

Triton X-100 Extraction of P815 Tumor Cells: Evidence for a Plasma Membrane Skeleton Structure

JOHN R. APGAR, STEVEN H. HERRMANN, JOHN M. ROBINSON, and
MATTHEW F. MESCHER

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115. Dr. Apgar and Dr. Mescher are currently with the Medical Biology Institute, La Jolla, California 92037.

ABSTRACT It has been shown that a Triton X-100-insoluble protein matrix can be isolated from the plasma membranes of P815 tumor cells and murine lymphoid cells (Mescher, M. F., M. J. L. Jose and S. P. Balk, 1981, *Nature (Lond.)*, 289:139-144). The properties of the matrix suggested that this set of proteins might form a membrane skeletal structure, stable in the absence of the lipid bilayer. Since purification of plasma membrane results in yields of only 20 to 40%, it was not clear whether the matrix was associated with the entire plasma membrane. To determine if a detergent-insoluble structure was present over the entire cell periphery and stable in the absence of the membrane bilayer or cytoskeletal components, we have examined extraction of whole cells with Triton X-100. Using the same conditions as those used for isolation of the matrix from membranes, we found that extraction of intact cells resulted in structures consisting of a continuous layer of protein at the periphery, a largely empty cytoplasmic space, and a nuclear remnant. Little or no lipid bilayer structure was evident in association with the peripheral layer, and no filamentous cytoskeletal structures could be seen in the cytoplasmic space by thin-section electron microscopy. Analysis of these Triton shells showed them to retain ~15% of the total cell protein, most of which was accounted for by low molecular weight nuclear proteins. 5'-Nucleotidase, a cell surface enzyme that remains associated with the plasma membrane matrix, was quantitatively recovered with the shells. Included among the polypeptides present in the shells was a set with mobilities identical to those of the set that makes up the plasma membrane matrix. The polypeptide composition of the shells further confirmed that cytoskeletal proteins were present to a very low extent, if at all, after the extraction. The results demonstrate that a detergent-insoluble protein matrix associated with the periphery of these cells forms a continuous, intact macrostructure whose stability is independent of the membrane bilayer or filamentous cytoskeletal elements, and thus has the properties of a membrane skeletal structure. Although not yet directly demonstrated, the results also strongly suggest that this peripheral layer is composed of the previously described set of plasma membrane matrix proteins. This article discusses possible roles for this proposed membrane skeletal structure in stabilizing the membrane bilayer and affecting the dynamics of other membrane proteins.

The plasma membrane of cells is composed of the lipid bilayer, integral membrane proteins, and peripheral proteins associated with, but not embedded in, the bilayer (1, 2). In the erythrocyte membrane, a set of peripheral proteins including spectrin, actin, band 4.1, and ankyrin interact to form a membrane skeleton associated with the cytoplasmic face of the membrane (3-5). This skeleton is a rigid layer that provides mechanical stability to the membrane (6), plays a role in determining morphology of the cell (6, 7) and appears to

influence cell surface protein mobility (8) and lipid distribution in the bilayer (9-11). Study of the mammalian erythrocyte membrane skeleton has been facilitated by the absence of other cytoskeletal structures in these cells. Thus, Triton X-100 (TX-100)¹ extraction of cells or membranes solubilizes the bilayer and most integral membrane proteins, leaving

¹ *Abbreviations used in this paper:* PBES phosphate-buffered Earle's salts; TX-100, Triton X-100.

only the spectrin matrix and associated proteins in an insoluble and thus readily isolated form (12).

In contrast, many nucleated cells have extensive cytoskeletal systems that include microtubules, microfilaments, and intermediate filaments (13, 14). There is some evidence for associations between transmembrane proteins and these cytoskeletal elements but it has remained unclear whether the plasma membranes of nucleated cells have associated with them a membrane skeleton distinct from the filamentous cytoskeletal systems. Organized cytoskeletal systems are readily apparent in adherent cells such as fibroblasts, and Triton extraction of such cells leaves the nucleus and filamentous cytoskeleton insoluble (15-17). Some studies have noted that the cytoskeleton of these preparations is covered by a lamina that appears to derive from the plasma membrane and includes some cell surface proteins (18-21). Whether these laminae represent membrane skeletal structures that are stable independently of the filamentous cytoskeletal network has not been determined.

Cells that normally grow in suspension, such as lymphocytes, lymphomas, and mastocytomas, do not have extensive filamentous cytoskeletal structure under normal conditions and may provide a simpler means of investigating the possible presence of a distinct membrane skeletal structure. It was previously shown (22) that Triton extraction of plasma membranes isolated from such cells yielded an insoluble residue consisting of a discrete set of membrane proteins. This insoluble fraction accounted for ~20% of the total membrane protein and included proteins with molecular weights of 70,000, 69,000, 38,000, and 36,000, as well as part of the membrane-associated actin and all of the 5'-nucleotidase. More recently, Davies et al. (23) reported the isolation and characterization of a Nonidet P-40-insoluble plasma membrane fraction from human lymphoblastoid cells and pig lymph node lymphocytes. The detergent-insoluble fraction from these membranes had properties and composition very similar to those of the insoluble matrix isolated from P815 and other murine cells (22).

The insoluble matrix of P815 cells was present as structures that had about the same size distribution as the membrane vesicles they were isolated from (22). The material appeared relatively amorphous in thin section and had little or no lipid bilayer structure remaining. Vectorial labeling studies indicated that this set of proteins was present at the inner (cytoplasmic) face of the membrane. The presence here and the properties of this matrix of proteins suggested that it might form a membrane skeletal structure. However, yields of plasma membrane upon purification are typically 20 to 40%. The possibility existed, therefore, that the membrane matrix might be associated with only some regions of the membrane, and not be continuous over the entire inner membrane surface.

This report presents evidence that demonstrates that detergent extraction of whole P815 tumor cells under appropriate conditions yields Triton shells with remnants of a nuclear structure, a largely empty cytoplasmic space, and a layer of protein at the periphery. Little or no lipid bilayer remains at the periphery and most cell surface and integral membrane proteins are extracted under these conditions. Morphological and biochemical evidence demonstrates the peripheral layer to be distinct from previously described cytoskeletal structures and strongly suggests that it is composed of the plasma membrane matrix proteins.

MATERIALS AND METHODS

Cells and Nuclei: P815, a murine mastocytoma of DBA/2 origin, was maintained by passage in ascites in (BALB/c × DBA/2)_{F1} (Cumberland View Farms, Clinton, TN) or (AKR × DBA/2)_{F1} (Jackson Laboratory, Bar Harbor, ME) mice.² Animals were injected intraperitoneally with 10⁷ cells in phosphate-buffered Earle's salts (PBES; Earle's salts buffered at pH 7.2 with 10 mM sodium phosphate). Peritoneal fluid was collected 6 d later and the cells (2-4 × 10⁶/mouse) were washed 3 times in PBES.

We prepared nuclei by lysing cells by nitrogen cavitation and then differentially centrifuging the lysate. Freshly harvested cells were washed, suspended in PBES at 10⁸ cells/ml, placed in a nitrogen bomb (Parr Instrument Co., Moline, IL), and equilibrated at 400 psi of nitrogen for 5 min. The lysate was then centrifuged at 3,600 g for 15 min to pellet the nuclei. This procedure results in 90 to 95% cell lysis and ~70 to 90% recovery of nuclei (22).

