High-resolution Three-dimensional Views of

Membrane-associated Clathrin and Cytoskeleton

in Critical-Point-dried Macrophages

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ABSTRACT   We obtained high-resolution topographical information about the distribution of clathrin and cytoskeletal filaments on cytoplasmic membrane surfaces of macrophages spreading onto glass coverslips by both critical-point drying of broken-open cells and preparation of rotary platinum replicas. Irregular patches of the adherent ventral surface of the plasma membrane were exposed in these cells, and large areas of these exposed membranes were covered with clathrin-coated patches, pits, and vesicles. Various amounts of cytoskeleton were attached to the plasma membranes of these spreading cells, either as distinct starlike foci, or as individual filaments and bundles radiating out from the cytoskeletal meshwork. In newly adherent cells a well developed Golgi-GERL complex, characterized by smooth, dish-like cisternae associated with rough endoplasmic reticulum, was observed. There were many coated vesicles budding off from the Golgi cisternae, and these were predominantly of the large type (150 nm) usually associated with the plasma membrane. In critical-point-dried samples, both cytoskeleton and membranes were preserved in detail comparable to that of quick-frozen samples, after appropriate fixation. Rotary replication of critical-point-dried cells provides a rapid, easily controlled, and generally easy to perform method for obtaining samples of exposed membrane large enough to permit quantification of membrane-associated clathrin and cytoskeleton under various experimental conditions.

Two subplasmalemmal structures that play a prominent role in such important functions as cell movement, capping, and endocytosis are cytoskeletal filaments and clathrin baskets (1–7). Although there is now a great deal of information about how these structures can be assembled in vitro from their major component parts (8–10), little is known about the mechanisms underlying their changing associations with different membranes in intact cells. For example, several studies have presented evidence for the dynamic association of microfilaments and clathrin basketworks with the plasma membrane during cell adhesion and spreading, but these have provided only a general view of the configuration of these attachment foci (1, 3–5, 11). We have recently used the quick-freeze, deep-etch, rotary replica techniques developed by Heuser (12) to obtain a high-resolution view of the inner aspect of the plasma membranes of phagocytosing macrophages (13). In addition to a more detailed view of filament associations with plasma membrane surfaces, these high-resolution studies have provided evidence for increased assembly of clathrin

basketworks on substratum-adherent membranes (13, 14). Because it has been difficult to obtain consistent sampling of cells under different experimental conditions using the quick-freeze, deep-etch method, we turned to the more reliable critical-point-drying process and developed a fixation protocol that allowed us to apply this technique to macrophages spreading onto IgG immune complexes. Replicas of these critical-point-dried cells, in most cases, showed ultrastructural detail comparable to the images obtained after quick freezing.

MATERIALS AND METHODS

Cell Culture: Resident macrophages were harvested from CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) by peritoneal lavage and plated onto (5 mm)^2 glass coverslips in Eagle's minimal essential medium containing 25 mM HEPES. In some experiments, coverslips coated with BSA/ anti-BSA IgG complexes were used (15). Spread macrophages were broken open in buffer (IB)[1] (100 mM KCl, 5 mM MgCl$_2$, 3 mM EGTA, and 20 mM

[1] *Abbreviations used in this paper:*   IB, inside buffer; TA, tannic acid; UAc, uranyl acetate.

HEPES, pH 6.8), by placing a poly-L-lysine-coated coverslip over them and pulling it away (13). An average of 20% of the cells were broken open by this procedure.

Quick-Freezing, Deep-Etching, and Rotary Replication: Broken-open cells were fixed at 22°C for 1 h in 1% glutaraldehyde/1% paraformaldehyde in IB (pH 6.8), rinsed briefly in water and 15% methanol, and then quick-frozen against a copper block that had been cooled to liquid helium temperature (4°K) (12). Frozen samples were stored in liquid N_2 before being loaded into a Balzers high-vacuum freeze-etch unit (Model BAF 301, Balzers High Vacuum Corp., Nashua, NH), in which they were deep-etched by warming them to −80°C at a vacuum of 2 × 10^{-6} torr or better for 1 h. Finally, they were rotary replicated with platinum-carbon at an angle of 25°. Replicas were removed from the glass coverslips with 30% hydrofluoric acid and washed in distilled water before cellular material was digested away in undiluted household bleach (1 h). Cleaned replicas were examined at 80–100 kV in a JEOL 100CX electron microscope. All micrographs are printed as negatives to give an appearance comparable to that of scanning electron micrographs.

