

Agorins: Major Structural Proteins of the Plasma Membrane Skeleton of P815 Tumor Cells

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Abstract. Plasma membranes of P815 mastocytoma cells contain a set of proteins that remain selectively insoluble upon extraction of the membranes with Triton X-100, and appear to form a membrane skeletal matrix independent of the filamentous cytoskeletal systems. EGTA treatment of the matrix was found to release ~25% of the protein as polypeptides of 70, 69, 38, and 36 kD, all of which appear to be peripheral components associated with the cytoplasmic face of the plasma membrane via divalent cation-dependent interactions. About 75% of the total matrix protein was recovered in the EGTA-insoluble fraction. Actin accounted for ~5% of the total protein in the EGTA-insoluble fraction. The rest was accounted for by two novel proteins of 20 and 40 kD which, despite their

relatively low molecular weights, do not enter SDS PAGE gels. Together these proteins account for ~15% of the total plasma membrane protein, and are thus present in much higher amounts than any other characterized protein of nucleated cell plasma membranes. Based on the extensive associations of these proteins to form very large detergent-insoluble structures, we propose that they may be named agorin I, the 20-kD protein, and agorin II, the 40-kD protein, from the Greek *agora* meaning assembly. The amount and properties of these proteins and the appearance of the EGTA-insoluble material in thin-section electron micrographs indicate that the agorins are the major structural elements of the membrane matrix, and thus of the putative membrane skeleton.

DETERGENT extraction of lymphoid (2, 3, 8, 9, 15, 22, 27) and nonlymphoid (6, 10, 20, 28, 31, 36) cells and isolated membranes has demonstrated the occurrence of detergent-insoluble components associated with the plasma membranes of a variety of nucleated cells. Partial characterization of the components of these detergent-insoluble fractions have led to suggestions that they represent complexes of cell surface transmembrane proteins interacting with cytoskeletal components, or submembranous cytoskeletal complexes associated with the membrane. Our studies of the detergent-insoluble plasma membrane fraction of murine tumor and lymphoid cells have led to the proposal that this material represents a membrane skeletal structure distinct from the filamentous cytoskeletal systems previously characterized (2, 27).

Extraction of purified plasma membranes from murine lymphocytes, lymphoid tumor cells, and P815 mastocytoma cells with nonionic detergents solubilizes 75–80% of the membrane protein (3, 27). The fraction remaining insoluble has been found to consist of a discrete set of proteins and phospholipids that remain associated, as assessed by density gradient centrifugation. In the case of P815 plasma membranes, the insoluble fraction includes proteins of 70, 69, 45, 38, and 36 kD, as visualized by protein staining on SDS PAGE. With the exception of the 45 kD protein, which has been shown to be actin, these proteins are present exclusively in the detergent-insoluble fraction. About half of the

membrane-associated actin is extracted by Triton X-100 (TX-100)¹, whereas the rest remains associated with the insoluble fraction. The plasma membrane origin of the matrix proteins was confirmed by the finding that 5'-nucleotidase, a cell surface glycoprotein, also remains selectively associated with the insoluble fraction (2, 27). In addition to the proteins, sphingomyelin remains associated with the detergent-insoluble matrix, whereas the other phospholipids are largely extracted by the detergent. Plasma membranes from other murine lymphoid cell lines (3, 27), bovine (3) and pig (9) lymphocytes, and a human lymphoblastoid cell line (9) have been found to have a detergent-insoluble fraction consisting of a similar set of components.

Examination of the insoluble fraction from P815 plasma membranes by thin-section electron microscopy showed the material to be present largely in the form of closed structures having about the same size distribution as the starting plasma membrane vesicles (27). The properties of the detergent-insoluble protein matrix suggested that it might be a membrane skeletal structure, and recent studies examining detergent extraction of intact cells have provided further support for this suggestion (2). It was found that extraction of P815 cells with TX-100 under the same conditions as those used for isolation of the membrane matrix resulted in structures

1. *Abbreviations used in this paper:* DTT, dithiothreitol; HPLC, high pressure liquid chromatography; TX-100, Triton X-100.

having a nuclear remnant, a largely empty cytoplasmic space, and a continuous layer of protein at the cell periphery. The peripheral layer was independent of both the membrane lipid bilayer and filamentous cytoskeletal components for its stability. The evidence available thus far very strongly suggests, but does not yet prove, that this peripheral skeletal layer is composed of the detergent-insoluble membrane matrix.

Further characterization of the composition of the insoluble matrix from P815 plasma membranes has shown the major components of this matrix to be novel 20 and 40-kD membrane proteins, as described in this paper. These proteins account for ~70% of the matrix and ~15% of the total plasma membrane protein, but had not been previously detected because they do not enter SDS PAGE gels despite their relatively low apparent molecular weights. The amount and properties of these proteins indicate that they are the major structural elements of the membrane matrix and of the putative membrane skeleton.

Materials and Methods

Plasma Membrane Purification

P815, a murine mastocytoma, was grown as an ascites in (AKR × DBA/2)F₁ mice (Jackson Laboratory, Bar Harbor, ME). Mice were injected i.p. with 10⁷ cells, the cells were allowed to grow for 6 d, and peritoneal fluid was then harvested yielding ~5 × 10⁸ cells per mouse. The cells were washed three times in Dulbecco's phosphate-buffered saline (D-PBS) and resuspended in the same media containing 0.2 mM phenylmethylsulfonyl fluoride and 10 mM iodoacetamide. Plasma membranes were then isolated, as previously described in detail (2, 21). Briefly, the cells were lysed by nitrogen cavitation and spun at 3,600 g to remove nuclei and unlysed cells. The pellet was washed twice more, and the combined supernatants were spun at 22,000 g to pellet the membranes. This material was loaded onto a sucrose step gradient and spun at 18,000 rpm for 16 h. The purified plasma membranes were collected at the 25–37.5% sucrose interface. This procedure results in highly enriched plasma membrane, as assessed by biochemical and morphological criteria (2, 21, 27). Essentially all (>90%) of the 5'-nucleotidase activity of P815 cells has been shown to be located on the cell surface (2, 27), and this enzyme thus provides a good marker for plasma membranes in these cells. Based on 5'-nucleotidase activities and protein determinations, plasma membrane yields of ~50% are obtained, and the plasma membrane accounts for 1.5–3% of the total cell protein (2, 21, 27).