Plasma Membrane Purification: Plasma membranes were purified as previously described (24) with minor modifications. In brief, cells were suspended at 10⁸ cells/ml in PBES containing 0.2 mM phenylmethylsulfonyl fluoride. Cells were lysed by nitrogen cavitation in a nitrogen bomb after equilibration at 400 psi for 5 min. The homogenate was centrifuged 15 min at 3,600 g and the resulting pellet was resuspended in PBES and again centrifuged at 3,600 g. The supernatants of the two low speed spins were combined and centrifuged for 30 min at 22,000 g. The resulting high speed pellet was resuspended in 10 mM Tris buffer, pH 7.2 and sucrose added to a final concentration of 37%. This suspension was placed in the bottom of centrifuge tubes and an equal volume of 25% sucrose in 10 mM Tris, pH 7.2, was layered on top. The tubes were then centrifuged for 16 h at 22,000 rpm in a Beckman SW 50.1 rotor (Beckman Instruments Inc., Palo Alto, CA). After centrifugation, the interface band (plasma membrane) was removed with a pipette and diluted with 5 vol 10 mM Tris pH 7.2. The pellet (endoplasmic reticulum fraction) was resuspended in the same buffer. The fractions were then pelleted by centrifugation for 120 min at 100,000 g and the final pellets were resuspended in PBES.

This procedure results in a 30- to 60-fold purification of plasma membrane based on enrichment of 5'-nucleotidase activity (24, 25). The yield of plasma membrane was found to depend heavily on the manner in which the 22,000 g membrane pellet was resuspended before sucrose density gradient centrifugation. Use of a Dounce homogenizer to resuspend this pellet, as done previously (24), results in recovery of ~25% of the plasma membrane (based on 5'-nucleotidase activity) at the gradient interface. We found that more vigorous resuspension of the 22,000 g pellet by short bursts of sonication in a bath sonicator increased the plasma membrane yield to ~50% without significantly changing the specific activity of 5'-nucleotidase in this fraction. There was a corresponding decrease in the amount of plasma membrane recovered in the pellet (endoplasmic reticulum enriched) fraction. We used this procedure for the plasma membrane isolations described in this report.

Triton Extraction: Cells or nuclei were suspended in PBES at 2 × 10⁷ cells/ml and an equal volume of 1% TX-100 (Sigma Chemical Co., St. Louis, MO) in PBES added with gentle mixing. Phenylmethylsulfonyl fluoride was present at 0.2 mM to inhibit proteolysis. In some cases, deoxyribonuclease I and ribonuclease A (Sigma Chemical Co.) were included at final concentrations of 0.5 mg/ml. Extraction on ice for 20 min was done with occasional mixing by inversion of the tube. We avoided vortexing and vigorous pipetting, as a decreased yield of Triton shells was obtained if the suspension was subjected to strong shear forces. After extraction, Triton shells were sedimented by centrifugation at 1,000 rpm for 8 min. Yields of Triton shells, as determined by light microscopy, were routinely 90 to 100% of the starting cell number.

Microscopy: Samples were extracted as described above, diluted 10-fold with extraction buffer, and made 1% in glutaraldehyde. After incubation for 20 min at 4°C, samples were pelleted by centrifugation for 8 min at 1,000 rpm and resuspended in extraction buffer. Intact cells and nuclei were treated identically with the exception that detergent was not included in the buffers. We examined samples immediately using either phase-contrast optics or Nomarski optics with a Zeiss, model UEM. In this case, videomicrographs were stored on videotape with a contrast enhancement camera (Dage-MTI Inc., Wabash, MI) and subsequently photographed from the monitor using Kodak Plus-X Pan film.

Whole cells and Triton shells (made using nucleases) were prepared for

² Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. (NIH) 78-23, revised 1978).

electron microscopy by fixation in 2% glutaraldehyde in PBS (pH 7.4) for 45–60 min at room temperature. Fixed specimens were washed three or four times in PBS before they were further processed. Specimens were osmicated in 2% OsO₄ in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Osmicated specimens were stained en bloc in 1% uranyl acetate or left unstained before dehydration. Routine dehydration was in a graded series of ethanol solutions. In some preparations the specimens were stained en bloc with 1% hafnium chloride. Hafnium staining was carried out in 100% acetone after dehydration in a graded series of acetone solutions (M. J. Karnovsky, personal communication). Another fixation protocol that includes tannic acid in the primary glutaraldehyde step as described by Maupin and Pollard (26) was also used. After dehydration, specimens were infiltrated and embedded in Epon 812. Thin sections were cut on a diamond knife, collected on bare copper grids, and stained with aqueous uranyl acetate and lead citrate. Sections were examined in the micrographs taken with a Philips 200 electron microscope operated at 60 kV.

Lactoperoxidase-catalyzed Iodination: Cell surface proteins were labeled with ¹²⁵I by lactoperoxidase-catalyzed iodination (27) as previously described in detail (28).

Analytical: We determined protein by the method of Lowry et al. (29) in the presence of 1% SDS using bovine serum albumin as the standard. 5'-Nucleotidase activity was assayed as described by Avruch and Wallach (30). SDS-polyacrylamide slab gel electrophoresis was done with the buffer system of Laemmli (31) on a running gel consisting of a 5–15% polyacrylamide gradient. Samples, in application buffer, were reduced with 10 mM β-mercaptoethanol, incubated for 3 min in a boiling water bath and made 20 mM in iodoacetamide before electrophoresis. Gels were stained with Coomassie Brilliant Blue after electrophoresis.

RESULTS

Morphology of Structures Remaining after Triton Extraction of Cells

P815 cells were treated with 0.5% TX-100 for 20 min at 4°C in PBES, the same conditions used to isolate the membrane matrix, and examined by phase-contrast microscopy. The treated samples had the appearance of relatively intact nuclei surrounded by an empty baglike structure (Fig. 1). Recovery of these structures was 90 to 100% of the starting cell number. In several instances, dense granules could be seen within the bags. These were free moving but clearly confined within the bag. The appearance of these Triton shells suggested that the cytoplasmic contents had largely been extracted but that a TX-100-insoluble structure remained at the cell periphery. Disappearance of this baglike structure upon treatment of the Triton shells with 1 mg/ml Pronase (Calbiochem-Behring Corp., La Jolla, CA) at 4°C showed the structures to be dependent on protein for stability.

Triton shells obtained by simple extraction with 0.5% TX-100 had variable amounts of amorphous material associated with them (Fig. 1B). Furthermore, they were relatively unstable. Upon standing at room temperature the nuclei swelled and lysed, resulting in our inability to visualize clearly the bag-like structure and in extensive aggregation of the material. Washing the shells by centrifugation removed some but not all of the amorphous material and did not improve the stability of the structures. We noted also that resuspension of the shells after pelleting was effective only if detergent was included in the buffer. In the absence of detergent, the structures remained extensively aggregated.

The instability of the nuclear structures within the Triton shells suggested that removal of the nucleic acid might result in more stable and cleaner preparations that have less amorphous material associated with them. When cells were extracted with TX-100 in the presence of DNase and RNase at 0.5 mg/ml, the resulting insoluble structures were barely, if at all, discernible by phase-contrast microscopy. However, when

examined by Nomarski optics with video enhancement, structures that had a nuclear remnant, a largely empty cytoplasmic space, and a continuous peripheral layer were seen (Fig. 2B). The average diameter of these structures was 30 to 40% less than that of intact (unextracted) cells (Table I), raising the possibility that this outer layer derived from the nuclear membrane and not from the cell periphery. A nuclear origin for the peripheral layer was argued against, however, by the observations that intact cells contained detergent-resistant droplets (presumably lipid droplets) within their cytoplasmic space and that these vesicles were clearly trapped in the space between the periphery and the nuclear remnant after extraction (indicated by arrowheads in Fig. 2, A and B). These droplets were relatively immobile within the cytoplasm of intact cells but moved freely within this space after extraction. In all cases where droplets were observed, this movement was clearly confined by the peripheral layer of the shells.

