TWO-DIMENSIONAL ELECTROPHORETIC ANALYSIS OF CONCANAVALIN-A BINDING COMPONENTS IN THE PLASMA MEMBRANES OF CHINESE HAMSTER FIBROBLASTS

Michael N. HORST, George BAUMBACH and R. Michael ROBERTS
Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, FL 32610, USA

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1. Introduction

Many of the polypeptide components of plasma membranes are glycoproteins which seem to fulfill a wide variety of important functions at the cell surface. Because glycoproteins frequently form highly specific associations with lectins, the latter molecules have assumed great importance as a means for probing the structures of membranes. In addition, lectins can be iodinated [1,2], coupled to horseradish peroxidase [3] or conjugated with fluorescent dyes [4] and used to detect glycoproteins on polyacrylamide gels. Here we describe a two-dimensional electrophoretic procedure for separating plasma membrane polypeptides and demonstrate by means of ^{125}I-labeled Con A 'staining' that the binding components are extremely numerous. In particular, we show that a major group of binding components for this lectin are acidic molecules of high molecular weight which stain faintly, if at all, with Coomassie blue. They may thus represent a new class of membrane glycoproteins whose presence is not revealed by conventional electrophoretic techniques.

2. Materials and methods

CHO cells were maintained as in [5]. For membrane isolation, cells were cultured in 1300 cm^2 borosilicate roller bottles containing 100 ml medium. Cells were washed with PBS and removed from the glass using 0.02% (w/v) disodium EDTA in Ca^{2+}–Mg^{2+}-free PBS. After centrifugation and washing, the cells were diluted to 10^7 ml in TBS, (0.05 M Tris–HCl + 0.15 M NaCl, pH 7.4). The cells were then broken using an air-driven disrupting pump (Stansted Fluid Power, Essex) by the methods in [6,7]. A back pressure of 300 lbs/in^2 and a flow rate of 35–40 ml/min was used to break over 90% of the cells, but disrupted few nuclei. Recoveries of nuclei and whole cells were estimated after trypan blue staining of the final preparation. Details of plasma membrane preparation and full criteria for purity will be published elsewhere. Briefly, the disrupted cell preparation was centrifuged, first at 300 × *g* (10 min) then at 5500 × *g* (15 min) to remove whole cells, nuclei and other large particles including mitochondria. The supernatant fraction was then centrifuged at 30 000 × *g* (45 min) to yield a pellet which was homogenized in 10 ml hypotonic Tris–HCl (0.01 M; pH 7.4) using 10 strokes of a tight-fitting Dounce homogenizer in order to release trapped cytoplasm [7]. Plasma membrane vesicles were then purified by sucrose density gradient centrifugation by the method in [7]. A fraction highly enriched in plasma membrane vesicles as assessed by marker enzymes [8] was recoved at the 25%/40% (w/v) sucrose interface. This preparation was 37- and 36-fold enriched over the cell homogenate in Na^+/K^+-stimulated ATPase and phosphodiesterase I, respectively. It contained no detectable succinate dehydrogenase and was only 2.3- and 1.4-fold enriched over the homogenate in NADH-cytochrome *c* reductase and glucose 6-phosphatase, respectively. Lactic dehydrogenase specific activity of the preparation was only 7% of the homogenate.

Plasma membrane was rendered soluble by low

Abbreviations: CHO, Chinese hamster ovary; Con A, concanavalin A; PBS, phosphate buffered saline; TBS, Tris–HCl-buffered saline

energy sonication in a 9.5 M urea–5 mM K_2CO_3 (pH 10.3) solution, followed by the addition of Nonidet P-40 (to 2% v/v) and dithiothreitol (to 0.5% w/v) [9]. This procedure released > 92% of the total protein in soluble form, as judged by centrifugation at 100 000 × *g*. The clear supernatant fraction was immediately subjected to isoelectric focusing by a modification of the method in [10], using *N,N'*-diallyltartardiamide rather than bis-acrylamide as the crosslinking agent [11]. After focusing, the gels were equilibrated briefly (7.5 min) in 0.065 M Tris–HCl (pH 6.9) containing 1% (w/v) sodium dodecyl sulfate and 1% (v/v) 2-mercaptoethanol, then sealed in position above the second dimension, 10% (w/v) polyacrylamide slab gel [12]. Following electrophoresis, slab gels were fixed and stained for protein by standard