Internal labeling of cells with ³H-amino acids was done by first incubating 5 × 10⁷ cells in Hank's media (Gibco, Grand Island, NY) for 1 h to deplete the cells of available amino acids. The cells were then incubated in 25 ml of Hank's media containing 5% dialyzed fetal calf serum and 1 mCi of ³H-amino acid mixture (New England Nuclear, Boston, MA). After 4 h at 37°C, 25 ml of RPMI 1640 (Gibco) containing 10% fetal calf serum was added for 1 h and the cells were then washed twice with RPMI 1640 and twice with D-PBS. The labeled cells were lysed by freezing in liquid nitrogen and thawing, and the plasma membranes were then prepared as described above.

Triton Extraction

The detergent-insoluble membrane matrix was prepared by suspending plasma membranes at 1 mg/ml in D-PBS containing 0.5% TX-100 (Sigma Chemical Co., St. Louis, MO) and incubating for 30 min at 4°C with occasional mixing. The suspension was then centrifuged at 100,000 g for 45 min to pellet the insoluble fraction. EGTA extraction was done by suspending the detergent-insoluble matrix pellet at 1 mg/ml in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) containing 5 mM EGTA. In some cases, 0.5% TX-100 was included in the buffer solution. Samples were left on ice for 30 min with occasional mixing and then centrifuged at 100,000 g for 45 min to yield an EGTA supernatant fraction and an EGTA-insoluble pellet.

Gel Filtration Chromatography

The EGTA-insoluble proteins were resuspended in 2% SDS containing 5 mM dithiothreitol (DTT) and placed in a boiling water bath for 3 min. After 15 min, iodoacetamide was added to a concentration of 10 mM. The reduced and alkylated EGTA-insoluble proteins were separated on a Sepharose CL-4B gel filtration column equilibrated in 0.1% SDS in 20 mM sodium phosphate, pH 6.5. Fractions were collected and aliquots were analyzed by high pressure liquid chromatography (HPLC; Beckman 341 system, Beckman Instruments, Inc., Palo Alto, CA) gel filtration on a Toya-Soda TSK-3000 SW column. The material was chromatographed in 0.1% SDS, 20 mM sodium phosphate, pH 6.5 at a flow rate of 0.5 ml/min. The effluent was monitored at a wavelength of 214 nm.

SDS Binding to Proteins by Equilibrium Dialysis

The EGTA-insoluble matrix fraction was produced as described above. The pellet was resuspended in a small volume of PBS and extracted with a large volume of chloroform/methanol (1:2) for 30 min at room temperature. The material was then pelleted by centrifuging at 1,500 rpm for 15 min. The pellet was resuspended in chloroform/methanol (2:1) for 30 min at room temperature followed by centrifugation. Analysis of the pellet by the organic phosphate assay of Ames and Dubbin (1) showed that the two organic extractions removed all of the phospholipids that are associated with these proteins.

The organically extracted protein was solubilized at a concentration of 10 mg/ml in 1% SDS, 50 mM phosphate, pH 11 buffer that contained 2% β-mercaptoethanol. The material was dialyzed against 0.1% SDS, 50 mM phosphate, pH 7.2, 0.02% sodium azide, and 0.1% β-mercaptoethanol with constant stirring at room temperature. Every 3 d the contents of the dialysis bags were assayed for protein by the method of Lowry (23) and for SDS using a modification of the method of Hayashi (12). The dialysis buffer was changed after each analysis. Equilibrium was reached by day 6 and the readings were continued every 3 d to obtain a more accurate average binding ratio. SDS binding to bovine serum albumin (BSA; Sigma Chemical Co.), ovalbumin (Sigma Chemical Co.), and β-lactoglobulin (Sigma Chemical Co.) was determined in parallel.

Analytical

Samples were solubilized for analysis in 2% SDS either at room temperature or by heating for 3 min at 100°C. Samples were reduced using 5 mM DTT and in some cases alkylated by addition of iodoacetamide to a final concentration of 10 mM, 15 min after addition of DTT. SDS polyacrylamide slab gels were run using the buffer system of Laemmli (17) with a 3% polyacrylamide stacking gel and a running gel consisting of a 5–15% polyacrylamide gradient. Standard proteins run in parallel on gels were from Bio-Rad Laboratories (Richmond, CA) and included phosphorylase B (92,500 D), BSA (66,200 D), ovalbumin (45,000 D), carbonic anhydrase (31,000 D), soybean trypsin inhibitor (21,500 D), and lysozyme (14,400 D). After electrophoresis, proteins were visualized by staining with Coomassie Blue or by the silver staining method of Merrill et al. (26). Details of materials and procedures used for gel filtration, size exclusion, and reverse-phase HPLC are given in the legends to the figures.

Protein concentration was determined using the method of Lowry et al. (23) or using a fluorescamine assay (37) with BSA as the standard. 5'-Nucleotidase activity was measured by the procedure of Avruch and Wallach (4), and organic phosphate was determined by a minor modification of the assay described by Ames and Dubbin (1).

Electron Microscopy

The EGTA-insoluble matrix fraction was isolated as described above and fixed in 3% glutaraldehyde in 1 M cacodylate buffer and osmicated in 2% osmium tetroxide in the same buffer for 2 h. Samples were then rinsed, dehydrated in a graded series of ethanol solutions, infiltrated, and embedded in Polybed 812. Thin sections were cut on a diamond knife, stained with aqueous uranyl acetate and lead citrate, and examined using a JOEL 100S transmission electron microscope.

Results

Treatment of plasma membranes of P815 with TX-100 at detergent to protein ratios higher than 5:1 results in solubiliza-

tion of ~75–80% of the membrane protein (27). SDS PAGE shows the insoluble matrix fraction to consist of a discrete set of membrane proteins of 70, 69, 45, 38, and 36 kD (Fig. 1, lane 3). Treatment of the Triton-insoluble matrix with 5 mM EGTA resulted in solubilization of the 70, 69, 38, and 36-kD proteins (Fig. 1, lane 4), and extraction of this set of proteins was equally effective in the presence or absence of 0.5% TX-100. Provided that EGTA was included in the elution buffer, gel filtration of the solubilized proteins in the absence of detergent resulted in their eluting in positions corresponding to monomers (not shown). Vectorial labeling using lactoperoxidase-catalyzed iodination had previously shown this set of proteins to be located at the inner face of the membrane (27). Thus, it appears that these are peripheral proteins bound to the cytoplasmic face of the plasma membrane and associated with the detergent-insoluble matrix via divalent cation-dependent interactions.