Critical-Point Drying: Broken-open cells were fixed at 22°C for 1 h with either 2% glutaraldehyde in IB or 1% glutaraldehyde/1% paraformaldehyde in IB. For some experiments 50 mM lysine (pH 7.2) was included during the first 15 min of fixation (16). Several additional fixation steps were tested for their ability to prevent extraction of membranes during alcohol dehydration. These postfixation steps were 1% OsO_4 in IB (4°C, 15 min), 1% tannic acid (TA) in water (Mallinkrodt, #1764, St. Louis, MO) (4°C, 1 h), and 1% uranyl acetate (UAc) in water (4°C, 1 h). No washing was required before OsO_4 postfixation, but extensive washing in cold water was required after all subsequent fixation steps. Fixed samples were dehydrated through cold, graded ethanols (20, 40, 60, 70, 80, 95, and 100%) (2 min each) and then critical-point dried out of "bone-dry" CO_2 (Matheson Gas Products, Newark, CA), using either a Bomar (Model SPC-900/EX, The Bomar Co., Tacoma, WA) or a Polaron (Model E3000 CPDA, Polaron Instruments, Inc., Hatfield, PA) critical-point dryer. To preserve delicate cytoplasmic structures during critical point drying, we found it necessary to remove all traces of water from the samples by repeated flushing with CO_2 (10–20 exchanges) and by using only anhydrous reagents. In addition, dehydrated samples were loaded into the critical-point dryer in ethanol to prevent accidental air drying which instantly and totally destroyed all ultrastructural detail. For each experiment all samples were mounted together on the rotary stage of the Balzers unit with double-stick tape and replicated. Ordinarily, experiments were carried out in a single day and samples were replicated immediately after critical-point drying; however, preliminary experiments indicated that critical-point-dried samples could be stored over Drierite for at least 18 h before replication, without noticeable loss of detail.

RESULTS AND DISCUSSION

Fixation of Macrophages

Rotary replicas of detergent-extracted cytoskeletons prepared by critical-point drying (5, 17) are comparable to quick-frozen, deep-etched ones (18), but critical-point drying has not been applied to isolated membrane preparations, primar-

ily because of membrane lipid extraction during the necessary dehydration steps. We tested a number of fixatives for their ability to stabilize macrophage membranes during alcohol dehydration (Table I) and found that the best preservation of membrane structure was obtained by employing an initial fixative containing lysine, glutaraldehyde, and formaldehyde, followed by postfixation with OsO_4, TA, and UAc. This protocol was very similar to the one used for en bloc staining for thin-section electron microscopy (13). When cytoskeletal morphology was assessed, it was found that the simpler fixation schemes were best. Microfilaments and clathrin baskets were sharp and crisp in lysine-fixed samples postfixed with OsO_4 alone. The subsequent addition of TA, or TA and UAc, caused some flattening of filaments and occasional collapse of cytoskeletal meshworks. In all UAc postfixed samples, the diameter of microfilaments was increased ~25% (from 10.7 to 13.5 nm). The protocol that gave consistently the best overall results was that employing OsO_4 and TA without UAc, but the conditions chosen for any individual experiment must depend on the specific structures to be analyzed.

Views of the Plasma Membrane

As has been previously described (13, 19), the outside surface of the plasma membranes of quick-frozen macrophages appeared as a smooth background, presumably of lipid, studded with numerous randomly distributed small bumps; this morphology was similar whether the cells were fixed in the presence (Fig. 1 *a*) or absence (Fig. 1 *b*) of lysine, and did not require postfixation with OsO_4. Preservation of membrane lipid after alcohol dehydration and critical-point drying was difficult to achieve, and in samples that were fixed with glutaraldehyde/formaldehyde and postfixed with OsO_4 the plasma membrane was largely extracted, leaving a residual structure that appeared cobbled rather than smooth (Fig. 1 *c*). This cobbled structure may represent a subsurface lamina underlying the plasma membrane of macrophages, similar to that described in detergent-treated fibroblasts (20). In cells fixed with lysine and aldehydes followed by OsO_4, the appearance of the plasma membrane was different from that of either quick-frozen or critical-point-dried cells fixed with aldehydes and OsO_4 alone. In these samples, cell surfaces were completely covered with small particles with no smooth lipid background apparent (Fig. 1 *d*). Preliminary measurements