To assess more directly the possibility of a nuclear origin of the peripheral layer, we examined isolated nuclei before and after extraction with TX-100 under the same conditions as those used for preparation of the Triton shells. We saw no peripheral layer after extraction of the isolated nuclei (Fig. 2C). Furthermore, the average diameter of the extracted nuclei was the same as that of the nuclear remnants seen within the Triton shells (Table I). Thus, the structures that remain visible by light microscopy after TX-100 extraction of

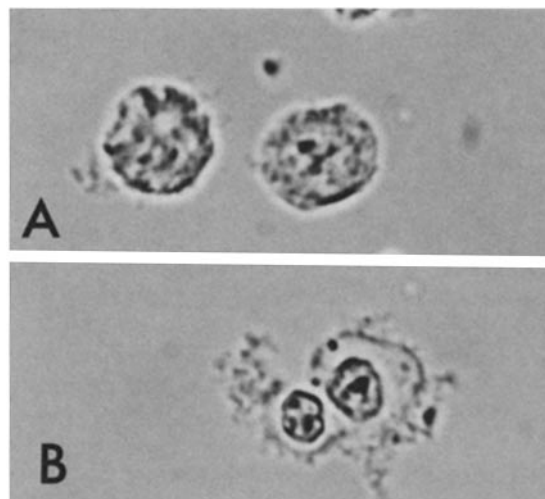


FIGURE 1 Phase contrast micrographs of P815 cells and Triton shells. Triton shells were prepared in the absence of nucleases, as described in Materials and Methods, resuspended in 0.5% TX-100 in PBES and examined directly. Whole cells were kept in PBES throughout. (A) Whole cells. (B) Triton shells.

TABLE I
Size (in Micrometers) of Cells, Shells, and Nuclei*

	Intact cell	Shell		TX-100-extracted nucleus
		Periphery	Nucleus [†]	
Expt 1	19.9 ± 1.9	14.7 ± 1.8	7.8 ± 1.5	ND
Expt 2	20.2 ± 4.0	13.3 ± 1.9	7.0 ± 1.3	7.5 ± 2.1

Expt, experiment. ND, not determined.

* Sizes were determined by measurement of images on the video monitor (see Materials and Methods) and conversion to micrometers by use of a reference grid. At least 50 measurements were made for each sample; the standard deviations of the values are shown.

[†] The diameter of the nuclear remnant present in the Triton shell.

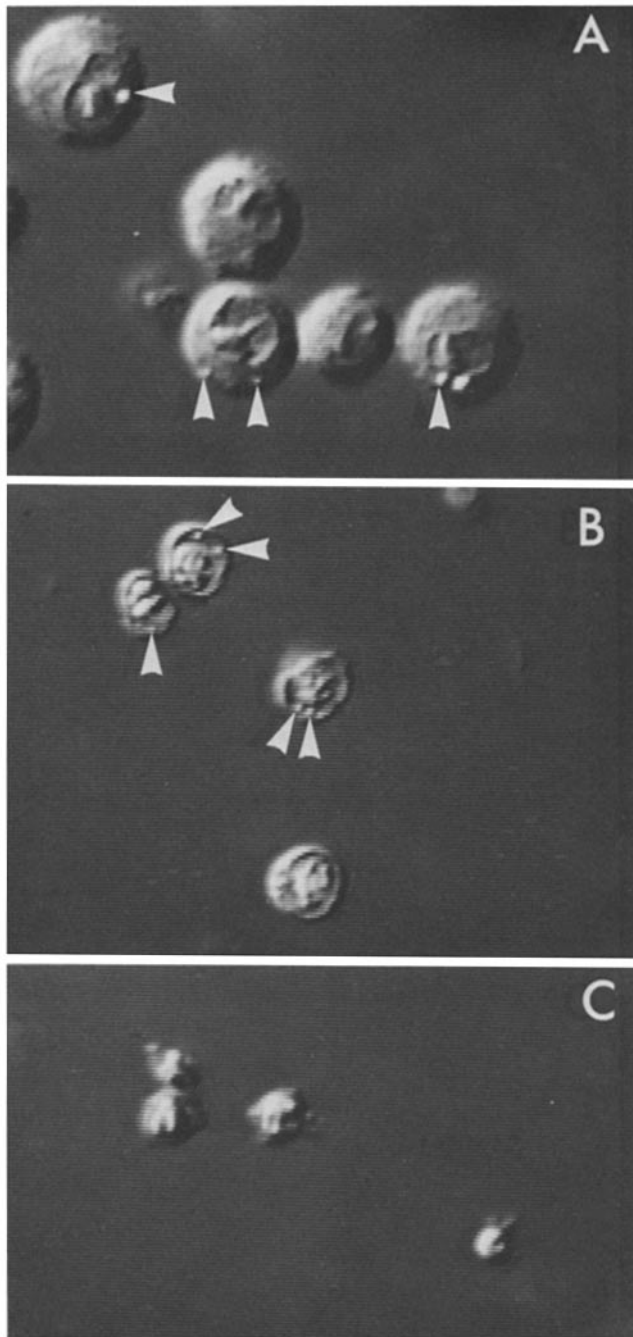


FIGURE 2 Light micrographs of cells, Triton shells, and Triton-treated nuclei. Samples were prepared and examined by use of Nomarski optics and a video enhancement camera as described in Materials and Methods. $\times 720$. (A) Intact P815 cells. (B) Triton shells prepared by TX-100 extraction of cells. (C) Isolated nuclei extracted with TX-100. Arrowheads indicate lipid droplets.

cells included the nuclear remnant, detergent-resistant droplets (lipid droplets) confined to the cytoplasmic space and a continuous shell which appears to derive from the cell periphery.

Examination of nuclease-treated Triton shells by thin-section electron microscopy confirmed the observations made by light microscopy and showed that the peripheral layer was uniformly present surrounding the nuclear structures (Figs. 3 and 4). Shells were examined after glutaraldehyde fixation, osmication, and staining with either hafnium chloride (Figure

3) or uranyl acetate (not shown) or after fixation by the procedure described by Maupin and Pollard (26) employing tannic acid (Figure 4). In every case, the Triton shells had an intact nuclear remnant, a relatively empty cytoplasmic space, and a continuous layer of material at the periphery. Except for occasional small regions, this peripheral layer did not have the appearance of a membrane bilayer structure. The difficulty in visualizing the peripheral layer by phase-contrast microscopy may be due to the small amount of material present in this layer. The cytoplasmic droplets that resist detergent solubilization were seen in the thin sections to be external to the nuclear remnant but clearly confined by the peripheral layer.

The continuity and uniform occurrence of the peripheral layer very strongly suggests that a detergent-insoluble structure is present at the cell periphery which persists as an intact structure after extraction. Whether all of the material seen at the periphery composes this structure, or whether some of it may be cytoplasmic- or nuclear-derived insoluble material associated with the structure is unclear. No detailed fine structure was apparent in the peripheral layer in these preparations.

Neither the occurrence nor the stability of the peripheral layer remaining after Triton extraction depended on filamentous cytoskeletal components. Few if any filamentous components were detected within the cytoplasmic space of the shells by thin-section electron microscopy (Figs. 3 and 4) although the conditions used for fixation and staining allowed visualization of these components, if present. The P815 cells used in these experiments were grown as ascites in mice and therefore were slightly contaminated with macrophage-like cells (<1–2%). One such cell is shown in Fig. 4. In marked contrast to the P815 cells, the plasma membranes of these cells were much less effectively extracted by Triton, and filamentous structures were seen in the cytoplasm (Fig. 4, arrowheads).

The absence of organized cytoskeletal elements after the Triton extraction used in these experiments is not surprising. P815 cells appear to have a less extensive cytoskeletal system than that present in adherent cells (Fig. 5). More important, the conditions used for extraction are unlikely to allow preservation of such structures. Microtubules would not be stable under these conditions, and the DNase present during the extraction probably will result in microfilament depolymerization. It is also possible that phenylmethylsulfonyl fluoride-insensitive proteases released during the extraction further contribute to the instability of cytoskeletal elements. No efforts have been made to find conditions that would preserve the filamentous systems, if present. The relevant point is that their absence after extraction, for whatever reason, demonstrates the stability of the detergent-insoluble peripheral layer to be independent of these components.

