Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virology 323 (2004) 189–196
VIROLOGY
www.elsevier.com/locate/yviro

Atomic force microscopy investigation of wild-type Moloney murine leukemia virus particles and virus particles lacking the envelope protein

Y.G. Kuznetsov, A. Low, H. Fan, and A. McPherson*

Department of Molecular Biology and Biochemistry, University of California-Irvine, Irvine, CA 92697-3900, USA

Received 10 October 2003; accepted 16 February 2004

Available online 22 April 2004

Abstract

Moloney murine leukemia virus (M-MuLV) lacking the gene for the envelope glycoprotein (env$^-$) was produced in NIH 3T3 cells and investigated using atomic force microscopy (AFM). The particles were compared with similarly produced wild-type virions, some of which had been exposed to a monoclonal antibody against the surface component of the envelope protein (SU protein). The env$^-$ particles generally exhibit a distinctly different external appearance suggesting only a low density of associated proteins that have an almost fluid, mechanically unstable character. The weakly associated proteins may be host cell membrane proteins that are incorporated into the viral membrane in place of or in addition to virus envelope protein. The amount of this non-viral protein on virion surfaces appears to vary from negligible in most cases to a substantial complement in others. It seems clear that the presence of the envelope protein, in a mechanical sense, significantly strengthens and stabilizes the virion envelope. Binding of monoclonal antibody to wild-type virions indicates that some particles expose a significant amount of antigen while adjacent virions may not. This suggests that the conformation of the envelope glycoprotein or the disposition of oligosaccharides may be different among particles, on some virions exposing the specific epitope, and others little or none.

Keywords: gp120 protein; SU protein; Retrovirus; Immunolabeling; AFM; Surface

Introduction

Current models for the architecture of both mature and immature retroviruses have been described (Bolognesi et al., 1978; Briggs et al., 2003; Vogt, 1997; Wilk et al., 2001; Yeager et al., 1998), as well as the structures of individual proteins of which they are composed (Frankel and Young, 1998; Turner and Summers, 1999). Some features of the models are particularly relevant to the work presented here.

Retroviral particles are bounded by a lipid membrane that is obtained by the budding of the internal components of the virion through the plasma membrane of the host cell. That membrane is likely to be locally enriched in certain lipid components such as cholesterol (Graham et al., 2003; Nguyen and Hildreth, 2000; Shiraishi, 2001). The envelope glycoprotein is associated with the viral membrane, which consists of a transmembrane domain (TM protein) embedded in the lipid, and a soluble, exterior, globular head called the SU protein. Although originally contiguous elements of a single polypeptide, cleavage by a cellular protease renders their association noncovalent (Dong et al., 1992; Freed et al., 1989; Perez and Hunter, 1987), though occasionally in Moloney mouse leukemic virus (M-MuLV) they are disulfide linked (Leamnson et al., 1977; Witte et al., 1977). In the interior of the virion, the carboxy-terminal tail of the TM protein likely makes contact with the viral matrix (MA) protein encoded by the *gag* gene (Gebhardt et al., 1984; Wyma et al., 2000).

There is persuasive evidence that in the viral envelope, the TM "stalks" associate to form a trimer based on threefold symmetry (Chan et al., 1997; Weissenhorn et al., 1997), though other data suggest that this symmetrical arrangement may exist only after receptor binding (see the discussion in Frankel and Young, 1998). Principally by inference from the transmembrane trimer and by homology with the envelope protein from human foamy virus (HFV) (Wilk et al., 2000), it is commonly assumed that the extra membrane "heads" of SU protein are also organized into

* Corresponding author. Department of Molecular Biology and Biochemistry, University of California-Irvine, 560 Steinhaus Hall, Irvine, CA 92697-3900. Fax: +1-949-824-5992.
E-mail address: amcphers@uci.edu (A. McPherson).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.02.023

threefold symmetrical trimers (Poignard et al., 2001; Turner and Summers, 1999; Vogt, 1997), though this has never been convincingly demonstrated. Another ubiquitous feature of the models is that the "stalk" and "head" trimers protrude away from the membrane and appear as "spikes". The evidence for these "spikes" is based on conventional transmission and cryoelectron microscopy (Gelderblom, 1990; Gelderblom et al., 1987; Yeager et al., 1998).

During the budding process, host cell membrane proteins may also be incorporated into the viral envelope, but the extent of this incorporation is not known (Ott, 1997; Vogt, 1997). There is also some controversy as to how many envelope proteins, putative trimers, or "spikes" are present on viral surfaces. Also uncertain is the mechanism by which viral envelope proteins are incorporated, whether this is by active recruitment by the budding virion contents, or whether it includes displacement and exclusion of cellular proteins by envelope protein. In the former case, a complement of cell membrane proteins would be supplemented by envelope proteins, while in the latter there would be depletion of host cell protein in the viral membrane by active displacement.

The structures of both transmembrane "stalks" (Chan et al., 1997; Weissenhorn et al., 1997) and globular "heads" (Fass et al., 1996; Kwong et al., 1998) have been investigated by X-ray crystallography for both human immunodeficiency virus (HIV-1) and murine leukemia virus (M-MuLV). In the cases of the ''stalks'', only truncated fragments corresponding to the ectodomains of the proteins could be crystallized and their structures determined. The same was true for the globular, extracellular SU "heads". In addition, the proteins investigated were substantially deglycosylated and heavily modified. Numerous exposed protein loops were deleted, and other mutations were necessary as well. While the transmembrane proteins crystallized as threefold symmetric trimers, neither M-MuLV SU nor HIV-1 gp120 crystallized as trimers. In addition, expression of gp120 in baculovirus systems produced a monomeric protein (Kwong et al., 1998). These observations cast some doubt on the presumed trimeric arrangement of the envelope protein in the native virus and its symmetrical arrangement.