Despite the fact that SDS PAGE indicated that most of the matrix protein was solubilized by EGTA (Fig. 1, lanes 4 and 5), examination of the protein content of the EGTA-soluble and -insoluble matrix fractions showed that ~75% of the starting protein remained in the insoluble fraction (Table I). 5'-Nucleotidase was also recovered in the EGTA-insoluble fraction (Table I). We have previously shown that ~40% of the membrane phospholipid remains associated with the TX-100-insoluble membrane matrix (3). The lipid association with the matrix is selective, in that essentially all of the membrane sphingomyelin is recovered with the matrix while the other phospholipids are largely extracted. The matrix-associated phospholipid was recovered in the insoluble fraction after EGTA extraction, regardless of whether TX-100 was present during the extraction.

The discrepancy between the measured protein content of the EGTA-insoluble fraction (Table I) and the gel patterns of the soluble and insoluble fractions led us to further examination of this material. Essentially identical values for protein content were found using the assay of Lowry et al. (23) and an assay using fluorescamine (37), and organic extraction of the insoluble fraction to remove the lipid before assay did not significantly change the values obtained. Furthermore, the fractionation of radiolabeled material was the same as that found for protein content when membranes from cells grown in ³H-amino acids were examined (Table I).

The 45-kD matrix protein, the only major band seen by Coomassie Blue (Fig. 1, lane 5) or silver staining (not shown) of the EGTA-insoluble fraction on SDS PAGE (27), has been shown to be actin by limited proteolysis peptide mapping (27) and by immunoprecipitation after solubilization with SDS (unpublished observations). Comparison of the EGTA-insoluble fraction and a series of known amounts of mouse muscle actin by SDS PAGE indicated that the actin could account for no more than 5–10% of the total protein in this fraction (not shown).

The only other material seen on gels of the insoluble fraction was a smear of staining at the top of the gel (Fig. 1, lane 5). Note that lane 3, the unfractionated matrix, has a similar smear at the top). No differences were seen if the samples were reduced and alkylated or left unreduced before electrophoresis. When the EGTA-insoluble fraction from membranes of cells grown in ³H-amino acids was run on SDS PAGE and the gel then sliced and counted, the only peak of

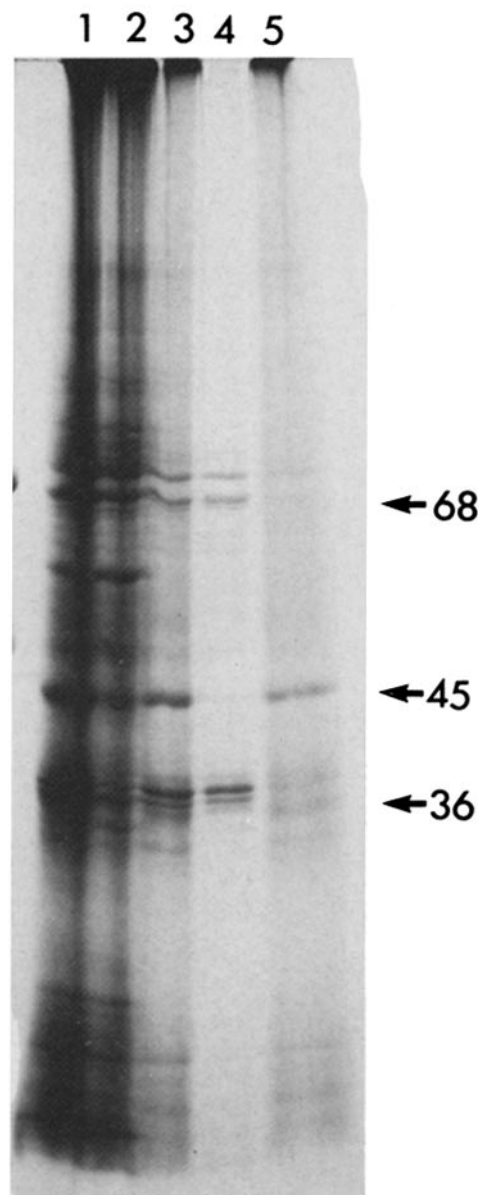


Figure 1. SDS PAGE analysis of P815 plasma membranes and fractions obtained on detergent and EGTA treatment. Purified plasma membranes were extracted with Triton and centrifuged to yield detergent soluble and insoluble (matrix) fractions. The insoluble matrix was then treated with EGTA and again centrifuged to yield EGTA soluble and insoluble fractions. Details of the extraction procedures are described in Materials and Methods. Samples derived from 200 μ g of starting plasma membrane were taken at each stage, subjected to electrophoresis as described in Materials and Methods, and the proteins were visualized by staining with Coomassie Blue. Molecular masses in kilodaltons, shown on the right, are based on standard proteins run on the same gel. Lane 1, plasma membranes (200 μ g); lane 2, Triton-soluble fraction (146 μ g); lane 3, Triton-insoluble (matrix) fraction (46 μ g); lane 4, EGTA-soluble matrix fraction (12 μ g); lane 5, EGTA-insoluble matrix fraction (35 μ g).

radioactivity was found at the top of the gel, and the results confirmed that actin accounts for ~5% of this material (not shown).

The above results suggested that the majority of the

Table 1. Protein and 5'-Nucleotidase Content of Plasma Membrane Fractions*

	Protein		
	Colorimetric [‡]	³ H-Amino acid [§]	5'-Nucleotidase
	%		%
Membrane fractions (% of starting plasma membrane)			
Plasma membrane	100.0	100.0	100.0
Triton supernatant	73.0	72.5	ND [†]
Matrix (Triton pellet)	23.2	19.5	120.8
EGTA soluble	6.1	5.3	0.2
EGTA insoluble	17.3	17.7	139.0
Matrix fractions (% of starting matrix)			
Matrix	100.0	100.0	100.0
EGTA soluble	26.3	27.1	0.2
EGTA insoluble	74.6	90.7**	114.9

* P815 plasma membranes were detergent and EGTA extracted as described in Materials and Methods.

[‡] Protein content determined by the method of Lowry et al. (23) in the presence of 1% SDS.

[§] Protein content based on distribution of radioactivity in fractions from plasma membranes prepared from cells grown in ³H-amino acids as described in Materials and Methods.

^{||} Determined by the method of Avruch and Wallach (4). As previously described (2), detergent treatment of the membranes results in an increase in 5'-nucleotidase activity.

[†] Not determined.

** Note that recovery in the two fractions totaled 117.8%. The EGTA-insoluble material accounts for 77% of the total recovered material.

