Envelopes of Mouse Mammary Tumor Virus Studied by Freeze-Etching and Freeze-Fracture Techniques

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As part of a study of the cell surface changes associated with the production of murine mammary tumor virus, the structure of the envelope of this virus has been examined by using freeze-fracture techniques. Both fracture and deep-etch surfaces were examined. The fracture faces contain 10-nm spheres comparable to those observed on fractured plasma membranes, although fewer in number. Surfaces exposed by etching possess a highly regular hexagonal array of pits 25 nm apart. By examining freeze-fracture and freeze-etch preparations of virus with ferritin covalently bound to its surface, it has been determined that the surface exposed by etching is the outer surface of the virus. The pitted exterior surface of the mammary tumor virus appears to be a unique surface structure.

The surface of the mouse mammary tumor virus (MTV) has been shown to be covered with a network of regularly spaced projections. These projections have been observed both with conventional embedding and sectioning techniques and with negative stain (9, 19). In negative-stain preparations, the projections appear as spikes with a head 4.5 nm in diameter attached to a 1- to 2-nm stalk extending from the surface of the virion in a regular hexagonal pattern with an interparticle spacing of 7.4 nm. These projections are rarely present on other oncornaviruses and are different from the projections coating various normal cell membranes (19).

Since the viral envelope is derived from the host cell membrane and yet is significantly different from it, the study of the structure and the morphogenesis of virus-related membrane has significant implications towards the understanding of how cells can modify their surface coats in response to changes in function or expression of specific characters. As a first step in the analysis, we have undertaken a study of the structure of the envelope of murine MTV (MuMTV) after freeze-fracture under conditions which permit either fracture or etch faces to be observed by a replica technique. It has been suggested (6, 18, 24) that freeze-fracturing in the presence of glycerol as a cryoprotective reveals internal membrane structures, whereas deep-etching in dilute dimethyl sulfoxide (DMSO) solutions or water provides images of the outer surface. We will distinguish in this paper between freeze-fracture, a process which yields only fractured surfaces, and freeze-etching, a process in which the fracture is followed by the sublimation of water, exposing true external surfaces. The rationale for this distinction will be discussed further in the paper. In this study, both freeze-fracture and freeze-etch images were obtained of MuMTV.

By covalently labeling the exterior of MuMTV with ferritin, it was confirmed that neither of the faces exposed by freeze-fracture is an exterior surface, whereas the face exposed by deep-etching is indeed the exterior of the virus. Preliminary accounts of this study have been presented (J. B. Sheffield, Proc. 64th Meet. Amer. Ass. Cancer Res. 14:71, 1973; 21).

MATERIALS AND METHODS

Density gradient-purified MTV from the milk of high-tumor strain RIII mice (12) was suspended in either 15% glycerol or 5% DMSO or distilled water, pelleted (32,000 rpm for 1 h in a Beckman SW39 rotor), and allowed to resuspend overnight in 0.1 ml of the same solution.

Samples of approximately 1 μl were applied to the apposed replica specimen holders of the Denton freeze-etch apparatus, and the samples were frozen in solid-liquid equilibrium mixtures of Freon 22 at -150 C and transferred to the apparatus. The material was fractured at -190 C, when the vacuum was 3 × 10⁻⁷ torr. Virus suspended in glycerol was not etched,
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whereas virus suspended in 5% DMSO was etched for 5 min at \(-100\) C. Platinum-carbon replicas were obtained from the fracture faces at \(-175\) C, with a shadowing angle of 45\(^\circ\), and were examined, after cleaning, with a JEM 100B electron microscope.