Clarification of the ambiguous features of the models for retrovirus surfaces has more than academic interest. A substantial amount of research is currently focused on the development of vaccines against retroviruses, particularly against HIV (Burton, 1997; Parren et al., 1999; Wyatt and Sodroski, 1998). Many, if not most, of these deal with the generation of antibodies directed against the surface antigens of the virions, principally the envelope proteins. Thus, it is essential that we better define the structures of the proteins, their oligomers, their modes of association, number, distribution, and general arrangement on the surfaces of virions. It is also important to know the importance and abundance of cellular-derived proteins in the viral envelope as well.

We have undertaken here to investigate the surface features, particularly those associated with the envelope protein, of M-MuLV. The technique we have utilized is atomic force microscopy (AFM) of cells infected with and displaying emerging M-MuLV at their surfaces. In doing so we examined both wild-type virus (env^+) and mutant virus particles (env^-) lacking the envelope protein. In addition, we utilized immunolabeling of virus particles with antibody conjugated gold clusters (Kuznetsov et al., 2002).

As we described in previous papers (Kuznetsov et al., 2001; Malkin et al., 1999; Malkin et al., 2003; Plomp et al., 2002), including ones dealing with M-MuLV (Kuznetsov et al., 2002) and HIV (Kuznetsov et al., 2003), AFM offers an attractive complement to electron microscopy. It has the virtue of imaging only the surfaces of objects and does not yield a two-dimensional projection of an entire specimen, which may be difficult to interpret. It is also particularly precise in measuring the heights of features above a background level, something frequently difficult with EM. Finally, it is valuable for imaging individual particles within a large population and identifying both common and unique structural features.

Results

Fig. 1 is a gallery of typical, wild-type Moloney-MuLV (M-MuLV) particles emerging from or leaving the surfaces of productively infected NIH 3T3 cells (43-D). Images of M-MuLV on cell surfaces were presented in an earlier paper

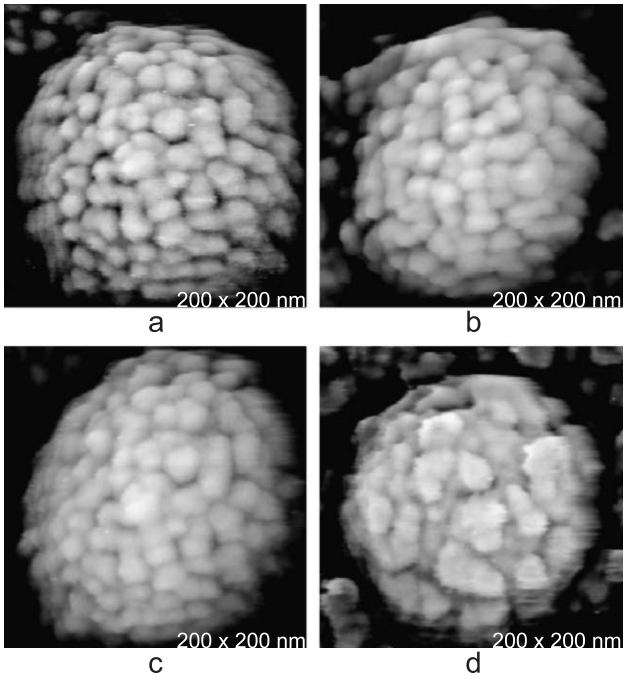

Fig. 1. In (a) through (c) are AFM images of the surfaces of wild-type env^+ M-MuLV virions exhibiting dense arrangements of protein "tufts" on their surfaces. These are the most common appearances of particles. In some cases, however, particles like that in (d) are observed, where the density of the protein "tufts" is perceptibly less, size diversity greater, and the arrangements more irregular.

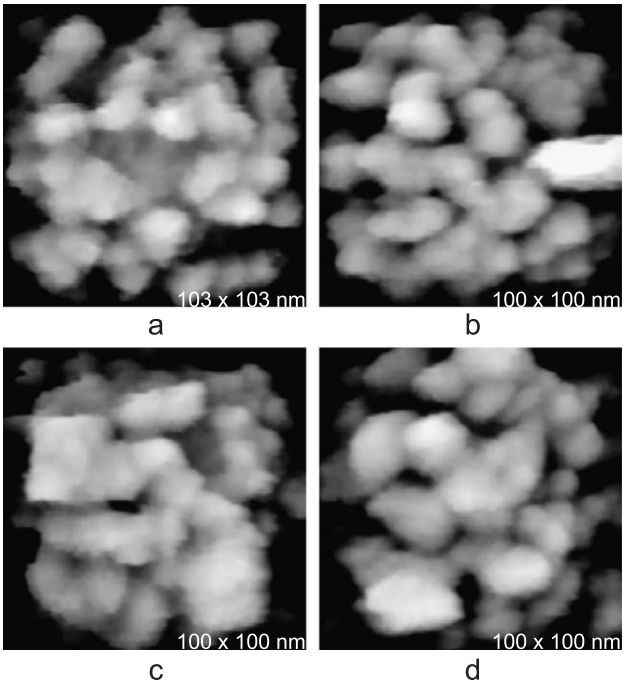

Fig. 2. High magnification AFM images of the surfaces of individual wild-type M-MuLV virions showing the varied shapes and sizes of the protein aggregates studding the viral membranes.