TABLE I

Fixation of Macrophages for Critical-Point Drying

Fixation						Preservation			
						Membranes			
G	F	L	O	TA	UAc	Outside	Inside	Clathrin	Cytoskeleton
Quick-freeze									
+	+	±	±	−	−	Excellent	Excellent	Excellent	Excellent
Critical point dry									
+	−	+	−	−	−	Fair	Poor	Excellent	Excellent
+	+	−	+	−	−	Poor	Poor	Excellent	Fair
+	−	+	+	−	−	Fair	Poor	Excellent	Excellent
+	+	+	+	−	−	Fair	Fair	Excellent	Excellent
+	+	+	+	+	−	Good	Good	Good	Good
+	+	+	+	+	+	Good	Excellent	Fair	Fair

Macrophages spreading onto IgG immune complexes were broken open and fixed as described in Materials and Methods using the fixatives indicated above. G, glutaraldehyde; F, paraformaldehyde; L, lysine; O, OsO_4; TA, tannic acid; UAc, uranyl acetate. The average preservation of membranes, clathrin baskets, and cytoskeleton (primarily microfilaments) in several experiments is ranked as excellent, good, fair, or poor.
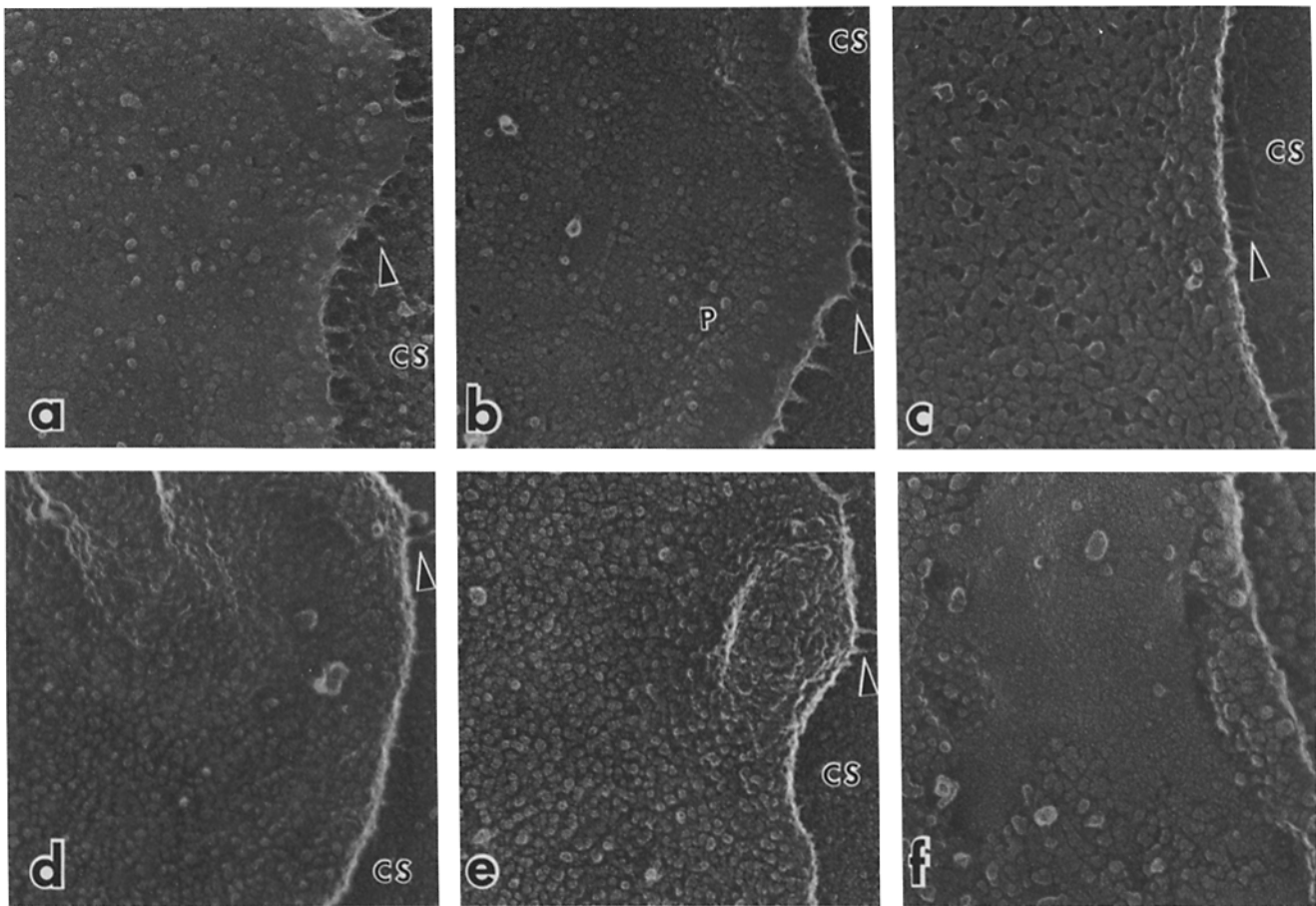
FIGURE 1 Replicas of the outside surface of macrophage plasma membranes. In a, b, and f particles are randomly distributed over a smooth background of lipid. In a–e the spreading edges of the cells are seen where they are in contact with the substratum via slender strands of material (arrowheads). CS indicates the surface of the coverslip. Samples were quick-frozen after fixation with (a) lysine and glutaraldehyde/formaldehyde, or (b) aldehydes alone, or critical-point dried after fixation with (c) glutaraldehyde/formaldehyde and OsO₄, (d) lysine, glutaraldehyde/formaldehyde, and OsO₄, (e) lysine, glutaraldehyde/formaldehyde, OsO₄, and TA, or (f) lysine, glutaraldehyde/formaldehyde, OsO₄, TA, and UAc. × 75,000.