EGTA-insoluble membrane protein might be present as very high molecular weight material even in the presence of SDS and reducing agents. To further examine this, the EGTA-insoluble protein was solubilized in 2% SDS at 100°C, reduced with DTT, alkylated with iodoacetamide, and chromatographed on a Sepharose CL-4B gel filtration column in 0.1% SDS. Rather than eluting in the high molecular weight region of the column, the majority of protein eluted between 70 and 14 kD, as determined by standards, with a peak at ~20 kD and a shoulder at ~45 kD (Fig. 2 I). Greater than 95% of the applied protein was recovered. Essentially the same profile was obtained when the EGTA-insoluble material was solubilized in SDS, iodinated using Iodobeads, and chromatographed under the same conditions (not shown). When the iodinated material was treated with Pronase before chromatography, all of the radioactivity eluted as a single peak in the completely included volume. Thus, it appeared that the bulk of the EGTA-insoluble fraction, and thus of the membrane matrix, consisted of relatively low molecular weight proteins that failed to enter SDS PAGE gels.

Examination of the Sepharose CL-4B fractions by size exclusion chromatography on a TSK-3000 SW HPLC column in SDS showed the majority of the protein to consist of 20 and 40 kD components (Fig. 2, IIb). Homogeneous appearing peaks were obtained, suggesting that each represented one or a small number of proteins. Attempts to examine the isolated 20 and 40-kD components by SDS PAGE again failed to reveal protein bands that had entered the gel. When the EGTA-insoluble fraction was chromatographed directly on the TSK-3000 column, without prior separation on Sepharose CL-4B, peaks were obtained at 20 kD, 40 kD, and in the excluded volume (>70 kD) (Fig. 2, panel IIa). Estimates of each species based on separation and protein quantitation for eight independent membrane preparations showed the composition (by weight) to be 44 ± 10% for 20 kD, 33 ± 6% for 40 kD, and 21 ± 7% for the excluded material. No indication of proteolysis of these proteins during isolation has been seen. Identical profiles were obtained

whether or not phenylmethylsulfonyl fluoride and iodoacetamide were included throughout, incubation of membranes at room temperature before isolation of the matrix did not change the profiles and, as mentioned above, essentially the same ratios of 20 kD, 40 kD, and excluded components were seen with multiple membrane preparations.

To rule out the possibility that the anomalous behavior of the 20 and 40-kD proteins was an artifact introduced by the TX-100 or EGTA treatments, purified plasma membranes were solubilized directly in 2% SDS at 100°C. Greater than 99.5% of the membrane protein is solubilized under these conditions. The solubilized membrane protein was chromatographed on a Sepharose CL-4B column in SDS (Fig. 3). Fractions were collected and examined for their protein content by SDS PAGE (Fig. 3, inset). The majority of the proteins eluted from the column in positions consistent with their apparent molecular weights as determined by SDS PAGE. However, fractions corresponding to the elution position of 20,000–50,000-D proteins had a large smear of Coomassie Blue staining material at the top of the gel (Fig. 3, inset, fraction 24); in addition to proteins running with the expected mobilities on the gels. Material was not detected at the top of the gel from fractions corresponding to higher or lower molecular weight elution positions. Thus, it appears very likely that the EGTA-insoluble matrix proteins chromatograph as 20 and 40-kD proteins but fail to enter SDS PAGE gels even when they have not been subjected to either TX-100 or EGTA.

A variety of SDS gel systems and conditions have been examined in an effort to resolve the EGTA-insoluble proteins. These have included: (a) the buffer system described by Laemmli (17, Fig. 1) using 5 or 10% gels; (b) the phosphate buffer system described by Weber and Osborne (38) with stacking buffer or with sample loaded in reservoir buffer so that concentration of the proteins did not occur; (c) phosphate-buffered gels run at pH 10.5 and (d) SDS-urea gels. Sample preparation before electrophoresis has included incubation in SDS at room temperature, 37°, or 100°C, and the

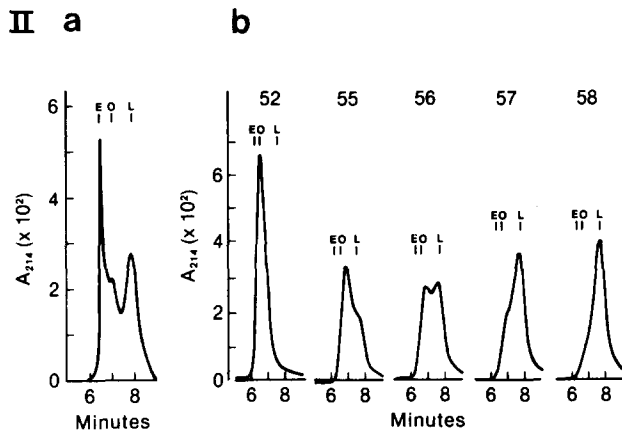
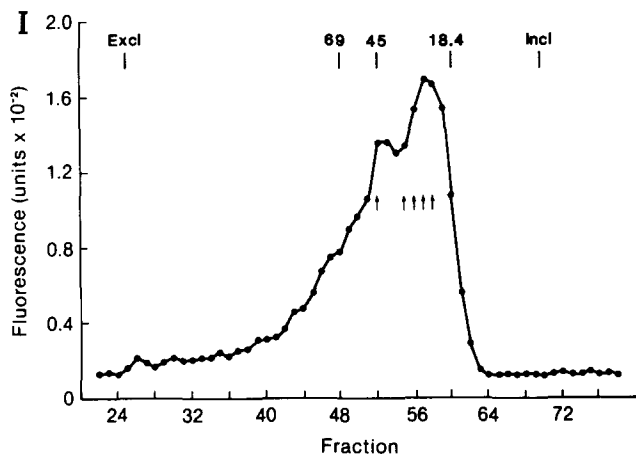