(Kuznetsov et al., 2002), and though the images presented here are qualitatively the same, the resolution of detail is somewhat better. The particles are, on average, 145 nm in diameter, but with a wide variation among individuals (Kuznetsov et al., in preparation). The surfaces of the virions, based on examination of many hundreds of particles, as in Fig. 1, are studded with "tufts" of protein that display a range of packing densities. Some, as exemplified by those in Figs. 1a–c, are densely packed with "tufts" of more or less uniform size of about 11–12 nm, though we frequently also see others, such as those in Fig. 1d, which have "tufts" of more varied size including some quite large in extent. As shown in our previous paper (Kuznetsov et al., 2002), and additionally below, monoclonal antibodies directed against the SU domain of the envelope protein do indeed bind to protein units on the virion surfaces. The "tufts" we believe to be aggregates, including perhaps monomers as well, of the SU protein. This would explain the observed size diversity and irregular forms. The images obtained using AFM are consistent with the reported pleiomorphic appearance of the particles based on cryo EM (Yeager et al., 1998).

In Fig. 2 are high-magnification AFM images of M-MuLV surfaces showing the protein aggregates at increased resolution. Careful examination of many hundreds of surface proteins revealed no symmetry or general pattern that would suggest a geometrically regular arrangement of protein subunits on the virion surface. The number of protein units or "tufts" on the virion surfaces was estimated to be 100 ± 20, but again, this varies a great deal among particles. None of the protein aggregates or their clusters on the surface exhibit any indication of threefold symmetry. We saw no protrusions from the virion surfaces consistent with protein molecules other than the "tufts" noted here, and no structures that we would describe as "spikes". The protein aggregates have a radial extent of about 6–10 nm.

14-11 cells are NIH 3T3 cells stably transfected with a M-MuLV provirus from which the env gene had been deleted (Feuerman, 1985). These cells produce virus particles that have no envelope protein (TM or SU). The phenotype of the particles, as judged from AFM images, is distinctly different than that of wild-type M-MuLV particles. Fig. 3 is a gallery of particles obtained from the 14-11 cells when scanned by AFM with the same force as was used to obtain the images of wild-type particles presented in Figs. 1 and 2. The protein "tufts" that were seen to densely stud the surfaces of wild-type virions are no longer present. Instead, we find an almost diaphanous display of membranous features. The appearance of these virions should not, however, be taken literally. Experience with other systems has shown that when lipid membrane lacking any reinforcement by a membrane skeleton is scanned at high resolution by AFM, this is the appearance one observes (Kuznetsov et al., 1997). The residual force of the AFM tip, though minimal in tapping mode, nonetheless creates wrinkles and waves in the deformable membrane whose transient and unstable nature results in the topological characteristics

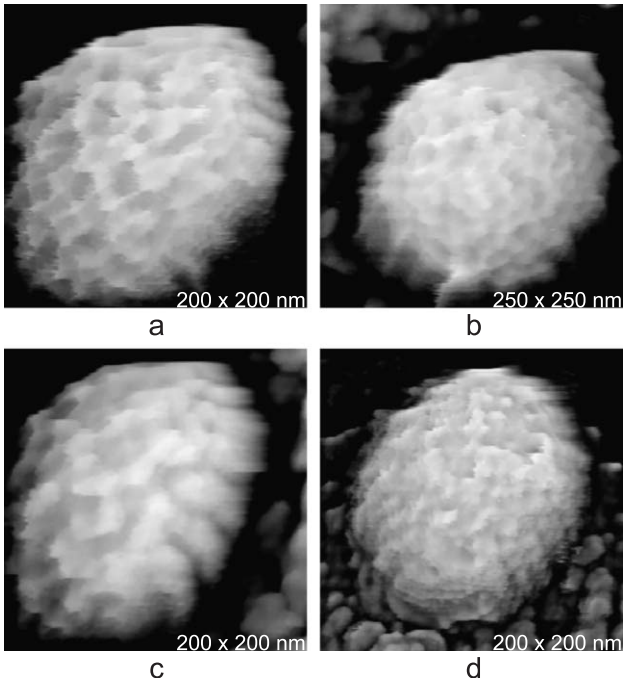

Fig. 3. M-MuLV virus entirely lacking the gene for the envelope protein (env $^{-}$) and therefore lacking envelope protein on their surfaces exhibits the distinctive appearance shown here. These we refer to as "bald" particles. The irregular surface character is a consequence of the deformable and unstable membrane surface in contact with the AFM tip. The scanning force used to obtain these images was the same as was employed to obtain the images of wild-type particles shown in Figs. 1 and 2.

