Localization of Structural Proteins in African Swine Fever Virus Particles by Immunoelectron Microscopy

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Seven African swine fever virus structural proteins were localized in the virion by immunoelectron microscopy. African swine fever virus-infected cells were incubated, before or after embedding and thin sectioning, with monoclonal antibodies specific for different structural proteins, and after labeling with protein A-gold complexes, the samples were examined in the electron microscope. Proteins p14 and p24 were found in the external region of the virion, proteins p12, p72, p17, and p37 were found in the intermediate layers, and protein p150 was found in the nucleoid and at one vertex. A monoclonal antibody that recognized protein p150 as well as p220, a virus-induced, nonstructural protein, could also bind to a component present in the nucleus of both uninfected and virus-infected cells.

African swine fever (ASF) virus is an icosahedral cytoplasmic deoxyvirus that multiplies in members of the Suidae family and Ornithodoros (Argasidae: Acari) (reviewed in references 9, 19, and 20). One of the most striking aspects of ASF is that the ASF virus-specific antibodies, even those from recovered or chronically infected pigs or from ASF virus-resistant animal species inoculated with the virus, do not neutralize the virus (6). The inability to produce neutralizing antibodies might be due to the nature of the virus rather than due to the host, since pigs recovered from virulent ASF virus infections or chronically infected animals respond normally to foot-and-mouth disease virus, producing neutralizing antibodies (6).

ASF virus particles consist of a nucleoid of about 80 nm surrounded by a lipid membrane, probably associated with the morphological units of the capsid (4). The extracellular virions have an external membrane derived by budding through the plasma membrane (1) and an external diameter of about 200 nm. The virion is built up by about 30 structural proteins, some of which are of host origin (5, 17). Knowledge of the protein composition of the different virion regions, mainly that of the envelopes, is important because those proteins might have immunological significance.

Localization of antigens in situ by the use of protein A-gold complexes is an accurate technique (14) which has been used to detect virus antigens in virions (7, 21) and tissues (2).

We show in this paper an analysis of ASF virus-infected cells by immunoelectron microscopy, using a collection of ASF virus-specific monoclonal antibodies (MAbs) (17) labeled with protein A-gold complexes. This study showed that proteins p14 and p24 were present in the external region of the virion, proteins p12, p72, p17, and p37 were in the intermediate layers, and protein p150 was in the nucleoid and one vertex. An MAb that recognized protein p150 and p220, a virus-induced, nonstructural protein, could also bind to a component present in the nucleus of both uninfected and virus-infected cells.

MATERIALS AND METHODS

Cells and virus. Vero cells (CCL81) were obtained from the American Type Culture Collection (Rockville, Md.). ASF virus adapted to grow in Vero cells and the conditions for plaque titration have been described previously (8). Extracellular ASF virions were purified as described elsewhere (5).

Immunoreagents. Serum against purified ASF virions were obtained as described by Carrascosa et al. (5). Hybrid cells secreting MAbs specific for ASF virus were obtained by fusing mouse myeloma and spleen cells from ASF virus-immunized mice (17). Protein A-Sepharose-purified immunoglobulin G from ascites was provided by J. Rey. The properties of the MAbs used in this work have been described previously (17).

Colloidal gold particles of about 5-nm diameter were prepared by the method described by Horisberger (10). The coupling of the colloidal gold to protein A from Staphylococcus aureus (Pharmacia, Uppsala, Sweden) was done as indicated by Roth (14).

Virus-infected cells. Vero cells were cultured in plastic plates in Dulbecco modified Eagle medium containing 10% calf serum to a density of about 10⁵ cells per cm² and infected with ASF virus at a multiplicity of infection of about 10 PFU per cell. After 2 h at 37°C, nonadsorbed virus was removed, and fresh medium with 2% calf serum was added for further incubation.

Immunolabeling and low-temperature embedding. (i) Labeling before thin sectioning. ASF virus-infected Vero cells were collected 24 to 36 h after infection, when they showed a strong cytopathic effect and many were permeable to the antibodies. The cells were washed and suspended in phosphate-buffered saline (PBS) containing 1% ovalbumin and different amounts of antibody. After 1 h at 25°C, the cells were washed and resuspended in PBS containing 1% ovalbumin and protein A-gold complex diluted 1/20th from a stock prepared as described by Roth (14). After 30 min at 25°C, the cells were washed with PBS, fixed with a solution

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containing 2% glutaraldehyde and 2% tannic acid in PBS (13, 18) for 1 h at 25°C, and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldrailburg, Federal Republic of Germany) as described below.