Figure 2. Gel filtration of the EGTA-insoluble matrix fraction in 0.1% SDS. (I) Chromatography on Sepharose CL-4B. 2 mg of the EGTA-insoluble matrix fraction was solubilized in 0.4 ml of 2% SDS at 100°C for 3 min and reduced and alkylated. The sample was then chromatographed on a Sepharose CL-4B column (0.9 cm × 60 cm) using 0.1% SDS in 20 mM sodium phosphate, pH 6.5. Fractions were collected and assayed for protein content using fluorescamine (37). Elution positions of standard proteins were determined in a separate run and are indicated in kilodaltons: 69, BSA; 45, ovalbumin; 18.4, β-lactoglobulin. *Excl*, excluded volume. *Incl*, completely included volume. *Arrows*, fractions examined by size exclusion HPLC (II b). (II a) HPLC size exclusion chromatography of the EGTA-insoluble fraction. 5 μg of EGTA-insoluble matrix was solubilized as described above and chromatographed on a TSK 3000-SW column (7.5 × 300 mm with a 7.5 × 100-mm guard column) using 0.1% SDS in 20 mM sodium phosphate, pH 6.5, for elution. Elution positions of standard proteins in kilodaltons (K) are indicated. *E*, excluded (>70K); *O*, Ovalbumin (45K); *L*, β-lactoglobulin (18.4K). (b) HPLC size exclusion chromatography of fractions from Sepharose CL-4B. Samples from the fractions collected in the experiment shown in I were chromatographed on a TSK 3000-SW column as described above.

samples either not reduced, reduced with β-mercaptoethanol or DTT, or reduced and alkylated. None of these gel systems or sample preparation procedures resulted in migration of the proteins into the gels. We have found that flat-bed electrophoresis in SDS on 1% agarose gels results in migration of the proteins but only very low resolution is achieved in these gels. The EGTA-insoluble proteins migrated to the region

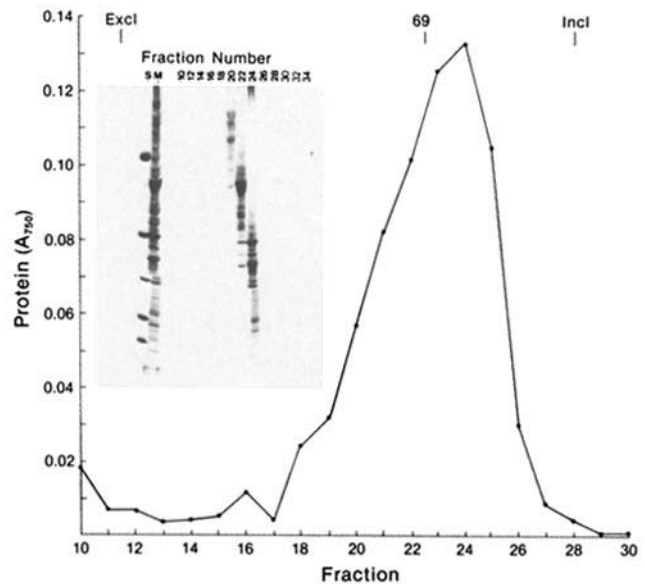


Figure 3. Gel filtration of plasma membrane proteins after direct solubilization in SDS. 2 mg of plasma membrane was solubilized in 2% SDS in a boiling water bath. The sample was then centrifuged for 45 min at 100,000 *g* and >99.5% of the protein was recovered in the supernatant. The supernatant was then applied to a Sepharose 4B column (1.0 cm × 45 cm) and elution done using 0.1% SDS. Fractions (0.3 ml) were collected and assayed for protein by the method of Lowry et al. (23). The excluded and 69-kD elution positions were determined using blue dextran and BSA. *Excl*, excluded volume. *Incl*, included volume. (*Inset*) Protein from even-numbered fractions was precipitated in acetone overnight, solubilized in SDS, reduced with β-mercaptoethanol, and run on a 5–15% SDS polyacrylamide gradient slab gel. Protein was visualized by staining with Coomassie Blue. Lane *S*, protein standards with molecular weights of 92,500, 66,200, 45,000, 31,000, 21,500, and 14,400. Lane *M*, 333 μg plasma membrane. Numbers above the remaining lanes indicate the Sepharose 4B fraction applied in that lane.

expected for proteins of 20–40 kD, in comparison to standard proteins, but were not resolved as two components. Increasing the percent agarose in the gels resulted in increased resolution of standard proteins. In contrast, the EGTA-insoluble proteins showed broadening of the stained region and smearing into the higher molecular weight region of the gel as the percent agarose was increased, and at 4–5% agarose the majority of protein again failed to enter the gel.

Binding of SDS to the EGTA-insoluble proteins was measured to determine if the anomalous behavior on gel electrophoresis might result from low SDS binding in comparison to most proteins. The EGTA-insoluble matrix fraction was extracted with chloroform/methanol to remove bound lipid, and SDS binding was determined by equilibrium dialysis (32, 33), as described in detail in Materials and Methods. Binding to several standard proteins was determined in parallel. Binding to the standard proteins was in the range of 1.2–1.4 mg SDS per mg protein (Table II), in reasonable agreement with previously reported values (32, 33), given that protein content was determined colorimetrically rather than by dry weight. Binding to the EGTA-insoluble proteins was found to be slightly higher than to the standards (Table II). The results strongly suggest that the anomalous behavior of the EGTA-insoluble proteins on electrophoresis in SDS

Table II. SDS Binding to the EGTA-insoluble Matrix Proteins*

	SDS/protein ratio [‡]
	mg/mg
BSA	1.30
Ovalbumin	1.23
β-Lactoglobulin	1.42
EGTA-insoluble proteins [§]	1.68

* Proteins at a concentration of 1.0% (wt/vol) were dialyzed against 0.1% SDS as described in Materials and Methods. Protein and SDS content were assayed every 3 d until equilibrium was reached.

[‡] Protein content was determined by the method of Lowry (23) and SDS content according to Hayashi (12). The equilibrium ratios shown here are the average SDS determinations on each of 5 d after equilibration.

[§] The EGTA-insoluble matrix material was prepared as described in Materials and Methods and extracted with chloroform and methanol to remove lipids.

cannot be accounted for by low SDS binding in comparison to other proteins.