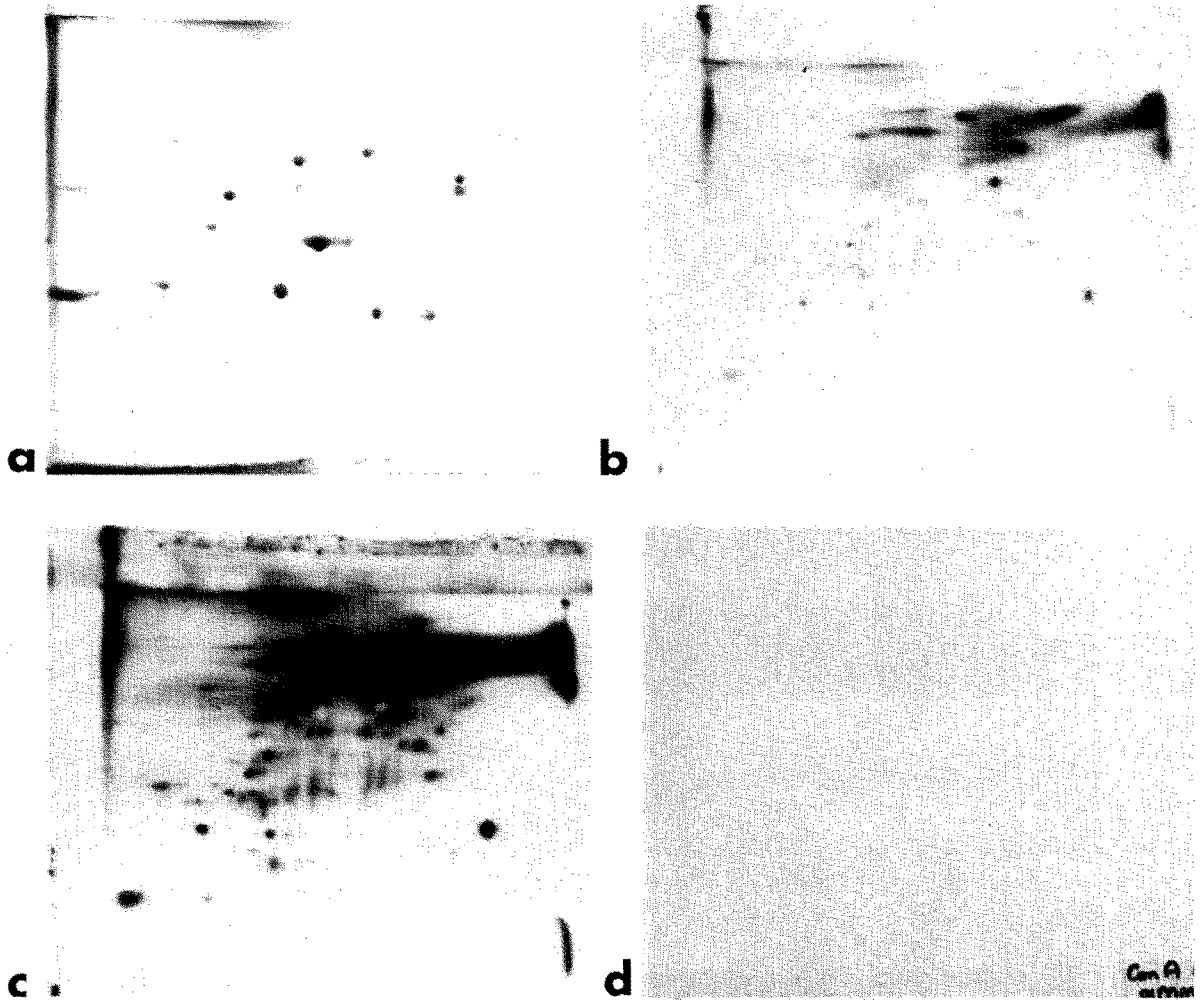

Fig.1a. Two-dimensional electrophoretic maps of CHO plasma membrane vesicles proteins as revealed by Coomassie blue staining. The pH gradient (left to right) was ~8–4. The second dimension slab gel separates proteins from mol. wt ~4.0×10^5 (top) to mol. wt 1.7×10^5 (bottom). These scales are shown in more detail in fig.2. Fig.1b. Autoradiography of membrane proteins shown in fig.1a, after 'staining' with ^{125}I-labeled Con A. The exposure time was 3 h. Fig.1c. Autoradiography of ^{125}I-labeled Con A-stained map (1a) using a 12 h exposure time. Fig.1d. Autoradiography of a duplicate membrane protein map to that shown in fig.1a–c which was stained with ^{125}I-labeled Con A in the presence of 0.5 M methyl α-mannoside and washed in TBS containing 0.5 M glucose. Exposure time was 12 h.

procedures using Coomassie brilliant blue R-250.

Gels were stained with ^{125}I-labeled Con A by a modification of the method in [1]. Con A was iodinated by the lactoperoxidase technique to spec. act. 10^8–10^9 cpm/mg protein. The labeled product was purified by affinity chromatography on Sephadex G-100 [1]. Polyacrylamide slab gels were equilibrated with TBS, sealed in plastic bags and incubated on a rocking platform for 24 h, with ~2.5 × 10^7 dpm ^{125}I-labeled lectin in 20 ml TBS containing 0.1% (w/v) hemoglobin. They were then washed in shallow pans with several changes of buffer until the ^{125}I in the wash reached background levels, dried and autoradiographs prepared.

3. Results

The plasma membrane preparation contained a large number of polypeptides covering a wide range of molecular weights and pI values (fig.1A). In order to make identification of components easier, we have constructed a composite map which has been divided arbitrarily by a series of vertical and horizontal grids into a series of alphabetically designed areas. Each polypeptide within such a rectangle has been assigned a number. The diagram shown in fig.2 is based on comparisons of at least 30 different gels. Certain spots have been stippled to indicate that they are generally detected by methods other than Coomassie blue staining.

