidase and an alkaline phosphodiesterase between the extract and the insoluble residue (Table 1). The addition of detergent to the membrane stimulated phosphodiesterase activity and 80% was recovered in the

Fig. 2. Polyacrylamide-gel electrophoresis densitometer tracings of mouse liver plasma-membrane proteins

Tracings are of gels of plasma membranes (a) or of a 1% deoxycholate–1% Triton X-100 extract of these membranes (b) stained for protein. The hatched areas represent glycoproteins stained by the periodate–Schiff procedure. The molecular-weight markers are: I, immunoglobulin G (150000); B, bovine serum albumin (69000); L, lactate dehydrogenase (33000); and reoviral proteins λ_1 (155000); μ_2 (72000); σ_1 (42000) and σ_3 (34000). The direction of electrophoresis was from left to right.

EXPLANATION OF PLATE I

Two-dimensional immunoelectrophoresis of alkaline-EDTA-soluble liver plasma-membrane proteins

The alkaline-EDTA-soluble fraction was analysed against whole antiserum (*a* and *a'*) or against antiserum which had been pre-absorbed with 400 μ g (*b*–*e*) or 800 μ g (*b'*–*e'*) of total plasma membranes (*b*), or alkaline-EDTA-insoluble membranes (*c*), or the pyridine-insoluble fraction (*d*) or the pyridine extract (*e*).

EXPLANATION OF PLATE 2

Two-dimensional immunoelectrophoresis of a 1% deoxycholate-1% Triton X-100 extract of mouse liver plasma membranes

Membranes were extracted with 1% deoxycholate-1% Triton X-100-5 mM-Tris-HCl, pH 8.9, and a portion of the extract was analysed against whole antiserum (a) or against antiserum which has been pre-absorbed with 400 μ g of whole plasma membranes (b), or alkaline-EDTA-insoluble membranes (c), or the pyridine-insoluble fraction (d) or the pyridine extract (e).

Table 1. *Extraction of protein, 5'-nucleotidase and phosphodiesterase activities from mouse liver plasma membranes with 1% deoxycholate-1% Triton-5 mM-Tris-HCl, pH 8.9*

The extractions were carried out at room temperature as described in the Experimental section.

Fraction	Protein (mg)	5'-Nucleotidase activity (%)	Phosphodiesterase activity (%)
Plasma membranes	24.8	100	100
Plasma membranes dispersed in 1% deoxycholate-1% Triton X-100	24.8	88	169
1% deoxycholate-1% Triton X-100 extract	12.0	27	137

soluble fraction; only 31% of the 5'-nucleotidase activity was recovered in the extract. Immunoelectrophoretic analysis of the detergent extract indicated the presence of six precipitation lines (Plate 2a). Pre-absorption of the detergent extract with the plasma membranes (Plate 2b) appeared to remove precipitation lines 1 and 2 completely, and 4 partially, thus indicating that these three antigens were active on the unperturbed plasma membrane. Precipitation lines 1, 2 and 4 were totally or partially removed by pre-absorption of the detergent extract with the alkaline-EDTA extract (Plate 2c), and also the increased heights of lines 3, 5 and 6 indicated that after the extraction these antigens became more active on the membranes. Further, of the six antigens extracted by the detergent mixture, precipitation line 5 persisted after pre-absorption with pyridine-insoluble membranes (Plate 2d) but appeared absent after pre-absorption with the pyridine-soluble extract (Plate 2e), thus indicating that this antigen was largely removed from the membranes by the aqueous pyridine treatment.