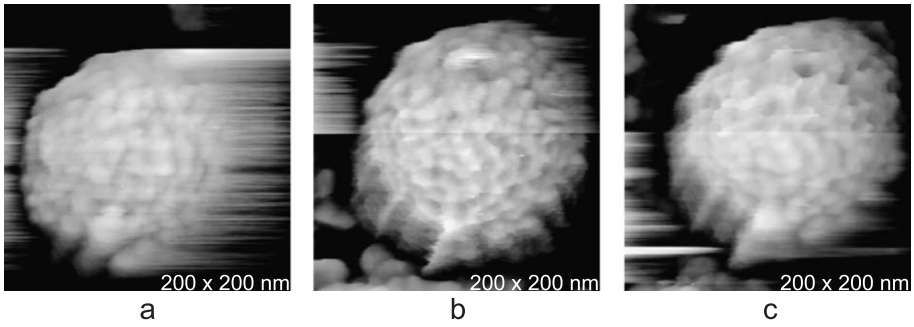

Fig. 4. Here, the same virus particle was scanned three times. In (a), the entire particle was scanned at low force. In (b), the top of the particle was scanned at low force, but the force was increased to that normally used, for example, for those images in Fig. 1, midway down the particle (hence the discontinuity), and the bottom of the particle scanned at normal force. In (c), the top of the particle was scanned at normal force, but the bottom at low force. In (b), the shift from normal to low force results in the appearance of the globular, presumably protein units on the particle surface, and in (c) the converse produces their erasure.

seen here. Repeated scans of the same particles show these deformations to be arbitrary and successive images highly variable. We refer to these env$^-$ particles as "bald" particles. In contrast, repeated scans of the same wild-type particles on 43-D cells showed identical images with the tufts in the same arrangements.

Examination of many virions on many cells, however, suggests a more complicated scenario than might be concluded from a simple side by side comparison of Figs. 1 and 3. A fraction of env$^-$ particles do exhibit some globular units on their surfaces when scanned at normal force. In most cases where the particles are not entirely "bald", these protein units are rather few and do not form anything resembling the closely packed arrays seen on the particle surfaces in Fig. 1. In only a few rare cases are particles seen that have a significant number of protein units.

If these "bald" particles are re-scanned but using very low force where tip–sample contact is only just sufficient to obtain an image, then it can be seen that more of the bald particles show indications of having globular features on their surfaces. An example is shown in Fig. 4 where a single particle is scanned in part at normal force and in part at low force. The diversity of sizes of the globular features is even greater than for wild-type particles, though the average size of the proteins is less, the overall density less, and the arrangement more scattered and less well packed than for wild-type env$^+$ particles. Because these virions contain no M-MuLV, if these globular features indeed represent proteins, they most likely represent host cell proteins incorporated into the viral membrane during budding.

It is not entirely evident why the surfaces of wild-type particles are so much more physically robust and stable, allowing reproducible high-resolution imaging of associated proteins, while those of the env$^-$ particles are more deformable and physically unstable. The difference, presumably, is a consequence of the presence of the envelope protein, and either some specific biophysical property it contributes to the surface, or perhaps simply to its greater packing density. It may also be true that the env protein is more firmly associated and fixed on the viral envelope than are host cell proteins.

Our attention having been drawn by the "bald" appearance of the env$^-$ particles on 14-11 cells, we retrospectively reviewed past recordings of wild-type virions on 43-D cell surfaces. In doing so, we identified several examples of "bald" or partially "bald" particles among populations of wild-type virus. An example is shown in Fig. 5a. These had generally escaped our notice previously or were simply attributed to poor image quality. This, however, was not the case. In Figs. 5b–c are two images of normal wild type-

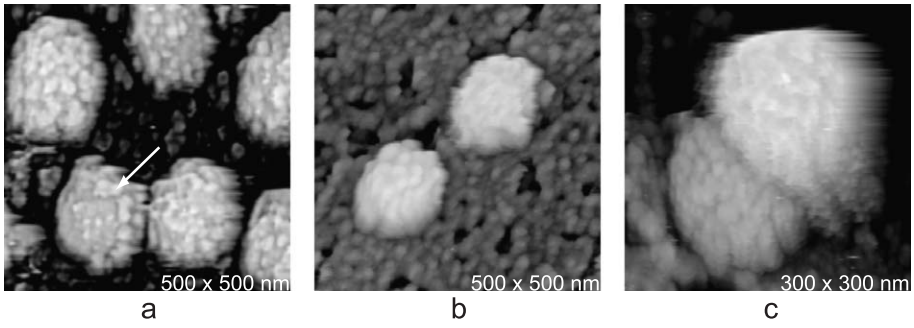

Fig. 5. In (a) is a partially "bald" M-MuLV wild-type particle that apparently lacks a normal complement of the envelope protein. In (b) and (c) are two images of ostensively wild-type M-MuLV that contain a normal "tufted", wild-type virion immediately proximal or touching a "bald" particle, the latter of which has presumably failed to incorporate envelope protein.

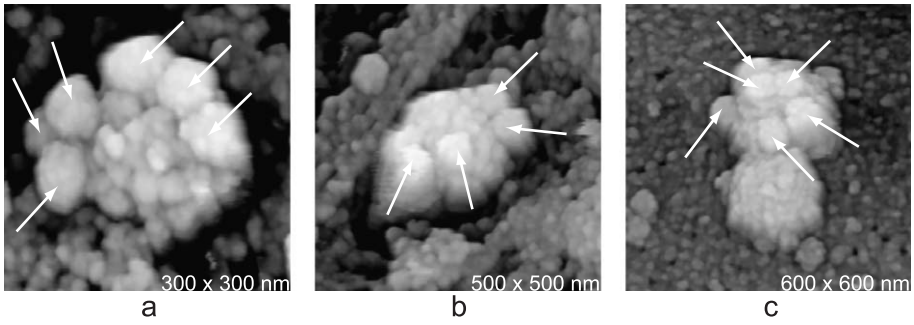

Fig. 6. In (a) and (b) are two M-MuLV virions in the process of emerging from the host cell surface, which have attracted monoclonal antibodies to the envelope protein. This is indicated by the secondary antibody conjugated gold clusters (marked with arrows) that subsequently bind to the primary antibody. In (c) are two M-MuLV particles immediately adjacent to one another, if not in contact. One of the virions is virtually covered with gold clusters, while the other has attracted none.