indicated that these particles were similar in size to those observed on the surfaces of quick-frozen cells (Fig. 1, a and b); thus, these samples may represent stabilization of membrane proteins by lysine cross-linking, here exposed by lipid extraction during critical-point drying. Postfixation of such samples with both OsO₄ and TA yielded identical results (Fig. 1 e). In contrast, further postfixation with UAc produced samples in which the smooth lipid bilayer appeared to be partially preserved (Fig. 1f). In these samples, areas of smooth membrane alternated with areas covered with granular material. This granular material was difficult to interpret and may represent either aggregation of the smaller particles present elsewhere on the membrane or precipitation of UAc that was not washed away after fixation. Although unambiguous interpretation of these various images of the plasma membrane surface must await further experimentation, it seems likely that useful information regarding the distribution and/ or organization of specific membrane proteins may be obtainable by this technique.

The appearance of the cytoplasmic surface of the plasma membranes of critical-point-dried macrophages was also compared to that of quick-frozen cells. As was seen on the outside surface of the plasma membrane, the cytoplasmic plasma membrane surface also displayed a smooth lipid background with scattered small particles after quick-freezing (Fig. 2a)

(13). Without added postfixation steps, the exposed plasma membranes of aldehyde-fixed cells were largely extracted by alcohol dehydration, leaving only clathrin basketworks, cytoskeletal filaments, and a residual meshwork of aggregated protein similar to that in Fig. 1c (not shown). However, postfixation treatment with OsO₄, TA, and UAc appeared to stabilize the plasma membrane quite well, so that the smooth background with small bumps typical of frozen samples was preserved (Fig. 2b). In some cases, postfixation with OsO₄ and TA without UAc yielded similar results (Fig. 2c). It is not clear why the lipid of the inner plasma membrane leaflet should be more easily stabilized than the outer leaflet, but this was consistent with the observation that other intracellular membranes, such as the Golgi apparatus, were also more resistant to alcohol dehydration (see below).