Composition of Structures Remaining after Triton Extraction

The conditions used above for Triton extraction of intact cells are identical to those previously shown to preserve the membrane matrix of purified plasma membranes (22). The occurrence of a continuous protein layer at the periphery of the extracted cells is consistent with this matrix being continuous over the entire plasma membrane and persisting as an intact macrostructure after removal of most of the membrane

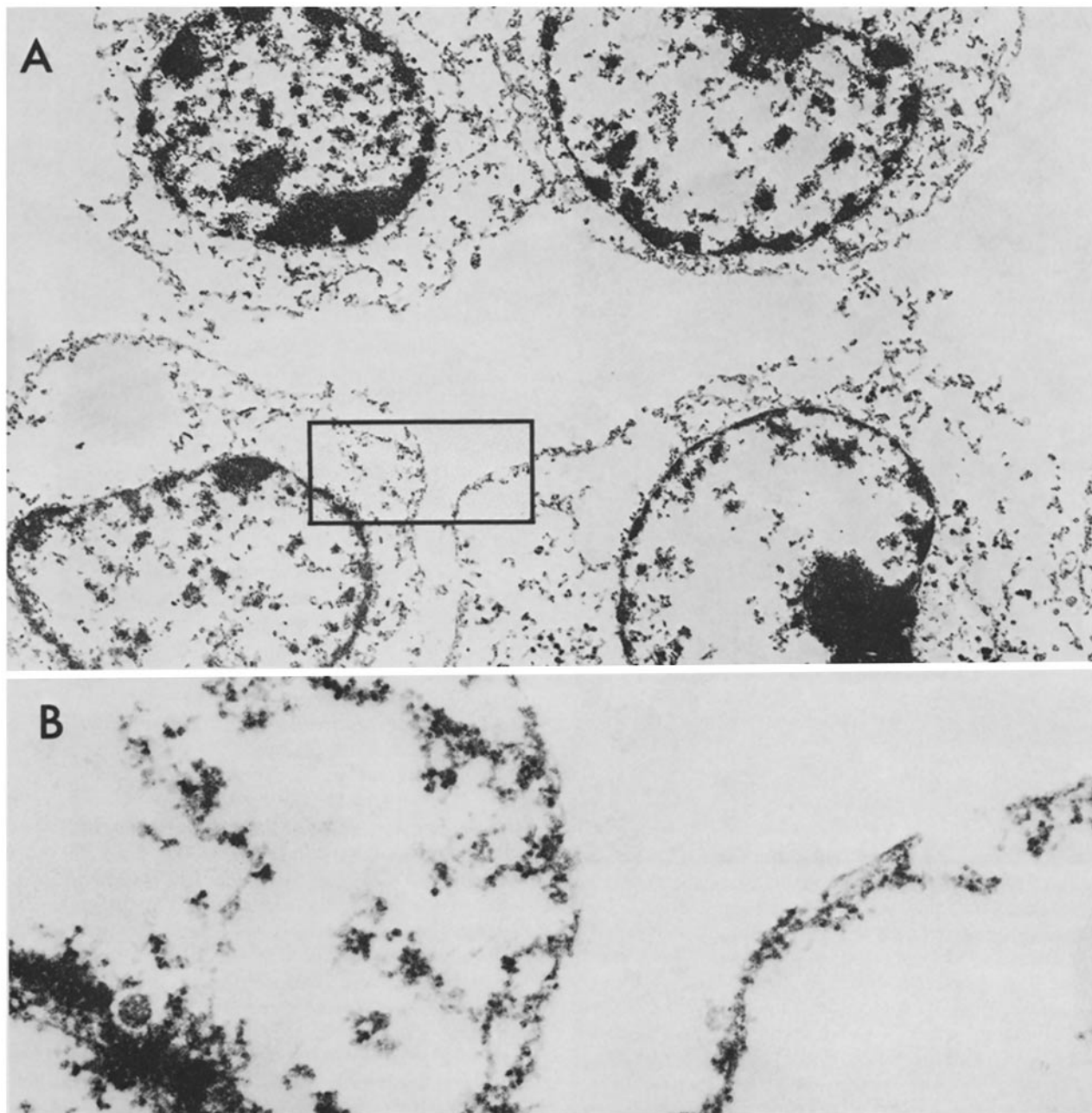


FIGURE 3 Thin-section electron micrographs of Triton shells. P815 cells were extracted with Triton in the presence of nucleases, prepared for microscopy as described in Materials and Methods, and stained en bloc with 1% hafnium chloride. (A) $\times 8,800$. (B) $\times 70,000$.

protein and lipid. At the same time, it is clear from the appearance of the extracted cells that other cellular components remain after extraction, including the nuclear remnants, which appear to account for the bulk of material in the shells (Figs. 3 and 4). To characterize further the Triton shells and determine if membrane matrix components are present, we examined the protein composition of the shells.

The conditions used here for detergent extraction are the same as those routinely used to solubilize transmembrane proteins such as H-2 or Ia antigens (32) or immunoglobulin (28) before immunoprecipitation or affinity purification. We confirmed effective solubilization of the membrane glycoproteins during preparation of the Triton shells by surface-labeling intact cells by lactoperoxidase-catalyzed iodination before

detergent treatment. Treatment of the ^{125}I -labeled cells with TX-100 resulted in the recovery in the supernatant of at least 85% of the label (Table II). Examination of the protein content of whole cells and Triton shells obtained from an equal number of cells showed that $\sim 15\%$ of the total cell protein was recovered in the shell preparation (Table II).

The polypeptide composition of subcellular fractions and of Triton shells was also examined in order to determine the origin of the shell components and to determine if proteins corresponding to those of the membrane matrix were present in the expected amounts. Fig. 6 shows the protein composition of Triton shells as compared with the composition of subcellular fractions obtained during membrane purification. The membrane fractionation scheme is summarized on the left