virions on cell surfaces with their characteristically closely studded "tufts" of SU protein, immediately adjacent to, or even in contact with a "bald" particle. These images demonstrate, first of all, that "bald" particles are not imaging artifacts, due to tip contamination, or due to any failure of the AFM technique, because they also contain, as internal standards, adequate renderings of normal, wild-type particles. The images further suggest that factors additional to the presence and availability of env protein in the host cell membrane may effect the incorporation of env protein into the lipid membrane of a virion. Some particles, it seems, are unable to acquire env protein even while their immediate neighbors can do so.

Fig. 6 illustrates another interesting observation. 43-D cells infected with wild-type M-MuLV were exposed to monoclonal antibody against SU protein and subsequently to 30 nm gold cluster-labeled secondary antibodies. In a and b are virions with gold particles attached to surface protein aggregates. In Fig. 6c, however, are two virions where one particle is almost covered with gold particles, while its immediate neighbor has no gold label bound to it, yet both virions have surfaces covered with protein "tufts". Both of these two particles appear to have a normal complement of SU envelope protein as judged by the presence of "tufts" of usual size and the stable physical surfaces. A possible explanation is that the specific epitope sought by the antibody is physically available for the reactive particle, while for the unreactive particle it is not. This differential binding of antibody was not unusual. We frequently observed a high affinity of some virions on the cell surfaces for antibody and virtually none by physically approximate neighbors.

Discussion

In these experiments, AFM was used to image wild-type M-MuLV virions associated with productively infected 43-3D cells, as well as M-MuLV virions lacking Env proteins produced from 14-11 cells. Comparison of the virions associated with the two cells under standard AFM conditions strongly supported the notion that the "tufts" of material visible on wild-type M-MuLV particles represent Env protein. It was interesting that no evidence for trimers or trimeric symmetry in the tufts was observed in the wild-type M-MuLV particles, which raises the possibility that envelope proteins in native M-MuLV particles may not exist as ordered trimers.

The protein tufts observed by AFM on the surfaces of wild-type M-MuLV virions have a radial extent of 6–10 nm, about what would be expected of SU protein aggregates. There are approximately 100 ± 20 tufts per virion. Given an average extent in the plane of the virion surface of 11–12 nm, then overall the tufts are rather globular and protrude only modestly from the virion surface. Thus it seems more appropriate to refer to SU "tufts" than "spikes".

The SU tufts in the AFM images presented here and in many more additional ones show no indications of being systematically composed of three discrete subunits, nor have we observed indications that tufts exhibit threefold symmetry. No systematic pattern of symmetrical arrangement of the tufts was evident either. Thus, the SU heads on M-MuLV envelope protein may be clustered in loose associations that may even be transient.

The lack of threefold symmetry of the SU proteins in M-MuLV virions was somewhat surprising because it might be assumed that the trimeric nature of retroviral envelope proteins would lead to threefold symmetry of all Env components in virions. However, direct evidence for threefold symmetry of SU trimers on M-MuLV or HIV virions has not been reported. The concept of threefold symmetry for SU originates primarily from the finding that the TM (gp41) domains of HIV-1 exhibit threefold symmetry when crystallized (Chan et al., 1997; Weissenhorn et al., 1997). Also, cryoelectron microscopy of human foamy virus particles indicates threefold symmetry of envelope protein (Wilk et al., 2000), although similar results have not been obtained for M-MuLV (Yeager et al., 1998). However, because the TM portion of Env protein is responsible for oligomerization (Doms, 1990; Earl, 1990; Einfeld and Hunter, 1994; Thomas, 1991), if the linkage between SU and TM is not rigid, then the attached SU protein might not

exhibit threefold symmetry. Indeed, when retroviral SU protein is expressed in the absence of TM, it is secreted from the cell in a monomeric form (Einfeld and Hunter, 1994), and the receptor-binding domain of M-MuLV SU and the core of HIV-1 gp120 (SU) crystallize as monomers (Fass et al., 1996; Kwong et al., 1998). Moreover, studies of the HIV-1 gp41 protein suggest that the trimer form may represent a ''fusion-active form'' that is created after binding of the virus to the cell receptor, and that it may not exist before binding (Frankel and Young, 1998).

On the other hand, the possibility must also be considered that the lack of detectable trimers in these studies could result from artifacts in the AFM technique. Possible artifacts related to tip shape or scanning force (that might theoretically distort protein shapes) must be under constant scrutiny, especially with biological specimens. Problems arising from unfavorable probe-surface interactions, particularly lateral force, are overcome to a great extent by the implementation of tapping mode, where the cantilever tip does not actually contact the specimen directly. With tapping mode, the probe tip rapidly oscillates up and down as it is scanned over the surface, essentially ''tapping'' its way and sensing the heights of features it encounters. A feedback mechanism adjusts, through a piezoelectric positioner, the vertical height of the sample surface to keep the amplitude of the probe oscillations a constant. Tapping mode minimizes, and may even eliminate, contact between cantilever tip and sample, and substantially reduces any distortion of soft samples. In the case of the virus and cell samples investigated here, which were considerably rigidified by glutaraldehyde fixation and dehydration, such distortion should not be significant. In fact, alteration of scanning force over a fairly wide range produces the same images for wild-type virions, and repeated scans of individual wild-type virions at the same force are consistently the same.