Coated Vesicles and Golgi Apparatus

One structure that has been dramatically visualized in rotary replicas of quick-frozen cells is the hexagonal clathrin basketwork associated with endocytic coated vesicles (19). In spreading macrophages, the abundant coated patches, pits, and vesicles were as well preserved after critical-point drying (Fig. 2, b and c) as after quick-freezing (Fig. 2a). We have found it difficult to visualize free triskelion arms at the edges
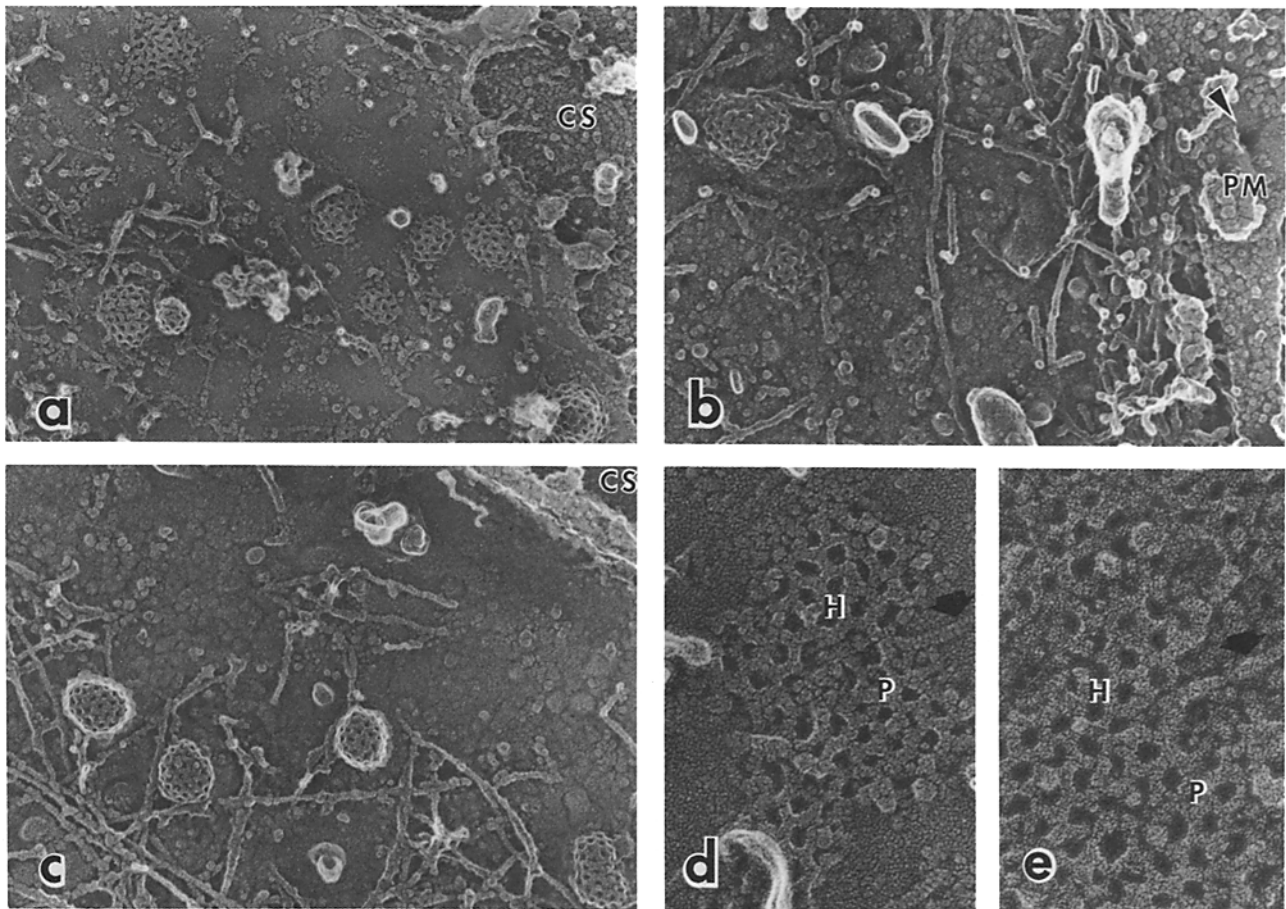
FIGURE 2 Replicas of the cytoplasmic surface of macrophage plasma membranes. In well preserved specimens, the membrane appears as a smooth background with small particles scattered about. Clathrin-coated pits and vesicles are easily identified on these surfaces. In b the outer plasma membrane surface (PM) is visible at the right with the opening to a coated pit indicated by an arrowhead. In d and e, flat patches of clathrin basketwork with hexagonal (H) and pentagonal (P) arrays are shown at high magnification. Cytoskeletal microfilaments (arrows) often approach the edges of such patches. In a and c, CS indicates the surface of the coverslip. Samples were (a and d) quick-frozen after fixation with glutaraldehyde/formaldehyde, or critical-point dried after fixation with (b) lysine, glutaraldehyde/formaldehyde, OsO₄, TA, and UAc, or (c and e) lysine, glutaraldehyde/formaldehyde, OsO₄, and TA. (a–c) × 50,000; (d and e) × 150,000.

of these patches, but pentagons and hexagons within the basket array were clearly delineated (Fig. 2, d and e). Even in samples that were not postfixed at all, in which the plasma membrane was largely extracted, coated structures were retained (not shown). One disadvantage of fixation in UAc (Fig. 2c) was that the clathrin struts appeared thicker than in quick-frozen (Fig. 2a) or TA-postfixed coated vesicles (Fig. 2b), and flat patches of basketwork were sometimes obscured.