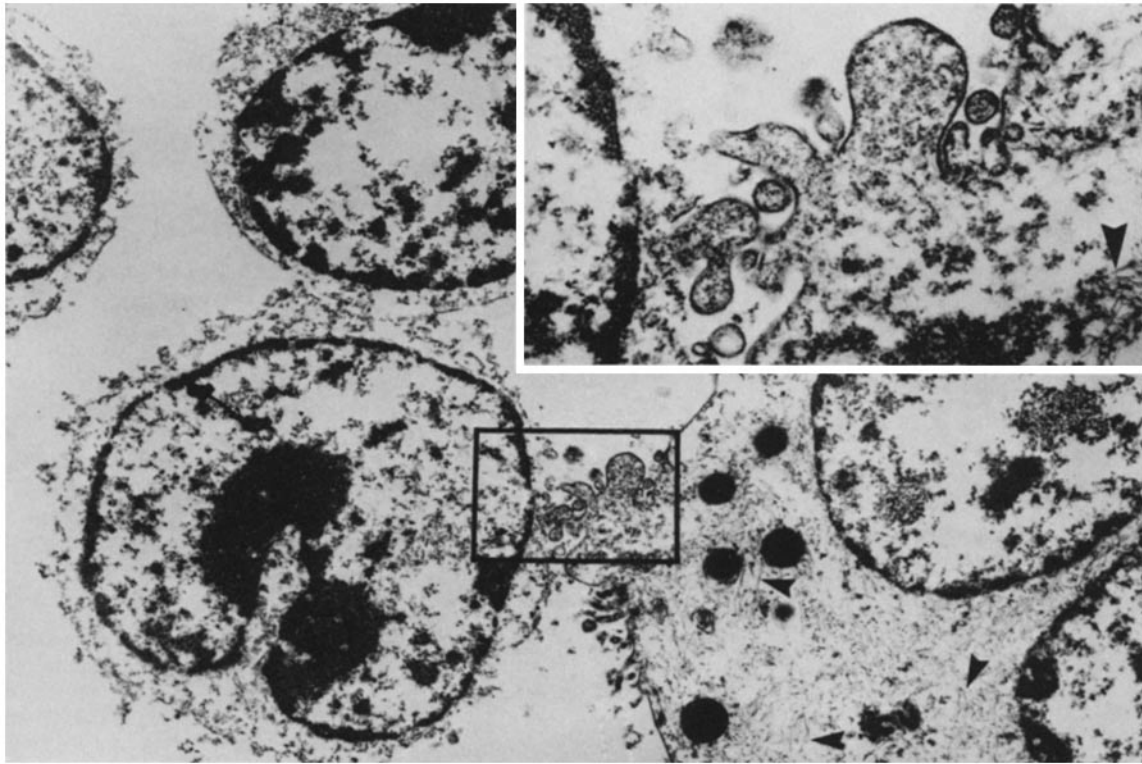


FIGURE 4 Thin-section electron micrographs of P815 Triton shells and a contaminating macrophage-like cell (*lower right*). Triton extraction was done in the presence of nucleases and sample was fixed by use of tannic acid in the primary glutaraldehyde step as described by Maupin and Pollard (26). Arrowheads indicate filamentous structures in the macrophage-like cell. $\times 8,500$. (*Inset*) $\times 34,000$.

side of Fig. 6. Aliquots of each of the numbered fractions obtained during purification were examined by SDS gel electrophoresis. The aliquots loaded on the gel contained material derived from the same number of starting cells to facilitate comparison (Fig. 6, lanes 1–7). A sample of Triton shells made from the same number of starting cells was run in parallel (Fig. 6, lane *A*). Triton shells retained ~15% of the cell protein (Table II), and most of this protein appeared to derive from the nucleus. The major proteins seen in the Triton shells (Fig. 6, lane *A*, regions *a* and *b*) are greatly enriched in the nuclear pellet which sediments at low speed upon cell fractionation (Fig. 6, lane 2). Comparison of the staining intensities of these proteins in Triton shells and low speed pellet indicates that most, if not all, of each of these proteins remains with the Triton shells. The low molecular weight proteins (region *b*) are probably histones.

Plasma membranes account for only 1.5 to 3% of the total cell protein and an amount of plasma membrane derived from the amount of cell homogenate in lane 1 (Fig. 6) gives only barely detectable protein bands (lane 7). A larger amount of purified plasma membrane was therefore compared directly with Triton shells. Plasma membrane from 3×10^6 cells (corrected for the yield obtained upon purification) was compared with Triton shells obtained from the same number of cells (Fig. 6, lanes *B* and *C*). Proteins of the same apparent molecular weights as the membrane matrix components are seen to be present in Triton shells. Furthermore, the staining intensities indicate that actin and the 38K and 36K proteins are present in amounts consistent with quantitative recovery of plasma membrane matrix in the Triton shells. This may also be the case for the 69K and 70K proteins but is less clear

owing to the occurrence of a more intensely staining protein of only slightly higher mobility in the Triton shells. It is interesting to note that the amount of actin that remained associated with the Triton shells could be accounted for by that associated with the plasma membrane matrix, consistent with the absence of microfilaments in these preparations.

Although most cell surface proteins are solubilized from membranes by TX-100, we found that 5'-nucleotidase was not. This glycosylated cell surface enzyme was completely recovered with the TX-100-insoluble pellet from plasma membranes (22). Co-migration of the 5'-nucleotidase activity and the bulk of the matrix protein on sucrose density gradients showed the enzyme to be associated with the matrix, and not simply insoluble under the extraction conditions (22). Although unlikely to be an important structural element, the enzyme is a convenient marker for the plasma membrane matrix. We therefore examined recovery of this matrix marker in the structures resulting from Triton extraction of intact cells. The total enzyme activity of intact whole cells (before extraction) and a particulate fraction obtained from an equal number of lysed cells was found to be essentially the same (Fig. 7). As cells are impermeable to 5'-nucleotides (33), these results further confirm that all of this enzyme is localized to the surface of P815 cells (22). More than 100% of the activity was recovered in Triton shells in comparison to whole cells or the particulate fraction (Fig. 7), and no activity was recovered in the Triton supernatant. Thus, it appears that all of the enzyme is present in Triton shells (Table II) where it has a somewhat higher specific activity, probably as a result of detergent treatment of the membranes. Attempts to localize the 5'-nucleotidase in the Triton shells by cytochemical stain-

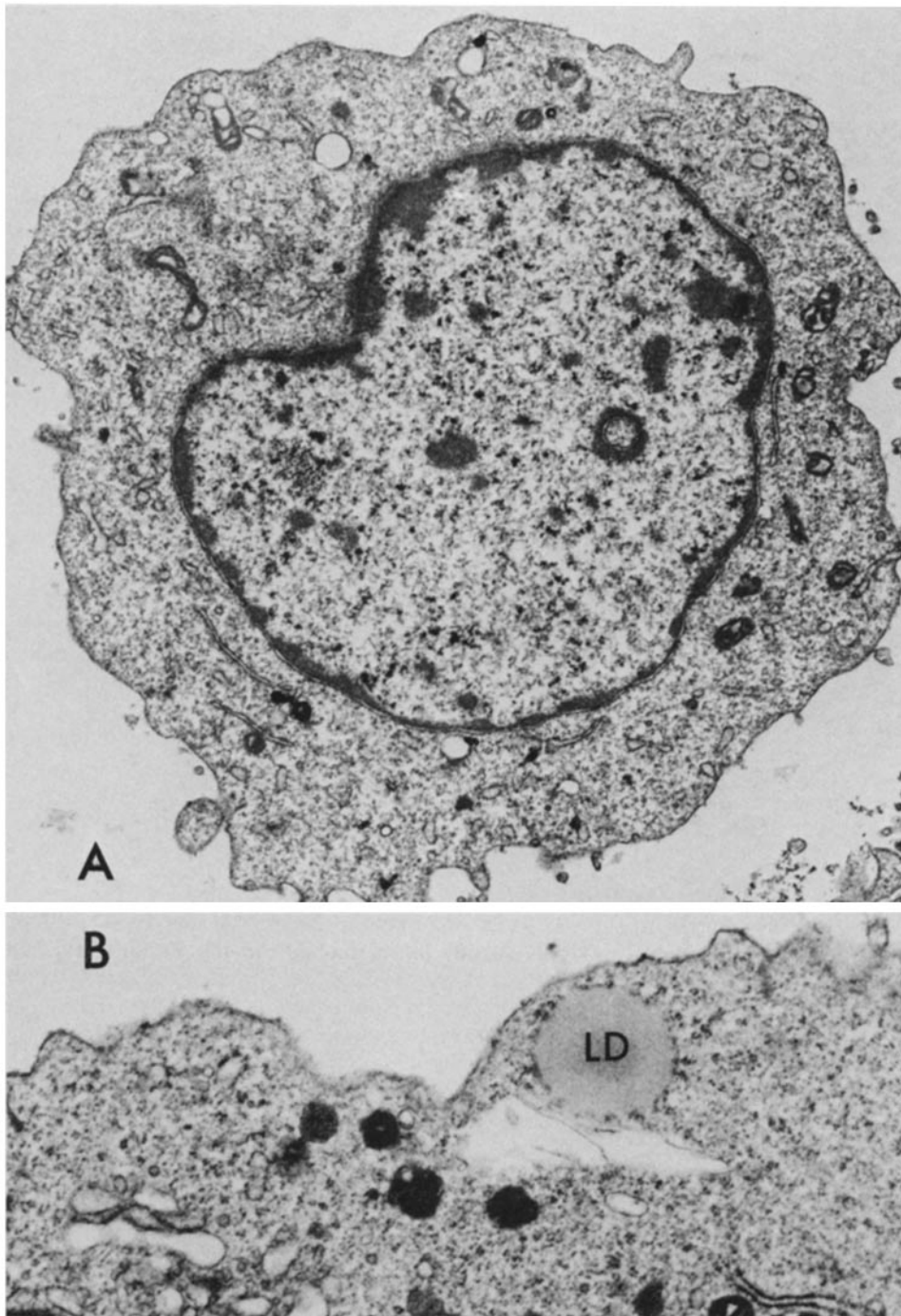


FIGURE 5 Thin-section electron micrograph of an intact P815 cell. LD, lipid droplet. (A) $\times 9,600$. (B) $\times 19,000$.