To prepare MuMTV covalently labeled with ferritin, glutaraldehyde was used as the coupling reagent (1). To 1 mg of purified virus suspended in 100 \(\mu\)l of distilled water, 100 \(\mu\)l of a solution containing 100 mg of ferritin per ml (Polysciences, Rydal, Pa.) was added. This mixture was allowed to incubate at room temperature for 5 min and then 0.5 ml of 2.5% glutaraldehyde in Millonig buffer was added. After 30 min, this material was centrifuged through a 0 to 60% sucrose gradient. The band at density 1.18 g/ml was collected, diluted with water, pelleted, and resuspended in 10 \(\mu\)l of distilled water. This material was then embedded for thin sectioning, examined by negative stain, or prepared for freeze-fracture and freeze-etching.

RESULTS

With both of the preparative techniques employed, the virions appear primarily as spheres. The maximum diameter observed for MTV was about 150 nm. We have not yet examined the nucleoids of these viruses, and all of the descriptions to follow refer to the appearance of the viral envelope.

Freeze-fracture. When glycerol-suspended virus is fractured and replicated in the absence of etching, two kinds of fracture faces can be observed (Fig. 1, 2). The first is that of a convex sphere protruding from the substrate (A in Fig. 2) and the second is a spherical concavity in the substrate (B). The surfaces exposed by the fracture appear irregularly rippled and often contain a variable number of spherical structures approximately 10 nm in diameter. These spheres appear singly or in clusters on the A surface and as a ring surrounding the B face. Occasionally, they are seen singly in the depression of the B face.

Freeze-etching. When virus is suspended in 5% DMSO prior to freeze-fracture and the fractured sample is warmed to \(-100\) C for 5 min prior to replication, additional structures are visible. The overall structure of the replica is highly complex due to the incomplete suppression of ice crystal formation by DMSO and the apparent separation of phases within the frozen matrix (Fig. 3). Virions can be found in both phases, with more fracture faces apparent in the smooth, presumably DMSO phase, and exposed etch faces commonly found in the rough, presumably water phase. The surface of MuMTV virions thus exposed by etching is covered with pits regularly distributed in a hexagonal array with a spacing of approximately 25 nm (Fig. 4). In occasional virions, it appears that the pits represent the intersection points of large subunits on the surface. Whereas the fracture procedure exposed concave and convex faces, which presumably match each other, there appear to be no concave faces corresponding to the pitted convex faces revealed by the deep-etching procedure.

Ferritin-conjugated virus. Ferritin-conjugated virus was examined by negative stain and thin section to determine the extent and location of the ferritin. Most of the virions contained from one to three ferritin molecules bound to the exterior surface (Fig. 5, 6). Ferritin was not observed within the virions. In freeze-fracture preparations the ferritin was not visible on either the A or B face, although occasionally a large sphere, presumably ferritin, could be seen adhering to the outer ring around the B face (Fig. 7). In freeze-etch preparations (Fig. 8–10), many of the pitted surfaces contained one large particle. This particle corresponded in size with ferritin molecules, as seen in other freeze-etch preparations (18), and in distribution with that in the negative-stain and thin-section preparations of labeled virus. Etch surfaces of virus which was fixed with glutaraldehyde in the absence of ferritin possessed pits and did not have large particles associated with them. It appears, then, that the surface exposed by deep-etching is the same surface to which ferritin is bound, and is the exterior surface.