While the 20 and 40-kD peaks appeared homogeneous on TSK-3000 chromatography (Fig. 2, panel *Ib*), this method does not provide sufficient resolution to allow the conclusion that the peaks represent single proteins. Attempts to obtain an estimate of the number of proteins present by amino acid sequencing have been unsuccessful, suggesting that the proteins are probably N-blocked. We therefore examined the gel filtration purified proteins by reverse-phase HPLC on a C3 column. Samples were applied to the column in 0.1% SDS and chromatography done using a 20–50% gradient of isopropanol in 0.1% phosphoric acid. The 20-kD material reproducibly eluted as a single sharp peak with a retention time of 16 min (Fig. 4 *A*). A second peak was seen at 22 min, but was present when sample buffer containing no protein was loaded and chromatographed. When the 20-kD protein was chromatographed and fractions collected and assayed, protein was only detected in the peak eluting at 16 min. Thus, only the 16-min peak represents protein applied to the column. Chromatography of the 40-kD material yielded a sharp peak at 17.2 min with a small shoulder at 16 min, as well as the 22-min nonprotein peak. Chromatography of the material excluded on the TSK column yielded a doublet with peaks at 16 and 17.3 min as well as the nonprotein peak. Chromatography of unfractionated EGTA-insoluble material yielded a single sharp peak at 16.1 min (Fig. 4 *D*), consistent with the 20-kD protein being the major component and the 40-kD protein being only slightly resolved from it. Results essentially identical to those shown in Fig. 4 have been obtained upon C3 chromatography of the EGTA-insoluble components using trifluoroacetic acid–isopropanol and trifluoroacetic acid–acetonitrile gradients (not shown).

These results strongly suggest that the 20-kD protein and probably the 40-kD protein are single protein species. Furthermore, the almost identical elution positions of the two, together with the fact that the excluded material consists of two peaks having identical elution positions as those of the 20 and 40-kD proteins, raises the possibility that the higher molecular weight components are closely related to, or possibly aggregates of, the 20-kD protein. Aggregation of the proteins in SDS after treatment at 100°C and reduction and alkylation would be unusual, but might explain the fact that these proteins fail to enter SDS PAGE gels despite their relatively low apparent molecular weights.

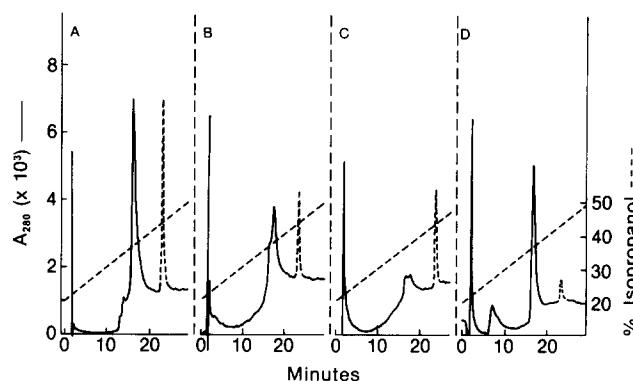


Figure 4. Reverse phase (C3) HPLC of the EGTA-insoluble matrix proteins. Samples were applied to a C3 column (4.6 × 75 mm) in 0.1% SDS, and the column was then eluted with a 20–50% gradient of isopropanol in 0.1% phosphoric acid (pH 2.2) in 30 min at a flow rate of 1 ml/min. Absorption of the effluent was continuously monitored at 280 nm. The peak eluting at 22 min (hatched) was present in all runs and did not represent protein, as determined by collecting fractions and assaying with fluorescamine (37). EGTA-insoluble matrix protein was first fractionated on a Sepharose CL-4B column, as shown in *A* and the proteins then run on the C3 column. K, kilodaltons. (*A*) 20K peak from Sepharose CL-4B. (*B*) 40K peak from Sepharose CL-4B. (*C*) Excluded peak from Sepharose CL-4B. (*D*) A sample of EGTA-insoluble matrix was dissolved in 2% SDS, reduced, and applied directly to the C3 column without prior separation.

It was previously shown that the detergent-insoluble membrane matrix is present largely in the form of closed structures having about the same size distribution as the plasma membrane vesicles they are isolated from (27). The properties and amounts of the 20 kD, 40 kD, and excluded proteins suggest that these are the major structural components of the matrix. Consistent with this, it was found that the EGTA-insoluble fraction has the same appearance as the unfractionated matrix when examined by thin-section electron microscopy (Fig. 5). The material is present in the form of discrete structures, many of them closed structures, and there is little if any lipid bilayer associated with the structures.

Discussion

The results reported here demonstrate the occurrence of novel, major membrane proteins having molecular masses of ~20 and 40 kD as determined by gel filtration. The proteins account for ~75% of the matrix protein and almost 15% of the total plasma membrane protein. They remain as insoluble macrostructures after detergent extraction of the membranes and subsequent treatment with EGTA, which releases a set of peripheral membrane proteins associated with the membrane skeleton. These peripheral proteins have molecular masses of 70, 69, 38, and 36 kD. The 70 kD, 69 kD doublet is very likely the murine equivalent of the 68-kD Ca⁺⁺-binding protein that Owens and Crumpton (29) have isolated from the detergent-insoluble fraction of plasma membranes from human and pig lymphocytes. The 45-kD protein, previously shown to be actin, remains associated with the 20 and 40-kD EGTA-insoluble proteins and accounts for ~5–10% of the EGTA-insoluble fraction.

While direct proof of the plasma membrane origin of the 20 and 40-kD proteins will only become possible when anti-

bodies specific for these proteins have been obtained, the available evidence indicates that this is almost certainly the case. The isolation procedure used to obtain the membranes for the experiments reported here results in $\sim 50\%$ yield of highly enriched plasma membranes, as assessed by biochemical and morphological criteria (2, 21, 27). Based on yields, the plasma membrane accounts for $\sim 1.5\text{--}3\%$ of the total P815 cell protein, a value in good agreement with those obtained for other lymphoid cells (21). For the 20 and 40-kD proteins, which account for $\sim 15\%$ of the membrane protein, to result from a contaminant would require that some other component of the cell be present in large amounts in the membrane preparation. Very strong evidence for the plasma membrane origin of these proteins is also provided by their association with 5'-nucleotidase. This enzyme provides a good plasma membrane marker for P815 cells, as $>90\%$ of the detectable activity has been shown to be present at the cell surface (2, 27). All of the 5'-nucleotidase activity remains associated with the isolated Triton-insoluble matrix, as evidenced by co-migration of the enzyme with the bulk of the protein on sucrose density gradients (27). Although we were not aware of the 20 and 40-kD proteins when those experiments were done (27), it is now clear that these represented the majority of protein being measured. Finally, the previously reported experiments examining Triton extraction of P815 cells (2; discussed in more detail below), lend further support to the conclusion that the 20 and 40-kD proteins are plasma membrane proteins, since these are the only detergent-insoluble membrane components present in sufficient amounts to account for the peripheral protein layer seen in the extracted cells. Whether the 20 and 40-kD proteins are present exclusively in the plasma membrane is not known.