ing have been unsuccessful. We have been unable to define fixation procedures sufficient to preserve the structures while mild enough to preserve activity of the enzyme.

The results described above indicate that the bulk of the protein recovered with the Triton shells derives from the nuclei, a finding consistent with the morphological observations that the nuclear remnant appears to account for the bulk of the material in these structures (Figs. 3 and 4). Also present in the Triton shells was the membrane matrix marker, 5'-nucleotidase, and a set of polypeptides with the same mobilities as those of the matrix. Furthermore, the amounts of these polypeptides were consistent with the matrix's being continuous over the entire plasma membrane and quantitatively recovered with the Triton shells. Thus, it appears very

likely that the previously described plasma membrane matrix accounts for the continuous peripheral structures present in the Triton shell preparations. Work is in progress to purify the matrix components and obtain antibodies specific for each of them. Once available, these antibodies will allow more direct demonstration of the identity of the peripheral layer and the membrane matrix.

DISCUSSION

Previous work showed that a Triton-insoluble matrix of protein was associated with the inner face of plasma membranes from murine tumor cells and lymphocytes (22). The location and properties of the matrix suggested that it might form a

membrane skeleton continuous over the inner plasma membrane face of these cells. Consistent with this suggestion, we have found that extraction of intact cells under the same conditions resulted in structures with a continuous layer of detergent-insoluble protein at the cell periphery. Confined within this peripheral layer was a nuclear remnant. The cytoplasmic space was largely empty and clearly lacked filamentous cytoskeletal elements. The failure to preserve the

filamentous cytoskeletal structures under these extraction conditions allowed the clear demonstration that the peripheral layer is independent of these structures.

The location and continuity of the detergent-insoluble peripheral layer indicated that it was a structure closely associated with, or a part of, the plasma membrane. It thus appeared likely that this peripheral layer was composed, at least in part, of the matrix associated with the plasma membrane. Support for this conclusion was provided by the demonstration that proteins with the same mobilities as those of the membrane matrix components were present in the Triton shells and were present in the amounts expected if the matrix forms the continuous peripheral layer. Although identity of these two sets of proteins has not yet been conclusively demonstrated, another component of the matrix, 5'-nucleotidase, was clearly shown to be present in the shells.

That the previously described set of detergent-insoluble membrane matrix proteins would also remain insoluble upon treatment of whole cells under the same conditions is not surprising. The results described here, however, demonstrate that quantitative recovery of the matrix is achieved upon low speed centrifugation to pellet the Triton shell structures and does not require the high speed centrifugation needed to pellet the matrix after its isolation from small purified plasma membrane vesicles (22). Thus, the matrix must either be present as very large, extended structures after cell extraction or be associated with large structures. Although it is not yet possible to demonstrate directly identity between the contin-

TABLE II
Protein and 5'-Nucleotidase Content of Triton Shell*

	Whole cell	Triton shell	% Remaining in Triton shell
Total protein [†] ($\mu\text{g}/10^6$ cells)	158	23	15
¹²⁵ I-surface protein [‡] (cpm/ 10^6 cell)	5.2×10^5	0.8×10^5	15
5'-Nucleotidase [§] (nmol/ 10^6 cell per h)	4.2	7.6	>100

* Triton shells were prepared as described in the text in the absence of nucleases. Recovery of shells was ~100%.

[†] Assayed by the method of Lowry et al. (29) in the presence of 1% SDS with bovine serum albumin as the standard.

[‡] P815 cells were surface-labeled with ¹²⁵I by lactoperoxidase-catalyzed iodination (28) and washed three times with PBS, and Triton shells were prepared.

[§] Values from data shown in Fig. 2 for 3×10^5 cells. In several experiments the activity recovered in Triton shells ranged from 120 to 190% of that obtained with whole cells.

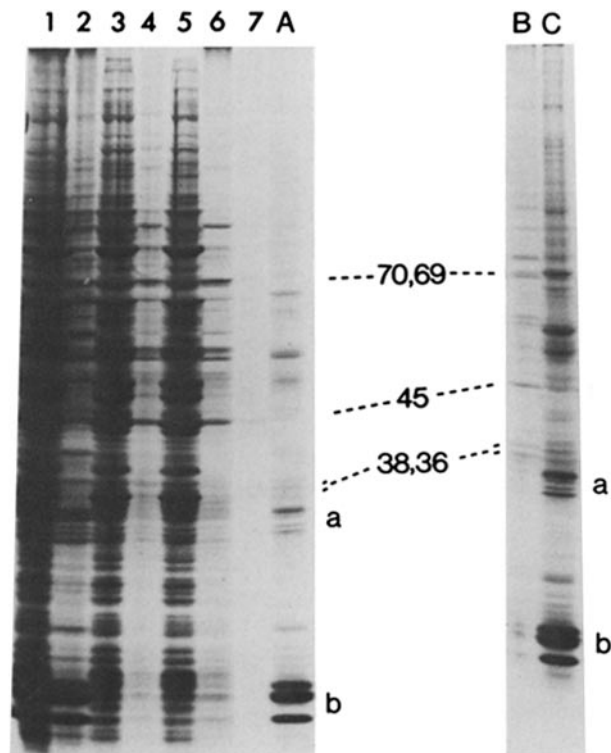
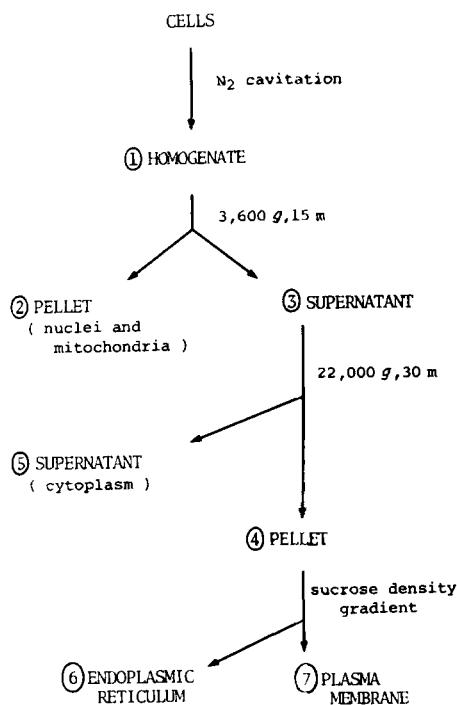


FIGURE 6 Protein composition of subcellular fractions and Triton shells. P815 cells were lysed and fractionated as described in Materials and Methods and as outlined at the left of the gel. An aliquot of each fraction corresponding to material derived from 1×10^6 cells was electrophoresed on a 5 to 15% polyacrylamide gradient gel in SDS. Triton shells were prepared as described in Materials and Methods and run in parallel. After electrophoresis, proteins were visualized by staining with Coomassie Blue. Lanes 1-7, subcellular fractions from 1×10^6 cells. Lane number corresponds to fraction number shown at left. Lane A, Triton shells from 1×10^6 cells. Lanes B and C, plasma membranes (B) and Triton shells (C) obtained from 3.5×10^6 cells. Positions of the major matrix proteins (22) are indicated as molecular weight $\times 10^{-3}$. Regions of the gels marked a and b are discussed in the text.