In addition to plasma membrane-associated basketwork, a number of cells revealed well developed Golgi apparatus with extensive coated vesicles budding off from the smooth or tubular cisternae (Fig. 3). Although some of the coated vesicles that we observed in these samples were of the small Golgi-related type (70 nm) (Fig. 3c) (21), many of them were of the larger type (150 nm) usually associated with the plasma membrane. The reason for these two classes of coated vesicles is unknown, but it may be functionally related to coated-vesicle shuttling between different membrane compartments (14, 22). It is interesting to note that Golgi membranes appeared to be well preserved even after postfixation with OsO₄ alone (Fig. 3, c and d). This could be due to differences in their protein composition, or perhaps to stabilization by Golgi contents fixed within the cisternae. Ribosome-studded

rough endoplasmic reticulum was often associated with the flattened Golgi cisternae (Fig. 3, b–d) and sometimes appeared to be continuous with the smooth membrane elements, suggesting that these should be classified as Golgi-GERL (23).

Cytoskeletal Filaments

Microfilaments, intermediate filaments, and microtubules have been identified in rotary replicas of detergent-extracted, quick-frozen mouse fibroblasts and their distribution described (18). In broken-open macrophages, all of the various filament types were observed, although their original distribution and interrelationships were sometimes altered by this procedure. Microfilament attachments to the ventral plasma membranes usually appeared as linear arrays stretching from the central cytoplasm to the cell periphery (Fig. 4a), or as distinct starlike foci (Fig. 4b) (4, 5, 13, 18). Microtubules (Fig. 4c) and intermediate filaments (Fig. 4d) were observed less frequently in association with the adherent cell bottoms, probably because they were torn away when the cells were broken open. In samples postfixed with UAc (Fig. 4f), microfilaments were flattened and thickened by ~25% compared to samples postfixed in OsO₄ or OsO₄ and TA (Fig. 4e),
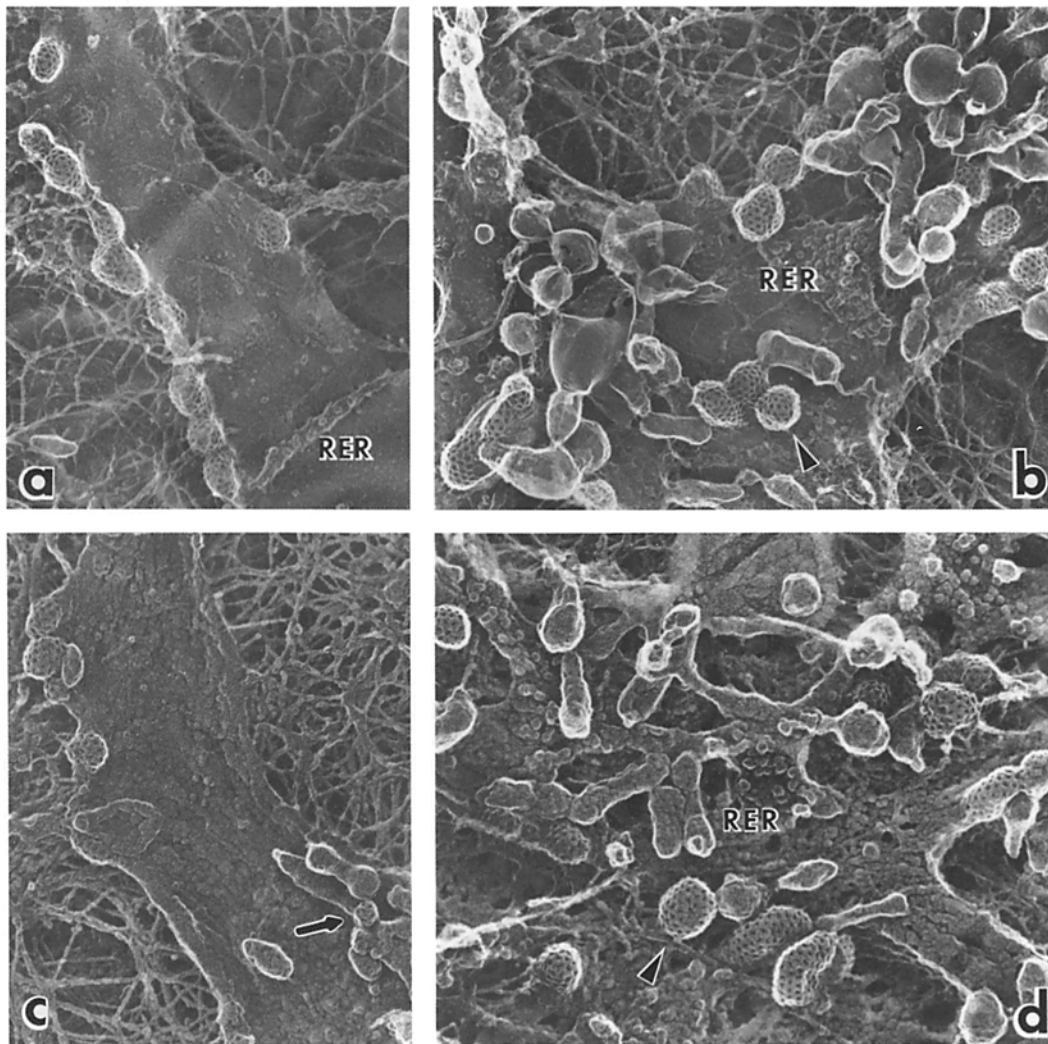
FIGURE 3 Replicas of the Golgi area in spreading macrophages. Coated vesicles of large (arrowhead; d) and small (arrow; c) sizes are observed budding off from flat (a and c) or more complex tubular (b and d) Golgi cisternae. Ribosome-studded rough endoplasmic reticulum (RER) is often closely associated with these cisternae, suggesting that these areas might be classified as Golgi-GERL. Samples were (a and b) quick-frozen after fixation with glutaraldehyde/formaldehyde or (c and d) critical-point dried after fixation with lysine, glutaraldehyde/formaldehyde, and OsO₄. × 40,000.