Despite their relatively low apparent molecular weights on gel filtration, the 20 and 40-kD proteins fail to enter SDS PAGE gels under a variety of conditions, which probably explains why these major membrane components have not been previously described. We do not yet know the explanation for this unusual behavior. It does not appear to result from an abnormally low level of SDS binding by the proteins (Table II). It may be the case that the 20 and 40-kD proteins undergo extensive aggregation, even in SDS, under the conditions used for electrophoresis. Some precedent for protein aggregation on SDS PAGE is provided by the gap junction protein, which runs as multiple higher molecular weight bands representing aggregates if treated at 100°C in SDS before electrophoresis. However, if the sample is solubilized in SDS at room temperature, then the protein runs at its monomer molecular weight (13). The 20 and 40-kD proteins fail to enter gels whether solubilized at room temperature or at 100°C and regardless of whether they are not reduced, reduced, or reduced and alkylated.

The 20 and 40-kD proteins, as well as the EGTA-insoluble material >40 kD, have very similar mobilities on reverse phase HPLC (Fig. 4). Furthermore, preliminary amino acid analysis of the proteins indicates that they have very similar amino acid compositions (unpublished data). These observations raise the possibility that some aggregation occurs even under the conditions used for gel filtration and that the 40-kD and higher molecular weight proteins might represent aggregates of the 20-kD protein. Inconsistent with this suggestion is the finding that the three components are found to be present in constant ratios when multiple independent prepa-

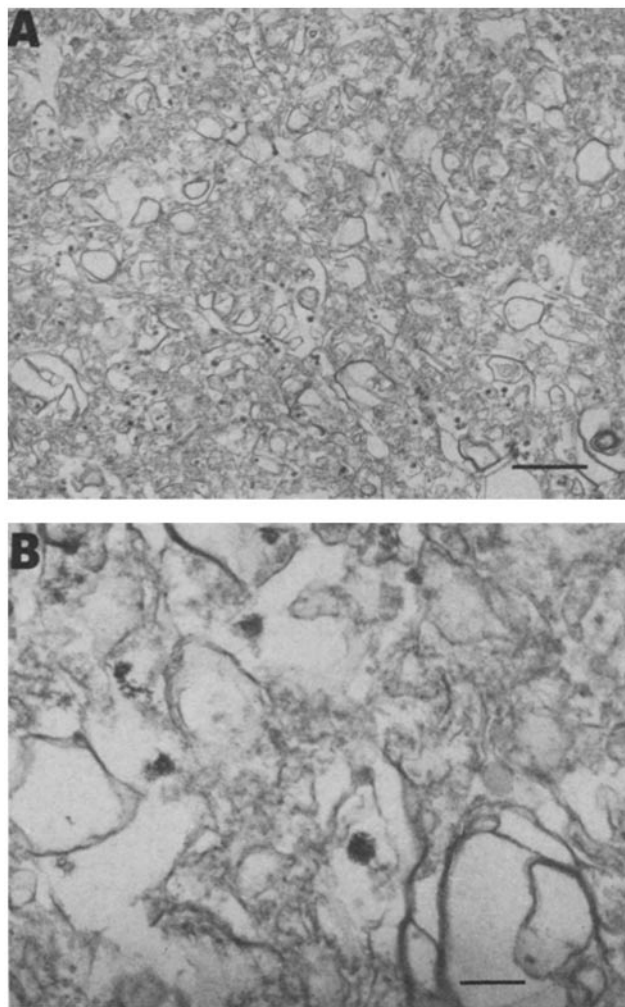


Figure 5. Thin-section electron micrographs of the EGTA-insoluble fraction of the plasma membrane matrix. Bars: (A) $1\ \mu\text{m}$; (B) $0.2\ \mu\text{m}$.

rations are examined and that re-chromatography of the isolated proteins does not result in appearance of any of the other forms.

We had previously suggested that the detergent-insoluble matrix of plasma membranes might represent a membrane skeletal structure composed of a set of interacting proteins that provide mechanical support for the membrane bilayer (2, 27). Further support for this suggestion has come from the demonstration that detergent treatment of intact P815 cells under the same conditions used to isolate the membrane matrix results in structures having a nuclear remnant, a largely empty cytoplasmic space and a continuous protein layer at the cell periphery (2). Stability of this peripheral protein layer did not depend on either the lipid bilayer, which was removed by detergent extraction, or the filamentous cytoskeletal components, which were not preserved under the extraction conditions used. Components of the plasma membrane matrix were present in the detergent-insoluble structures derived from cells but it has not been directly demonstrated that they make up the peripheral layer. However, alternative explanations appear unlikely, as they would imply the existence of an additional detergent-insoluble

Table III. Protein Composition of the TX-100-insoluble Plasma Membrane Matrix*

	Weight %	Estimated mole %
EGTA-insoluble components		
Excl (>70 kD)‡	15	6.8§
40 kD	24	19.5
20 kD	31	50.3
Actin	5	3.6
EGTA-soluble components		
70 kD	4	1.8
69 kD	1	0.4
38 kD	10	8.5
36 kD	10	9.0

* Estimates are based on protein determinations, staining intensities on SDS PAGE relative to known amounts of standard proteins, and on apparent molecular weights from SDS PAGE and gel filtration.

‡ Excl, excluded material.

§ Based on assuming an average molecular mass of 70 kD.

structure at the cell periphery that is not comprised of the known filamentous cytoskeletal components and does not copurify with plasma membranes upon subcellular fractionation.

In support of the idea that the matrix proteins form a membrane skeleton, Owens et al. (29, 30) have purified and prepared an antibody specific for the 68-kD component of the detergent-insoluble membrane matrix of human and pig lymphocyte plasma membranes. Using this antibody, they have demonstrated that the 68-kD protein is present in both lymphoid and nonlymphoid cells and that it was present in a lamina-like network in detergent-extracted 3T3 cells. Based on these observations they have also suggested that the 68-kD protein is part of a submembranous cytoskeletal complex in a variety of cell types (30).