In addition, an artifact might also arise from histological treatments of samples before imaging. However, our fixation and dehydration conditions were virtually the same as those used by transmission electron microscopists to image viruses, including HIV and M-MuLV. They were also the same as those used in the EM visualization of another retrovirus, human foamy virus (HFV), where the distinctive trimeric symmetry of Env protein on those virions was preserved and recorded (Wilk et al., 2000). Previously, we investigated a diverse array of plant viruses under these histological conditions, with virions ranging in size from only 17-nm diameter to 50 nm or more, and we were able to show that the observed virion structures were consistent with those obtained by transmission and cryoelectron microscopy, and in some cases X-ray crystallography (Kuznetsov et al., 2001; Lucas, et al., 2001). In particular, the symmetrical dispositions of capsomeres in viral capsids were maintained. On the surfaces of 3T3 cells in culture, M-MuLV particles were imaged that exhibited both low and high densities of surface proteins, with tufts of proteins on the former separated by smooth areas of membrane. This was additional evidence that fixation with glutaraldehyde, osmium tetroxide, and dehydration yields rigid particles that exhibit no membrane deformation.

Finally, particles were scanned left to right, and then right to left, and no essential differences were observed for wild-type virions. This suggests that deformation due to tip pressure was not a concern with wild-type particles with envelope proteins embedded in their lipid membrane. In this investigation, many different cantilever tips were used that displayed a range of tip shapes, acuities, and aspect ratios, and no significant differences were noted in virion features except for their resolution with a particular tip. A variety of scanning frequencies and oscillation frequencies within the permissible and appropriate ranges were also used, but with only the anticipated effects.

In contrast to wild-type M-MuLV virions, the env$^-$ virions on 14-11 cells had a ''bald'' appearance when imaged under standard conditions. This represented greater flexibility of the proteins present on the surface, and potentially lower concentrations of protein as well. When 14-11 cells are imaged at normal tip pressures, the virion surfaces are variable and only become marginally reproducible when the scanning force is significantly reduced. Thus, for these particles, the standard tip force does deform the particles. Presumably, the proteins present on the surface env$^-$ particles are host cell-derived proteins.

The inclusion of host cell membrane proteins in retroviral virions has been extensively documented and reviewed (Ott, 1997; Vogt, 1997). Incorporation of host cell surface proteins such as CD4 and MHC proteins (Arthur et al., 1992; Graham et al., 2003) has been demonstrated for HIV and M-MuLV, and heterologous viral envelope proteins can also be incorporated (pseudotyping). These, it is pointed out, are present in virions in quantities not very different from those of the viral Env protein (Arthur et al., 1992). Similar findings are reported as well for several other host cell proteins by Graham et al. (2003). Our results make no attempt to identify and quantify the proteins on the 14-11 particles, but do suggest that the total amount of these proteins is likely to be significant, at least in the absence of env protein, consistent with the findings of Graham et al. (2003) and others. It is also unclear if the density of the cellular proteins in these particles is reflective of the density in wild-type particles or if the presence of M-MuLV Env protein might affect the presence of host-derived envelope proteins.

As indicated, a noteworthy difference between the wild-type and env$^-$ M-MuLV particles was that the Env protein in wild-type virions had a more stable configuration. This stabilization could simply reflect increased protein density and better physical packing by bona fide M-MuLV Env protein. Alternatively, stability could arise from specific interaction of the internal C-terminal tail of TM with MA protein that underlies the viral membrane (Vogt, 1997). Crosslinking studies have indicated that the C-terminal domain of HIV-1 makes direct contact with MA in virions (Wyma et al., 2000). Thus, each copy of envelope protein on

the virion may serve as a reinforcing rod to rigidify the viral membrane through interaction with the MA shell inside. Cellular proteins incorporated into virions would probably not specifically interact with the MA shell and thus not stabilize the viral membrane.

We also observe that occasional "bald" particles appear even on cells infected with wild-type virus. This could arise from aberrant targeting of some emerging immature virions to areas of the cell membrane devoid of envelope protein. It has recently been reported that transport of M-MuLV RNA to the cell surface requires interactions with both Gag and Env proteins (Basyuk et al., 2003). Thus, intracellular interactions between Gag and Env proteins may be necessary for normal virion budding and morphogenesis.

We observe that viral particles do not all bind SU-specific MAb with the same affinity, even when the virions are physically close together, appear to have the same surface characteristics, and have emerged through proximal areas of cellular membrane. This suggests that the MAb 238-specific epitope is less accessible on some particles than others, even when similar densities of SU tufts are present. A possible explanation is that the structure of the env protein or its attached oligosaccharides on the virus surface may not be static, but assumes different conformations, or forms different oligomeric combinations with neighboring protein units, that then confer different levels of protection against antibodies.