probably because of heavy metal deposition. In other experiments, detergent-extracted macrophage cytoskeletons were prepared by critical-point drying (not shown). These cytoskeletons showed details comparable to those of broken-open cells and were virtually identical to quick-frozen samples (18). One major advantage of preparing such isolated cytoskeletons by critical-point drying is that this method avoids such possible artifacts of quick-freeze, deep-etch sample preparation as the formation of filament-like salt crystal lattices (24).

Advantages of Critical-Point Drying

Our data show that sample preparation by critical-point drying can produce results equivalent to quick-freezing for many experimental purposes. This is especially true for studies of clathrin basketworks or cytoskeleton, both of which are well preserved in critical-point-dried samples. For example, there has been a great deal of recent interest in identifying proteins that bind actin filaments to the plasma membrane (5, 8), and the preparations that we have described may provide an experimental tool for locating such proteins in situ. We have not, so far, been able to identify any character-

istic structure at sites where microfilaments approach the plasma membrane, but with the use of specific labeled probes this may be possible in the future. Quick-freezing has been used to obtain a unique view of cytoplasmic structures (12), and it would appear that certain subcellular compartments, such as the Golgi apparatus, can be studied equally well in critical-point-dried samples.

There are several important technical advantages to using the combination of rotary replication and critical-point drying, provided that appropriate fixation procedures are used. The equipment and expertise for carrying out these experiments are widely available. Experiments