The fact that the matrix remains present as intact macrostructures (Fig. 5) after EGTA extraction to remove the peripheral proteins indicates that the 20 and 40-kD proteins are the major structural elements of the matrix, and thus of the putative membrane skeleton. Based on protein determinations, relative staining intensities on SDS PAGE, and ratios from gel filtration profiles, estimates can be made of the relative amounts of the various matrix components and their mole percent occurrence in the matrix. These results are shown in Table III. The novel 20-kD, 40-kD, and excluded proteins clearly constitute the bulk of the matrix, accounting for ~70% of the total matrix protein by weight and close to 15% of the total plasma membrane protein. Thus, the amounts of the 20 and 40-kD proteins in the membrane are consistent with their being the major structural elements of the putative membrane skeleton. On a molar basis, these proteins account for ~78% of the proteins present in the matrix. Using values obtained for total cell protein and membrane yields during purification (21), it is estimated that the plasma membrane accounts for 1.5–3% of the cell protein and it can be calculated that there are $10\text{--}20 \times 10^6$ copies of the 20-kD, 40-kD, and excluded proteins per cell. The EGTA-soluble proteins are present in relatively small amounts and do not appear to be necessary for the structural stability of the matrix.

It appears likely that a similar set of proteins is a common feature of lymphoid cell plasma membranes. Examination of plasma membranes from RDM-4, a murine lymphoma, and

a human lymphoid hybridoma cell line have shown both to have a detergent-insoluble, EGTA-insoluble fraction that accounts for ~15–20% of the total membrane protein (unpublished). In both cases, solubilization of this material in SDS followed by size exclusion chromatography gave elution profiles essentially the same as that shown in Fig. 2, II A for the insoluble fraction from plasma membranes of P815 cells. A similar set of proteins may also be present in association with the plasma membranes of other cell types. Several reports of examination of detergent-resistant cytoskeleton preparations of adherent cells have noted the occurrence of a lamina that overlies the filamentous cytoskeletal meshwork and appears to derive from the membrane (6, 10, 20, 36). Also, as noted above, Owens et al. (30) have found that a protein cross-reactive with an antibody made against the 68-kD protein of the human lymphocyte plasma membrane matrix stains a lamina-like network on detergent-extracted fibroblasts.

The 20-kD, 40-kD, and excluded proteins clearly interact extensively to form large macrostructures, at least after detergent extraction of membranes, and may undergo some aggregation even in the presence of SDS. Based on these properties, we propose that they be named agorins from the Greek *agora*, which means assembly and is a cognate of gregarious, meaning “tending to associate with others of one’s kind.” The 20-kD protein will be referred to as agorin I and the 40-kD protein as agorin II.

The evidence available thus far indicates that the agorins are exposed at the cytoplasmic face of the plasma membrane, based on their association with the set of peripheral, EGTA-soluble proteins and on the fact that they are labeled upon lactoperoxidase-catalyzed iodination of isolated membranes but not of intact cells (unpublished). The association of these proteins with some of the membrane phospholipid after detergent extraction suggests that they may also be integral membrane proteins. This is also suggested by the finding that mixing of isolated membrane matrix with lipids in deoxycholate and dialyzing to remove the detergent results in selective association of the reconstituted lipid bilayers with the matrix structures (14).

The fact that the agorins are so extensively associated with each other after detergent extraction of either membranes or cells, together with the large amounts of these proteins present in the membrane, strongly suggests that they are interacting to form a membrane skeletal structure whose stability is independent of either the lipid bilayer or filamentous cytoskeletal components. The possibility exists that the extensive interactions to form the isolated macrostructures do not occur in the native membrane, and only form upon detergent extraction to remove the other membrane components. This would appear unlikely, as it would require that the associations form sufficiently rapidly and extensively to result in the continuous peripheral protein layer seen when intact cells are detergent extracted (2), but this possibility cannot be completely ruled out at the present time. A detailed understanding of the nature of the associations of the agorins in both the detergent-insoluble structures and in the native plasma membrane will be necessary to fully address this question.

While the membrane matrix of P815 cells bears some resemblance to the spectrin-containing skeleton of erythrocytes (7, 24, 25), in that it can be isolated by removal of the

other membrane components with nonionic detergents (39), it clearly differs with respect to its composition. Spectrin and several other red blood cell cytoskeletal proteins have been found in a wide variety of nonerythroid cells (5, 11, 19). Proteins that cross-react with antibodies against spectrin, ankyrin, and band 4.1 are also present in P815 cells, but none of these proteins copurify with the plasma membrane under the conditions we use for membrane isolation (35; and Spiegel, J., S. Lux, J. Apgar, and M. Mescher, unpublished results). If the agorins are integral membrane proteins, as their association with some of the membrane lipid might suggest, then the membrane matrix of P815 cells may also differ from that of red cells in being a true component of the membrane, rather than a submembranous skeleton anchored to the bilayer via interactions with transmembrane proteins, as is the case for the spectrin-containing skeleton. Despite these differences in composition, and possibly in organization with respect to the membrane bilayer, the P815 membrane matrix may play some of the same roles as the erythrocyte matrix, such as providing mechanical stability to the bilayer (18) or influencing the mobility of transmembrane proteins at the cell surface.

The fluid mosaic model of biological membranes (34) has proved useful in understanding many of the properties of membrane proteins and lipids, but does not explain the observations that, in general, diffusion of lipids is two or more orders of magnitude faster than that of the membrane proteins. Based on determinations of lateral diffusion coefficients of lipids and proteins in normal and spectrin-deficient spherocytic mouse erythrocytes, Koppel et al. (16) have presented a mathematical description of a matrix-control model of membrane protein diffusion. In this model, the dominant factor in controlling diffusion of transmembrane proteins is steric hindrance by a matrix, rather than direct binding of the proteins to cytoskeletal elements. Spectrin and its associated proteins provide the matrix of erythrocytes, and the proposed model fits the data available on erythrocyte ghosts. The authors speculated that the model might also apply to surface proteins on other cell types. The agorins would appear to be very good candidates for making up such a diffusion controlling membrane matrix.

Irrespective of the putative membrane skeletal role of the agorins, their quantity and location in the membrane make them interesting candidates for mediating membrane-cytoskeleton interactions and being involved in associations with cell-surface proteins. A portion of the membrane-associated actin is tightly associated with the matrix (27) and sediments with the agorins after EGTA extraction (Fig. 1). This actin might serve as an attachment site for microfilaments to the cytoplasmic face of the membrane. It is also possible that the observed associations of cell surface glycoproteins with detergent-insoluble fractions might result from interactions of these proteins with the agorins of the matrix (3, 10, 34, 36).

We thank William Hamilton, Childrens Hospital Medical Center, Inc., Boston, for performing the electron microscopy.

This work was supported by National Institutes of Health Grant CA-30381. Dr. Apgar was supported by a Postdoctoral Fellowship from the Leukemia Society of America, Inc. This work was performed in part at the Department of Pathology, Harvard Medical School.

Received for publication 16 October 1985, and in revised form 24 April 1986.

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