DISCUSSION

The oncornaviruses are produced by budding from the surface membrane of their host cell. In this process they modify the host cell membrane, converting it to a viral membrane, with virus-specific antigens and structures. This viral membrane is still osmotically active, and under conditions of hyperosmotic stress, shrinks down around the enclosed viral nucleoid to form a “head and tail” configuration. This is seen most commonly after preparation of the virus for negative stain, in which the procedure involves dehydration of a viral suspension in a solution of a salt (sodium phosphotungstate) to which the virus is impermeable. This leads to an increase in the salt concentration exterior to the virus, which dehydrates the structure, causing shrinkage which produces the familiar head and tail form of the virion. When the permeability barrier is destroyed, as by fixation, or when the virus is not dehydrated, as in the freeze-fracture techniques described here, the virus maintains a spherical shape. We have not yet determined the exact osmotic strength which is required to cause this configurational change, but such a study is in progress.
**Fig. 1.** Survey micrograph of freeze-fracture preparation of MuMTV suspended in 15% glycerol. Both convex (A) and concave (B) faces are indicated. In this and succeeding micrographs of shadowed material, the direction of the shadow is indicated by the circled arrow. ×44,000. The bar represents 0.5 μm.
Fig. 2. Details of the appearance of freeze-fractured MuMTV. The arrows indicate the small spherical structures. x270,000. The bar represents 0.1 μm.
Fig. 3. Survey micrograph of freeze-etch preparation of MuMTV suspended in 5% DMSO. The separation into smooth (S) and rough (R) phases is evident. Both fractured (F) and etched (E) virions can be seen. Fractured virions are found predominantly in the smooth phase, whereas etched virus surfaces are found predominantly in the rough phase and also in the smooth phase. ×25,000. The bar represents 1 μm.
Fig. 4. Details of etched surfaces of MuMTV. Concave faces are absent, and the exposed convex faces possess pits (P) in hexagonal array with a spacing of 25 nm. ×240,000. The bar represents 0.1 μm.
Freeze-fracture in the presence of large amounts of glycerol is believed to split cellular membranes at or within the lipid bilayer (3). A similar phenomenon appears to be the case with the oncornaviruses. The A face represents the internal side of the inner membrane leaflet, whereas the B face represents the internal side of the outer leaflet. In view of the complex and regular exterior surface of the virus, it is surprising that the B face, which represents the inner portion of that exterior surface, should be so free of regularities. It appears that the structures on the surface do not penetrate the membrane to a sufficient extent to be detected at this level of fracture. This might imply that the external subunits are attached at the outer protein layer of the viral membrane or that their inner extensions are not easily visible. The spherical structures which are seen on both the A and B faces of the virus membrane have been described on freeze-fractured membranes of many types (3, 13). In general, the A face of the viral membrane seems to possess more of these spheres than the B face. This is in agreement with observations on cellular plasma membranes (3). The nature of these structures is unknown.

The most surprising result of this study is the finding of the highly ordered array of pits on the surfaces of the MuMTV virion. We feel that the surfaces exposed by deep-etching after suspension in either dilute DMSO or distilled water are indeed the outer surface of the virion. This interpretation is supported by several kinds of evidence: (i) the experiments reported here in which ferritin, covalently bound to the exterior of the virion, was exposed only after deep-etching and was not observed in fractured virions. (ii) When virus is prepared by deep-etching, the convex surfaces possessing pits do not appear to be matched by corresponding concave impressions. The pits appear only on convex surfaces. If these impressions were to be the result of a fracture process, one would expect to obtain corresponding sides of the fracture. This does not appear to be the case, and we conclude that the convex surfaces which are seen are exposed by the removal of water from above them. (iii) If this interpretation were correct, the image obtained after deep-etching would be similar to that obtained by freeze-drying the virus and then shadowing it. This is, indeed, the case. Freeze-dried and shadowed virus, although collapsed, possess the same
pattern of surface pits that were described here for freeze-etched samples (N. H. Sarkar, personal communication). (iv) Furthermore, there is now ample evidence that deep-etching does expose external membrane surfaces in other systems. Freeze-etching of red cell ghosts covalently labeled with ferritin (18) or f actin (24) revealed the markers only when deep-etching was performed on samples suspended in dilute solutions or water. Natural surface markers such as ribosomes on nuclear membranes have been seen only after deep-etching (25), and similar observations have been made on thylakoid membranes (17). A more detailed discussion of the origin of the images in freeze-etching can be found in recent papers by Park (16) and Branton (3).

Although there have been several reports of the structure of the outer surface of cellular membranes (3, 16, 18, 24, 26), a pattern of depressions similar to that in the surface of MuMTV has not been described. It appears, then, that this pattern is unique to the surface of the MuMTV viruses and is absent even from other oncornaviruses such as MuRLV (15; J. B. Sheffield, unpublished data).