can be completed within a single day and the cost is greatly reduced, because neither liquid N₂ nor liquid He is required. The rate of sample loss is very low, permitting examination of several variables within an experiment of manageable size. Replicas of critical-point-dried samples are very stable, and the entire replica (25–30 mm²) can usually be recovered for viewing in the electron microscope. We estimate that the high rate of sample recovery, together with the degree of replica stability, increases the area of usable replica by 10–20-fold compared
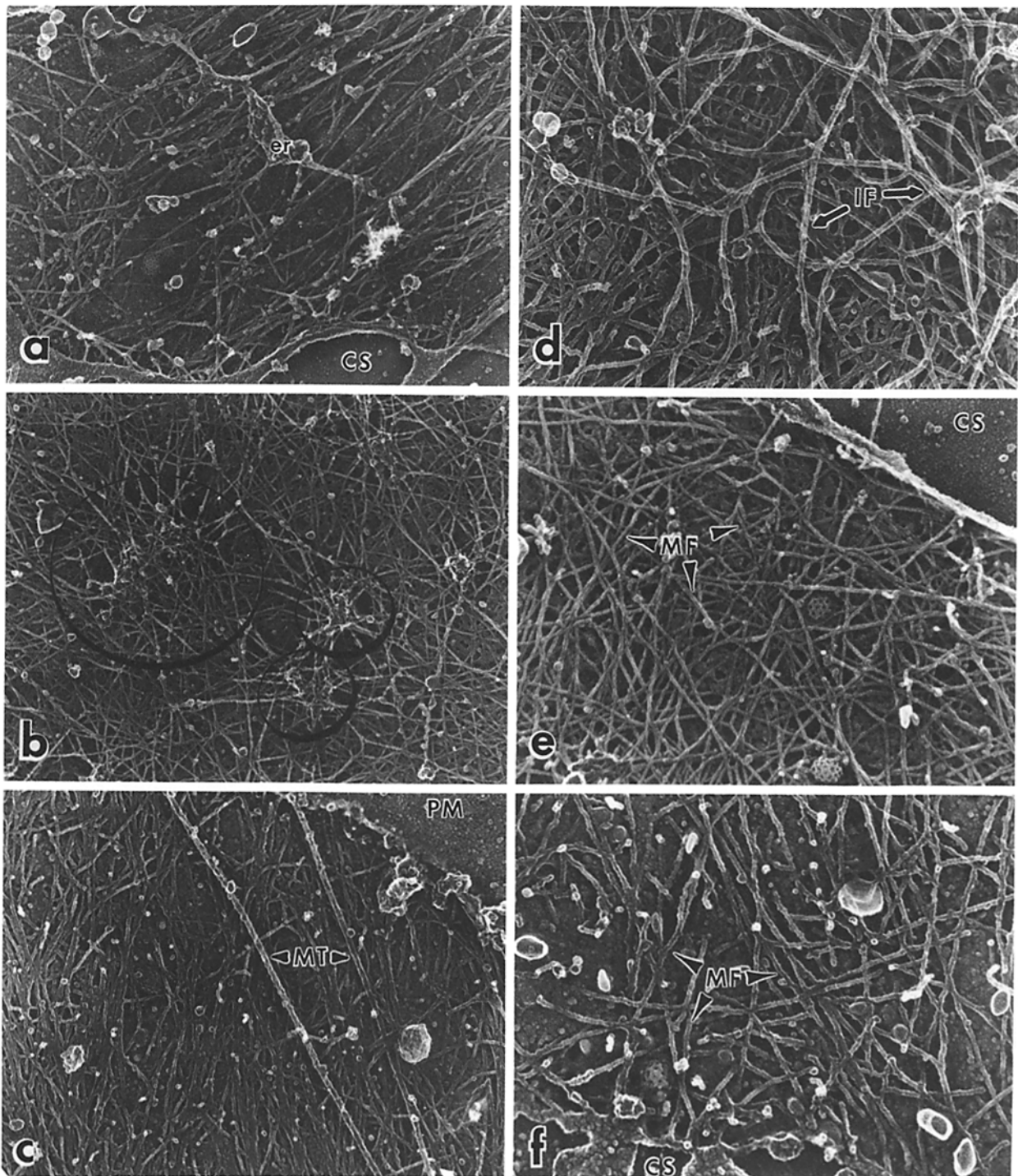
FIGURE 4 Replicas of critical-point-dried macrophage cytoskeletons. Microfilaments are usually attached to the plasma membrane as irregular linear arrays (a) or as starlike foci of radiating filaments (circles, b). Microtubules (MT) are occasionally observed crossing the cell (c). Intermediate filaments (IF) are abundant in the perinuclear area where they form a dense tangle (d). When microfilaments (MF) were compared after different fixation protocols, their diameter was found to be increased from 10.7 nm in OsO_4 or TA-postfixed cells (e) to 13.5 nm in cells postfixed with UAc (f). Fragments of rough endoplasmic reticulum (er) are also observed. Samples were critical-point dried after fixation with lysine and glutaraldehyde/paraformaldehyde, and postfixation with OsO_4 and TA (a and e); with OsO_4 only (b); or with OsO_4, TA, and UAc (c, d, and f). In a and e, CS indicates the surface of the coverslip. In c, PM indicates the outer surface of the plasma membrane. (a–c) × 20,000; (d–f) × 40,000.

to quick-freezing. Finally, the combination of critical-point drying and rotary replication produces high resolution images comparable to quick-freeze, deep-etch sample preparations.

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