Discussion

The present experiments study the distribution of antigenic components in liver plasma-membrane fractions and provide indirect information about the spatial distribution of these components within the membrane. With antisera raised against a purified liver plasma-membrane fraction, two quantitative techniques were used to estimate the distribution of antigens among the fractions, i.e. polyacrylamide-gel electrophoresis of iodine-labelled immunoprecipitates and two-dimensional immunoelectrophoresis of membrane extracts. The premise underlying the experiments is that a membrane antigenic determinant must be exposed on the surface before it can react with its corresponding antibody. It was shown that the mouse liver plasma-membrane fraction contained about seven major antigenic components by two complementary techniques. Immunoprecipitation and subsequent polyacrylamide-gel electrophoresis of the alkaline-EDTA extract of the

membranes, which although containing only 20% by weight of the membrane protein, accounted for most of the antigenic activity of the intact membrane (Gurd *et al.*, 1972b), indicated that one of the antigens was a major glycoprotein previously identified in this fraction (Gurd *et al.*, 1972a). This glycoprotein (apparent molecular weight 120000) has also been shown to be exposed on the surface of the liver plasma membrane by labelling with iodine (W. H. Evans, unpublished work). Although present results do not permit the allocation of the immunoelectrophoretic bands to specific membrane components or enzymes, two of the six bands extracted from membranes by Triton X-100-deoxycholate probably correspond to the membrane enzymes 5'-nucleotidase and alkaline phosphodiesterase, which have been shown to interact with the antisera used in the present experiments when positioned on the membrane, or when highly purified in soluble form in detergent-containing media (Evans *et al.*, 1973; J. W. Gurd & W. H. Evans, unpublished work).

The number of antigenic determinants obtained in the present experiments falls short of the 18 identified by Blomberg & Perlmann (1971a,b) on rat liver plasma membranes. The plasma membranes used by Blomberg & Perlmann (1971a,b) were not washed with iso-osmotic saline and therefore the possibility exists that antibodies to soluble hepatic components adsorbed to the membranes were also studied by them. The antigenic determinants studied in the present work may be present on either the inner or outer aspects of the membrane. The similarity of antigenic properties between a 'light' liver plasma-membrane subfraction composed exclusively of vesicles (Evans, 1970), in which only the outer aspect of the membrane would be available for interaction with antibodies, and of a 'heavy' subfraction containing strips of membrane in addition to vesicles (Gurd *et al.*, 1972a) suggests that the antigenic determinants are primarily associated with the outside surface of the membrane.

The results indicate, in agreement with previous studies (Gurd *et al.*, 1972b), that selective extraction of the plasma membranes with aqueous or

organic solvents results in an increased antigenicity of the remaining membrane-bound components. Most of the antigenicity of the membranes was associated with the alkaline-EDTA fraction (Gurd *et al.*, 1972b) and the present results also show by two-dimensional immunoelectrophoresis that this fraction, which may be considered to contain the more loosely bound 'extrinsic' proteins of the membrane, possessed most of the antigenic determinants present in the unperturbed membrane. However, certain antigens are unmasked after membrane perturbation. This was shown to be the case with the alkaline-EDTA-soluble antigen 3 (Plate 1) and the deoxycholate-Triton X-100-soluble antigen 5 (Plate 2), which were more completely adsorbed by both the alkaline-EDTA-insoluble membranes and the pyridine extract than by the plasma membranes, suggesting that these antigens become fully expressed after removal of membrane components. The phenomenon of unmasking of cryptic sites or receptors appears to be a general one with surface membranes, and has been shown to occur also with hormone receptors in the liver, e.g. for insulin and glucagon, especially after treatment of membranes with hydrolytic enzymes (Cuatrecasas, 1971). A similar unmasking of antigenic sites was also found to occur with rat liver plasma membranes after a brief papain digestion (Sheffield & Emmelot, 1972).

The present studies, which used antisera against a mouse liver plasma-membrane fraction in combination with chemically defined fractions, have shown the possibility of the use of antibodies as probes for the distribution of protein antigens within the membrane. The use of antibodies obtained against purified membrane glycoproteins, e.g. 5'-nucleotidase and phosphodiesterase, should provide further information on the molecular architecture of the liver cell surface membrane.

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