There is a marked resemblance between the AFM images of M-MuLV virions shown here and AFM images of HIV particles presented previously (Kuznetsov et al., 2003). The patterns of protein tufts on the surfaces are similar, as are the densities and distribution of sizes of the tufts. There are two differences, however. First, M-MuLV virions are about 20% larger than those of HIV. Second, M-MuLV particles tend to appear as individual particles more or less evenly distributed over entire surfaces of infected fibroblasts. HIV virions, on the other hand, tend to appear in dense masses on only a few cells of an infected population, the remainder exhibiting on their surfaces only a few particles (Kuznetsov et al., 2002, 2003).

Materials and methods

Cell culture

43-D cells are NIH 3T3 cells productively infected with a molecular clone of M-MuLV and have been described previously (Lander et al., 1999). 14-11 cells are NIH 3T3 cells that were stably transfected with a cloned M-MuLV provirus, from which the *env* gene had been deleted, and have been described previously (Feuerman, 1985). Both cell lines were grown in Dulbecco-modified Eagle's medium supplemented with 10% calf serum. For the AFM measurements, the cells were grown on glass cover slips. For immunogold detection, a monoclonal antibody specific for M-MuLV Env protein (Mab 238) was used (Lander et al., 1999).

Atomic force microscopy

The methods employed for AFM visualization of retroviruses on cells have been described elsewhere (Kuznetsov et al., 2001, 2002, 2003; Malkin et al., 2003) and are fundamentally the same as those used here. Cells and virions were fixed with 0.05% glutaraldehyde, postfixed with OsO_4, dehydrated in stages, and imaged under ethanol. To determine the change in dimensions of viruses and viral features because of dehydration, M-MuLV particles were also imaged under buffer alone where they were fully hydrated and dimensions compared with those imaged at low resolution under ethanol. Dehydration with ethanol produces a particle shrinkage of 15 $\pm$ 2%. Therefore, all dimensions reported here have been corrected by increasing the measured values by 15%. For immunolabeling, also described previously (Kuznetsov et al., 2002), paraformaldehyde-fixed cells, which preserve antigenicity, were similarly fixed and dehydrated after exposure to mouse primary monoclonal antibody against the SU protein (Mab 538), followed by reaction with a goat anti-mouse IgG antibody conjugated to 30 nm gold particles (Ted Pella, Redding, CA).

Specimens adhering to glass cover slips were mounted on a J-piezoscanner of a Nanoscope IIIa atomic force microscope (Digital Instruments, Santa Barbara, CA). Oxide-sharpened silicon nitride tips were used in the 75-μl fluid cell. AFM images were collected in tapping, height mode at frequencies of about 9.2 kHz with a scanning frequency of 1 Hz. The force employed was the same as used in previous studies of wild-type env^+ virions (Kuznetsov et al., 2002). For env^- virions, however, in some experiments, lower forces were used to observe proteins on the unstable membrane surfaces.

A precise assessment of the average size for protein aggregates on virion surfaces is complicated by cantilever tip shape and its effective radius of curvature, the sphericity of the virions, the failure of the tip to reach background between features, and the irregular shapes and sizes of the topological features. To obviate these problems, in well-resolved images, an average size for the protein aggregates on particle surfaces was obtained by choosing circular areas of defined size near the centers of particles (where there was the least amount of distortion due to tip and particle shape) and simply dividing the areas by the number of aggregates they contained. These results were then averaged for several particles. If tufts are assumed to then have a uniform cross section, their average diameter can be calculated.

Acknowledgments

The authors wish to thank Mr. Aaron Greenwood for his assistance in producing the figures. This work was

supported by grants CA32455 (HF) and GM58868 (AM) from the NIH and NASA NAS8-00017 (AM). Support of the UCI Cancer Research Institute and the Chao Family Comprehensive Cancer Center is acknowledged.

References

Arthur, L.O., Bess Jr., J.W., Sowder, R.C., Benveniste, R.E., Mann, D.L., Chermann, J.C., Henderson, L.E., 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science 258, 1935–1938.

Basyuk, E., Galli, T., Bertrand, E., 2003. Dev. Cell 5, 161–174.

Bolognesi, D.P., Montelaro, R.C., Frank, H., Schafer, W., 1978. Assembly of C-type oncornaviruses. A model. Science 199, 183–186.

Briggs, J.A.G., Wilk, T., Welker, R., Krausslich, H.-G., Fuller, S.D., 2003. Structural organization of authentic, mature HIV-1 virions and cores. EMBO J. 22 (7), 1707–1715.

Burton, D.R., 1997. A vaccine for HIV type 1: the antibody perspective. Proc. Natl. Acad. Sci. U.S.A. 94 (19), 10018–10023.

Chan, D.C., Fass, J.M., Berger, E.A., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89, 263–273.

Dong, J.Y., Dubay, J.W., Perez, L.G., Hunter, E., 1992. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. J. Virol. 66, 865–874.

Fass, D., Harrison, S.C., Kim, P.S., 1996. Retrovirus envelope domain at 1.7 angstrom resolution. Nat. Struct. Biol. 3 (5), 465–469.

Feuerman, M., 1985. J. Virol. 54, 804–816.

Frankel, A.D., Young, J.A.T., 1998. HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. (67), 1–25.

Freed, E.O., Myers, D.J., Risser, R., 1989. Mutational analysis of the cleavage sequence of the HIV type 1 envelope glycoprotein precursor gp 160. J. Virol. 63, 4670–4675.