When MuMTV virions are examined in the electron microscope after negative stain, the most prominent feature of the virus is the presence of what appear to be external spikes arranged in a highly ordered hexagonal array with an interspike distance of 7.4 nm (19). These spikes are absent from similarly prepared MuRLV and appear to be unique to the mammary tumor virus. We have been wrestling with the problem of how to reconcile these two sets of disparate observations on what should be the external surface of the same particle. In both cases, structures have been observed which are characteristic of MuMTV and which are absent from other viruses and membranes. We have been led to several hypotheses which are presented here with whatever data exist to support them, in the hope of stimulating further discussion and hopefully some experiments to clarify this complex issue.

**Hypothesis 1.** The spikes are present on the surface of the particle but are not visualized by the freeze-etch procedure because the shadowing technique cannot resolve the individual spikes. The pits result from regular holes in the lattice, where a spike may be missing. In support of this there are empty hexagons which could be holes in the lattice which are seen on occasional MTV virions (Fig. 11) after negative stain. However, this pattern appears only on occasional apparently damaged virions, and the space between the pits is approximately 13 nm.

The difference between the 25-nm spacing seen in freeze-etch and this 13-nm pattern seen in negative stain is too great to be accounted for by the differences in preparative technique. Nermut and Frank (14) report a difference of about 30% in their comparison of freeze-cleaved and negative-stained influenza virus. The possibility of the shadowing obscuring the spikes cannot be eliminated, but other closely packed subunit structures such as the 9-nm particles of gap junctions have been easily visualized (8, 11, 23).

**Hypothesis 2.** The deep-etch procedure exposes a surface which is exterior to the spikes. That is, the spikes are covered by an additional layer of material which is not detected by either negative stain or thin sectioning and which is present after freeze-fracturing and deep-etching and so is detected in the replica. A coating of this sort could obscure the details of spike structure so that they would not be seen after deep-etching. One conceivable candidate for coating material would be carbohydrate, perhaps a mucopolysaccharide. There is, to date, no information about the carbohydrate composition of the mammary tumor virus, although it has been shown that a significant proportion of Sindbis, Rauscher leukemia, or Rous sarcoma viruses is glycoprotein (2, 4) and that there are differences in glycoprotein composition between viruses released from transformed or nontransformed cells (10). In addition, there is evidence

![Fig. 11. MuMTV suspended in 5% DMSO and negatively stained with neutral sodium phosphotungstate. The surface of the intact virion (V) is covered with spikes, whereas the fragment possesses a hexagonal lattice on its surface. The distance between the centers of these hexagons is 13 nm. ×175,000. The bar represents 0.1 µm.](http://jvi.asm.org/)
that plant lectins such as concanavalin A and wheat germ agglutination are able to induce agglutination of purified MuMTV (J. B. Sheffield, unpublished data) and, in fact, can be detected histochemically at the surface of the virus (5). Furthermore, in a recent study (22), it was shown that MuMTV binds a significant amount of the acid mucopolysaccharide stain Ruthenium Red. A carbohydrate coat would not be readily detectable by conventional electron microscopy techniques and could be revealed only by special treatments (20). If there were a coat on the surface of the virus, the reported observations of the resistance of the surface spikes to enzymatic digestion could be understood. Proteases would be unable to penetrate the outer surface to reach the spikes, whereas polysaccharide-reactive enzymes would remove the coat but not affect the spikes, so the final appearance in both cases would be unchanged.

In this hypothesis, the surface polysaccharide would be arranged in subunits so that their intersections would be at intervals of 25 nm and would be the source of the pitted pattern which is seen in deep-etching. The spikes would be arrayed beneath this coat and would not contribute to the image. It is possible, however, that there is a more direct relationship between the spikes and this proposed surface polysaccharide coat, such that the origin of the subunit appearance would be a substructure of clusters of spikes. There is, at the present time, no evidence to indicate this substructure, and the suggestion must be considered entirely speculative.

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