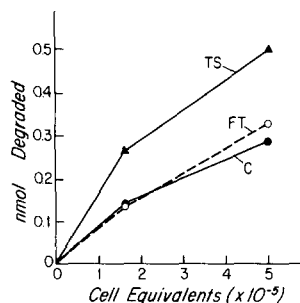


FIGURE 7 5'-nucleotidase activity of cells and Triton shells. Triton shells were prepared as described in the text, in the absence of nucleases. A particulate fraction from whole cells was prepared by subsection of unextracted cells to three cycles of freeze-thaw, centrifugation for 45 min at 100,000 g, and resuspension of the pellet in PBES. The indicated number of intact cells or material derived from that number

of starting cells was assayed for 5'-nucleotidase activity by the method of Avruch and Wallach (30). The reaction was carried out in PBES for 10 min at 37°C with 60 μ M AMP. —●—, intact cells (C). ---○---, particulate fraction (FT). —▲—, Triton shells (TS).

uous peripheral layer of the shells and the plasma membrane matrix, this appears almost certainly to be the case. The matrix, which accounts for 20% of the plasma membrane, is clearly present in the shells and has properties consistent with its forming the peripheral layer. To suggest an alternative origin for the peripheral layer would imply the presence of an additional detergent-insoluble, protein-dependent structure that is continuous over the entire cell periphery but that does not co-purify with plasma membranes upon subcellular fractionation. Furthermore, it would require that this structure, like the membrane matrix, be stable in the absence of the membrane bilayer and most of the membrane proteins and stable under conditions that do not preserve the filamentous cytoskeletal structures.

Detergent extraction of adherent cells under the appropriate conditions has been used extensively to study the architecture of the filamentous cytoskeletal systems of these cells (15–21). In some cases, it has been noted that these cytoskeletal preparations are covered by a lamina of material that appears to derive from the plasma membrane and includes some cell surface proteins (18–21). In these cases, the presence of the extensive cytoskeletal meshwork makes it impossible to determine if this lamina results from a stable interacting set of components that form a continuous peripheral layer, independent of the underlying cytoskeletal, and the laminae have not been isolated or the components characterized. As a result of P815 cells' having a less extensive cytoskeletal system, the use of conditions that would not favor preservation of the cytoskeletal, or both, it has been possible to demonstrate clearly the occurrence of a continuous, detergent-insoluble structure at the periphery of the cells and to show that its stability is independent of either a membrane bilayer or the filamentous cytoskeletal. The absence of a cytoskeletal meshwork after extraction probably accounts, at least in part, for the relative fragility of the Triton shell structures. Preservation of the structures was most effective when mechanical forces were avoided during the detergent extraction. Extensive vortexing, high speed centrifugation, or repeated pipeting resulted in fragmentation of the structures. When these were avoided, Triton shells were recovered with a 90 to 100% yield.

The matrix accounts for ~20% of the protein of purified P815 plasma membranes and includes components with apparent molecular weights of 70,000, 69,000, 42,000, 38,000, and 36,000, as well as 5'-nucleotidase (not seen on SDS gels owing to the low amounts of the enzyme that are present). Components of the same molecular weights have been found

to comprise the detergent-insoluble fraction of plasma membranes from murine (22) and bovine (unpublished) lymphocytes and a variety of murine lymphomas (reference 22 and unpublished results). Davies et al. (23) have recently examined Nonidet P-40 extraction of plasma membranes from human lymphoblastoid cells and pig lymphocytes. They too found a detergent insoluble fraction that accounted for 10 to 20% of the membrane protein and consisted of a discrete set of major polypeptides with apparent molecular weights of 68,000, 45,000, 33,000, and 28,000. In addition, there were prominent higher molecular weight components of 120,000 in the human and 200,000 in the pig. The specific activity of 5'-nucleotidase was enriched about fourfold in the insoluble membrane fractions. These results are very similar to our findings with P815 and murine and bovine lymphoid cells, with the exception that we have not observed prominent components with molecular weights > 70,000.

The only murine matrix components identified thus far are the 42K protein, which is actin (22), and 5'-nucleotidase. The functional role of cell surface 5'-nucleotidase is not well understood, but it provides a convenient marker for the matrix. The 69K and 70K proteins of matrix have about the same molecular weights as fimbrin, a cytoskeletal protein in microvilli of intestinal epithelial cells (34). However, immunoblotting showed no reactivity of these matrix proteins with an antiserum that labeled fimbrin of murine intestinal epithelial cells (A. Bretscher, Cornell University, personal communication). The 68K protein present in the insoluble fraction of human and pig lymphoid cell membranes was recently isolated and shown to have a high affinity calcium binding site (35). It may be that the 69K and/or 70K matrix proteins are the murine equivalent of this protein. Proteins related to spectrin and other components of the erythrocyte membrane skeleton were recently detected by antibody cross-reactivities in a variety of cell types (36–38). Preliminary experiments (in collaboration with J. Spiegel and S. Lux, Department of Pediatrics, Harvard Medical School) have shown that proteins cross-reactive with anti-spectrin, anti-ankyrin, and anti-band 4.1 antisera are present in P815 cells, but none of these co-purifies with plasma membranes. Work is in progress to isolate and characterize the P815 membrane matrix components and study their interactions to form the extended membrane skeletal structure.

To elucidate the role of the plasma membrane skeleton of these nucleated cells will clearly require much additional work. The location and properties of the skeleton suggest a number of possibilities. It seems likely that the skeleton provides the plasma membranes with greater mechanical stability than could be obtained with the lipid bilayer and integral membrane proteins, which are not known to form extended networks of interacting protein. We have found that sphingomyelin (which accounts for ~17% of the membrane phospholipid) remains selectively associated with the matrix upon detergent extraction of membranes, whereas the other phospholipids are effectively extracted (unpublished data). If this reflects an association that occurs in the native membrane, then the matrix may have significant influence on the properties of the lipid bilayer. There is some evidence for a role of the spectrin matrix of erythrocytes in maintaining the lipid asymmetry in these membranes (9–11), and the matrix of nucleated cells may have a similar role.

Cell surface proteins that span the membrane bilayer may interact with the membrane skeleton at the cytoplasmic face,

and their distribution and mobility on the surface may be affected by the interaction. Such interactions might, for example, retard the lateral motion of the surface proteins and account for the lower rate of diffusion observed for many membrane proteins in comparison with the lipids (39). One approach to the investigation of possible interactions is suggested by the observation that H-2, a transmembrane glycoprotein, and isolated matrix can be mixed with lipid in deoxycholate and reconstituted by dialysis to yield vesicles that contain both H-2 and matrix (40). It appears that a lipid bilayer forms on the matrix during the reconstitution and H-2 is inserted into the bilayer. H-2 in these matrix-containing reconstituted vesicles is more effectively recognized by lymphocytes than is H-2 in vesicles made using just lipid (40).

The membrane matrix also appears to be a candidate for mediating interactions between the membrane and the filamentous cytoskeleton. If such interactions occur it appears that they are not maintained under the conditions we have used for membrane isolation. In contrast, Moore et al. (41) found that Triton extraction of plasma membranes from sarcoma 180 cells treated with Zn^{++} resulted in an insoluble fraction which included actin-binding protein, α -actinin, and actin. Treatment with Zn^{++} before membrane isolation may stabilize interactions between the filamentous cytoskeleton and the plasma membrane skeleton.