Gebhardt, A., Bosch, J.V., Ziemiscki, A., Friis, R.R., 1984. Rous sarcoma virus p19 and gp 35 can be chemically crosslinked to high molecular weight complexes. An insight into virus assembly. J. Mol. Biol. 174, 297–317.

Gelderblom, H.R., 1990. Morphogenesis, maturation and fine structure of lentiviruses. In: Pearl, L.H. (Ed.), Retroviral Proteases: Maturation and Morphogenesis. Stockton Press, New York.

Gelderblom, H.R., Hansman, E.H., Ozel, M., Pauli, G., Koch, M.A., 1987. Fine structure of HIV and immunolocalization of structural proteins. Virology 156, 171–176.

Graham, D.R.M., Chertova, E., Hilburn, J.M., Arthur, L.O., Hildreth, J.E., 2003. Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with B-Cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts. J. Virol. 77 (15), 8237–8248.

Kuznetsov, Y.G., Malkin, A.J., McPherson, A., 1997. Atomic force microscopy studies of living cells: visualization of motility, division, aggregation, transformation, and apoptosis. J. Struct. Biol. 120 (2), 180–191.

Kuznetsov, Y.G., Malkin, A.J., Lucas, R.W., Plomp, M., McPherson, A., 2001. Imaging of viruses by atomic force microscopy. J. Gen. Virol. 82 (Pt 9), 2025–2034.

Kuznetsov, Y.G., Datta, S., Kothari, N.H., Greenwood, A., Fan, H., McPherson, A., 2002. Atomic force microscopy investigation of fibroblasts infected with wild type and mutant murine leukemic virus (MuLV). Biophys. J. 83, 3665–3674.

Kuznetsov, Y.G., Victoria, J.G., Robinson, W.E.J., McPherson, A., 2003. Atomic force microscopy imaging of human immune deficiency virus (HIV) and HIV infected lymphocytes. J. Virol. 77 (22), 11896–11909.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393, 648–659.

Lander, 1999.

Leamnson, R.N., Shander, M.H.M., Halpern, M.S., 1977. A structural protein complex in Moloney leukemia virus. Virology 76, 437–439.

Malkin, A.J., Kuznetsov, Y.G., Lucas, R.W., McPherson, A., 1999. Surface processes in the crystallization of turnip yellow mosaic virus visualized by atomic force microscopy. J. Struct. Biol. 127 (1), 35–43.

Malkin, A.J., McPherson, A., Gershon, P.D., 2003. Structure of intracellular mature vaccinia virus visualized by in situ atomic force microscopy. J. Virol. 77 (11), 6332–6340.

Nguyen, D.H., Hildreth, J.K., 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. J. Virol. 74, 3264–3272.

Ott, D.E., 1997. Cellular proteins in HIV virions. Rev. Med. Virol. 7, 167–180.

Parren, P.W., Moore, J.P., Burton, D.R., Sattentau, Q.J., 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. AIDS 13 (Suppl A), S137–S162.

Perez, L.G., Hunter, E., 1987. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein that block processing to gp85 and gp37. J. Virol. 61, 1609–1614.

Plomp, M., Rice, M.K., Wagner, E.K., McPherson, A., Malkin, A.J., 2002. Rapid visualization at high resolution of pathogens by atomic force microscopy: structural studies of herpes simplex virus-1. Am. J. Pathol. 160 (6), 1959–1966.

Poignard, P., Saphire, E.O., Parren, P.W., Burton, D.R., 2001. GP120: biologic aspects of structural features. Annu. Rev. Biochem. 19, 253–274.

Shiraishi, T., 2001. Myristoylation of human immunodeficiency virus type 1 gag protein is required for efficient env protein transportation to the surface of cells. Biochem. Biophys. Res. Commun. 282, 1201–1205.

Turner, B.G., Summers, M.F., 1999. Structural biology of HIV. J. Mol. Biol. 285, 1–32.

Vogt, V.M., 1997. Retrovirusal virions and genomes. In: Coffin, J.M., Hughes, S.H., Varmus, H.E. (Eds.), Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 27–69.

Weissenhorn, W., Dessen, A., Harrison, S.C., Skehel, J.J., Wiley, D.C., 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature 387, 426–430.

Wilk, T., de Haas, F., Wagner, A., Rutten, T., Fuller, S.D., Flugel, R.M., Lochelt, M., 2000. The intact retroviral env glycoprotein of human foamy virus is a trimer. J. Virol. 74 (6), 2885–2891.

Wilk, T., Gross, I., Gowen, B.E., Rutten, T., Felix, D.H., Welker, R., Krausslich, H.-G., Boulanger, P., Fuller, S.D., 2001. Organization of immature human immunodeficiency virus type 1. J. Virol. 75 (2), 759–771.

Witte, O.N., Tsukamoto-Adey, A., Weissman, I.L., 1977. Cellular maturation of oncornavirus glycoproteins: topological arrangement of precursor and product forms in cellular membranes. Virology 76, 539–553.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens and immunogens. Science 280, 1884–1888.

Wyma, D.J., Kotov, A., Aiken, C., 2000. Evidence for a stable interaction of gp41 with Pr55 Gag in immature human immunodeficiency virus type 1 particles. J. Virol. 74 (20), 9381–9387.

Yeager, M., Wilson-Kubalek, E.M., Weiner, S.G., Brown, P.O., Rein, A., 1998. Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms. Proc. Natl. Acad. Sci. U.S.A. 95, 7299–7304.