The major spot (N4) is in the middle of the gel in fig.1A and maps fairly close to rabbit muscle actin. This protein does not stain with ^{125}I-labeled Con A (fig.1B). However, depending upon the time of exposure (fig.1B,1C), up to 100 spots did bind the lectin. Staining in presence of methyl α-mannoside and extensive washing in presence of 0.5 M D-glucose eliminated all binding (fig.1D).

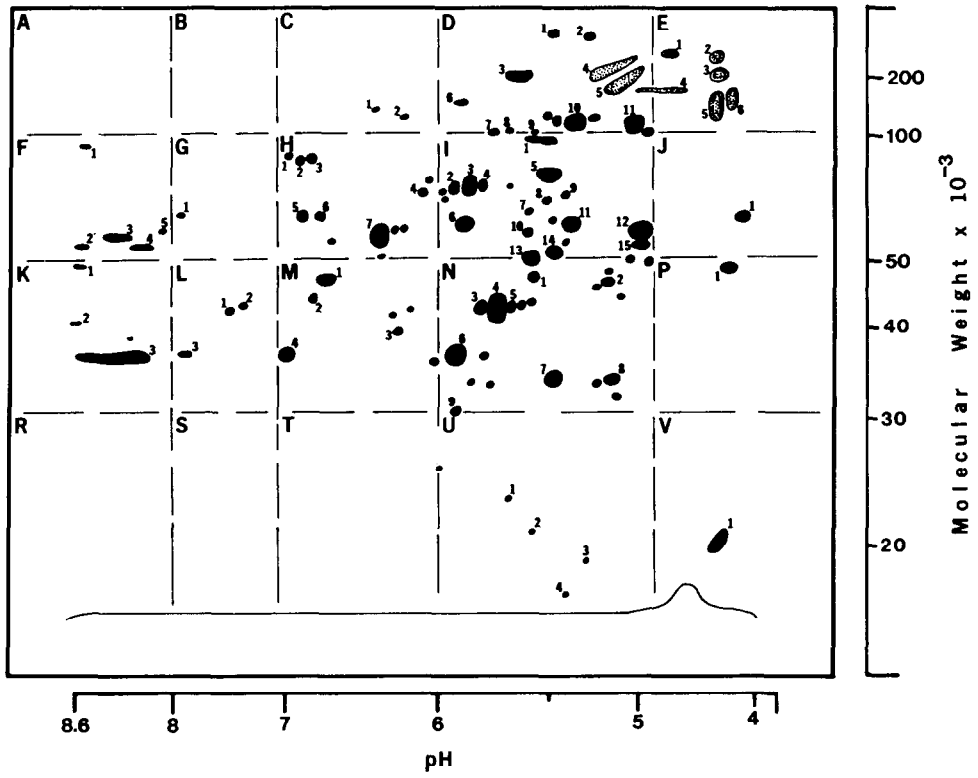

Fig.2. Composite map of CHO plasma membrane vesicle proteins as revealed by Coomassie blue staining (solid areas). The stippled areas indicate the positions of the major Con A-staining proteins. These fail to stain with Coomassie blue. Vertical lines represent pH values, horizontal lines molecular weights.

Several of the major Con A-binding species are located in the upper right-hand corner of the slab (rectangles D and E of fig.2). The most acidic of these, E2, 3, 5 and 6) focused very close to the bottom of the IEF gel and have pI < 5. They are mol. wt $1.3-2.5 \times 10^5$. Two other components (D4 and D5) map in a diffuse area very close to each other near rectangle E. They are mol. wt $\sim 2.0 \times 10^5$ and they have app. pI ~5.3. Only on heavily overloaded gels can they be detected even faintly by Coomassie blue staining when they can be seen as closely contiguous zones, sloping slightly upwards towards the more acidic end of the gel. These two proteins overlap in molecular weight with protein D3, which also labels with ^{125}I-labeled Con A. Other major binding components on the map include D 10, I9 and N8 plus a myriad of minor spots.

4. Discussion

This report shows that Con A can bind to a very large number of polypeptides of the CHO plasma membrane. Such complexity, particularly among the high molecular weight components, is not evident when electrophoretic analysis in a single dimension is employed. The major binding polypeptides in the preparation are of high molecular weight and do not stain with Coomassie blue. They become labeled after growing cells in presence of D-[^{3}H]glucosamine (unpublished results) and are not, therefore, adsorbed serum contaminants. Two of the polypeptides (D4 and D5) have molecular weights which overlap that of a component that stains with Coomassie blue (D3). It is in this molecular weight range that the so-called LETS protein is normally detected [13]. Several other Con A-binding proteins are distinguishable from unlabeled components only on the basis of their pI values and molecular weights together and not by one property alone. The lack of staining of D4, D5, E2, E3, E5, and E6 may indicate that, like glycophorin of the erythrocyte, they are extensively glycosylated. Because these glycoproteins are difficult to identify on one-dimensional gels, they may represent a class of membrane components whose presence has hitherto been unsuspected.

Acknowledgements

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