Further investigation will be needed to determine which, if any, of these suggested roles the membrane skeleton performs. It will also be interesting to determine if cell types other than those we have examined have a membrane skeleton associated with their plasma membrane. The laminae seen to overlie cytoskeletal preparations of adherent cells (18–21) appear to derive from the plasma membrane and an antibody made against the 68K matrix protein of pig lymphocyte membranes cross-reacted with a component of a lamina-like network of detergent extracted fibroblasts (42). These laminae may be structures analogous to the membrane skeleton described here and be composed of the same or related proteins.

We thank Dr. Morris J. Karnovsky for many helpful discussions and for the use of the Zeiss microscope and video equipment (supported by National Institutes of Health grant I SIO RR 01878-01 to Dr. Karnovsky).

This work was supported by NIH grants CA-30381 (to Dr. Mescher) and AI-17945 (to Dr. Robinson). Dr. Apgar was supported by a Postdoctoral Fellowship from the Leukemia Society of America, Inc.

Received for publication 13 July 1984, and in revised form 10 January 1985.

REFERENCES

- Singer, S. J., and G. L. Nicolson. 1972 The fluid mosaic model of the structure of cell membranes. *Science (Wash. DC)*. 175:720–731.
- Bretscher, M. S., and M. C. Raff. 1975. Mammalian plasma membranes. *Nature (Lond.)*. 258:43–49.
- Lux, S. E. 1979. Dissecting the red cell membrane skeleton. *Nature (Lond.)*. 281:426–429.
- Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell*. 24:24–32.
- Marchesi, V. T., H. Furthmayr, and M. Tomita. 1976. The red cell membrane. *Annu. Rev. Biochem.* 45:667–697.
- Lange, Y., R. A. Hadesman, and T. L. Steck. 1982. Role of the reticulum in the stability and shape of the isolated human erythrocyte membrane. *J. Cell Biol.* 92:714–721.
- Lux, S. E. 1979. Spectrin-actin membrane skeleton of normal and abnormal red blood cells. *Semin. Hematol.* 16:21–51.
- Shotton, D., B. Burke, and D. Branton. 1979. Molecular structure of spectrin. *J. Mol. Biol.* 131:303–329.
- Haest, C. W. M., G. Plasa, D. Kamp, and B. Deuticke. 1978. Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. *Biochem. Biophys. Acta*. 509:21–32.
- Lubin, B., D. Chiu, J. Bastacky, B. Roelfson, and L. L. van Deenan. 1981. Abnormalities in membrane phospholipid organizations in sickled erythrocytes. *J. Clin. Invest.* 67:1643–1649.
- Williamson, P., J. Bateman, K. Kozarsky, K. Mattocks, N. Hermanowicz, H. R. Choe, and R. A. Shlegel. 1982. Involvement of spectrin in the maintenance of phase-state asymmetry in the erythrocyte membrane. *Cell*. 30:725–733.
- Yu, J., D. A. Fishman, and T. L. Steck. 1973. Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. *J. Supramol. Struct.* 1:233–248.
- Nicolson, G. L. 1976. Transmembrane control of the receptors on normal and tumor cells. I. Cytoplasmic influence over surface components. *Biochem. Biophys. Acta*. 457:57–108.
- Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.)*. 283:249–256.
- Osborn, M., and K. Weber. 1977. The detergent resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. *Exp. Cell Res.* 106:339–349.
- Brown, S., W. Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119–130.
- Lehto, V.-P., I. Virtanen, and P. Kurki. 1978. Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. *Nature (Lond.)*. 272:175–177.
- Ben-Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton: a lamina derived from plasma membrane proteins. *Cell*. 17:859–865.
- Fulton, A. B., J. Prives, S. R. Farmer, and S. Penman. 1981. Developmental reorganization of the skeletal framework and its surface lamina in fusing muscle cells. *J. Cell Biol.* 91:103–112.
- Streuli, C. H., B. Patel and D. R. Critchley. 1981. The cholera toxin receptor ganglioside GM1 remains associated with Triton X-100 cytoskeletons of BALB/c-3T3 cells. *Exp. Cell Res.* 136:247–254.
- Lehto, V.-P., T. Vartio, R. A. Bdeley, and I. Virtanen. 1983. Characterization of a detergent-resistant surface lamina in cultured human fibroblasts. *Exp. Cell Res.* 143:287–294.
- Mescher, M. F., M. J. L. Jose, and S. P. Balk. 1981. Actin-containing matrix associated with the plasma membrane of murine tumor and lymphoid cells. *Nature (Lond.)*. 289:139–144.
- Davies, A. A., N. M. Wigglesworth, D. Allan, R. J. Owens, and M. J. Crumpton. 1984. Nonidet P-40 extraction of lymphocyte plasma membrane. *Biochem. J.* 219:301–308.
- Lemonnier, F., M. Mescher, L. Sherman, and S. Burakoff. 1978. The induction of cytolytic T lymphocytes with purified plasma membranes. *J. Immunol.* 120:1114–1120.
- Crumpton, M. J., and D. Snary. 1974. Preparation and properties of lymphocyte plasma membrane. *Contemp. Top. Mol. Immunol.* 3:27–55.
- Maupin, P., and T. D. Pollard. 1983. Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes and other cytoplasmic structures by tannic acid-galderaldehyde saponin fixation. *J. Cell Biol.* 96:51–62.
- Morrison, M. 1974. The determination of exposed proteins on membranes by the use of lactoperoxidase. *Methods Enzymol.* 32:103–109.
- Mescher, M. F., and R. R. Pollock. 1979. Murine cell surface immunoglobulin: two forms of delta heavy chain. *J. Immunol.* 123:1155–1161.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
- Avruch, J., and D. F. H. Wallach. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochem. Biophys. Acta*. 233:334–347.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.
- Mescher, M. F., K. C. Stallcup, C. P. Sullivan, A. P. Turkewitz, and S. H. Herrmann. 1983. Purification of murine MHC antigens by monoclonal antibody affinity chromatography. *Methods Enzymol.* 92:86–109.
- Fleit, H., M. Conklyn, R. D. Stebbins, and R. Silber. 1975. Function of 5'-nucleotidase in the uptake of adenosine from AMP by human lymphocytes. *J. Biol. Chem.* 250:8889–8892.
- Bretscher, A., and K. Weber. 1980. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335–340.
- Owens, R. J., and M. J. Crumpton. 1984. Isolation and characterization of a novel 68,000-M_rCa²⁺-binding protein of lymphocyte plasma membrane. *Biochem. J.* 219:309–316.
- Bennett, V. 1979. Immunoreactive forms of human erythrocyte ankyrin are present in diverse cells and tissues. *Nature (Lond.)*. 281:597–599.
- Geiger, B. 1982. Proteins related to the red cell cytoskeleton in nonerythroid cells. *Trends Biochem. Sci.* 7:388–389.
- Lazarides, E., and W. J. Nelson. 1982. Expression of spectrin in nonerythroid cells. *Cell*. 31:505–508.
- Cherry, R. J. 1979. Rotational and lateral diffusion of membrane proteins. *Biochem. Biophys. Acta*. 559:289–327.
- Herrmann, S. H., and M. F. Mescher. 1981. Secondary cytolytic T lymphocyte stimulation by purified H-K^k in liposomes. *Proc. Natl. Acad. Sci. USA*. 78:2488–2492.
- Moore, P. B., C. L. Ownby, and K. L. Carraway. 1978. Interactions of cytoskeletal elements with the plasma membrane of sarcoma 180 ascites tumor cells. *Exp. Cell Res.* 115:331–342.
- Owens, R. J., C. J. Gallagher, and M. J. Crumpton. 1984. Cellular distribution of p68, a new calcium-binding protein from lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:945–952.