(ii) **Labeling after thin sectioning.** ASF virus-infected cells were collected at 16 to 20 h after infection, suspended in 0.5% glutaraldehyde in PBS and 2% tannic acid (13, 18), and incubated for 5 min at 25°C with gentle stirring. After low-speed centrifugation, the samples were dehydrated with ethanol and embedded in Lowicryl K4M by the procedure of Carlemalm et al. (3). The first steps of the dehydration process were carried out at 4°C, and after the 50% ethanol step, the samples were kept at −20°C. The resins were polymerized by irradiation with 360-nm UV light for 48 h at −20°C and 24 to 48 h at room temperature. Thin sections, obtained with a Sorvall Porter-Blum ultramicrotome, were adhered to nickel grids, which were floated on a drop of 1% ovalbumin in PBS for 15 min at 25°C. After the grids were transferred to a drop of the appropriate dilution of the antibody, they were incubated at 4°C for 12 to 16 h. The antibody was washed off with a stream of PBS, and the grids were treated with 1% ovalbumin and protein A-gold complexes in PBS for 1 h at 25°C, washed with water, dried, stained with saturated uranyl acetate, and finally washed with water.

**Electron microscopy.** Samples were examined in a JEOL 100B electron microscope. Measurements were done on electron micrographs of virus sections showing a hexagonal outline. The radial distribution of the gold granules was calculated by determining, for each virus section, the center, the radius of the particle, and the distance of each gold granule to the center, in a writing table connected to a PDP11-45 minicomputer. The radial distribution of each labeled component was then expressed as the average ratio of the distances to the radii. The number of virus sections and gold granules counted are indicated in each experiment.

**RESULTS**

**Labeling of ASF virus particles with serum against purified virions.** ASF virus-infected cells obtained at 24 to 36 h postinfection showed a strong cytopathic effect and were permeable to the immunoglobulins from an antiserum against purified ASF virions. After the unbound antibodies were removed by washing, the bound immunoglobulins were labeled with protein A-gold complexes, and the cells were fixed, embedded in resin, and thin sectioned. Figure 1 shows that the antibodies labeled the periphery of the virus particles. Occasionally, gold particles were seen inside the virions, probably owing to superimposition of the antibody-gold complexes on the surface of sliced virions present within the sections.

To detect antigenic determinants inside the virus particle, ASF virus-infected cells were fixed with 0.5% glutaraldehyde and embedded under low-denaturation conditions to preserve as much as possible the antigenic properties of the structural components of the virion. Thin-sectioned cells, which revealed the presence of ASF virus particles in different states of maturation, were labeled with sera against purified ASF virus particles and tagged with protein A-gold complexes. A clear labeling was found with an ASF virus-
FIG. 2. Postembedding immunolabeling of ASF virus particles with serum against purified virions. (a) ASF virus-infected Vero cells, collected at 24 h after infection, were fixed, embedded in Lowicryl, thin sectioned, labeled with an ASF virus antiserum and protein A-gold complexes, and contrasted with uranyl acetate. (b) As a control, ASF virus-infected cell sections were labeled with a preimmune serum and protein A-gold complexes. Bar, 200 nm.

specific antiserum (Fig. 2a) but not with a preimmune serum (Fig. 2b).

Labeling of structural proteins in ASF virus particles with MAb. The data described above indicated the possibility of localizing the positions of the structural proteins of ASF virions by immunolabeling with MAb specific for different structural proteins.

(i) Proteins p14 and p24. ASF virus-infected cells were incubated with several MAb specific for different ASF virus proteins, tagged with protein A-gold complexes, fixed, thin sectioned, and processed for electron microscopy. Immunolabeling of the virion was only seen with MAb specific for proteins p14 and p24 (Fig. 3). This indicated that these proteins were present in the virion periphery. In the case of the MAb specific for protein p24 (Fig. 3b), the label was seen in many cases following the side of the virion that was in contact with the outer part of the viral factory. This suggests an accumulation of the protein at that interface.

(ii) Proteins p72, p12, p17, and p37. Immunolabeling of thin sections of ASF virus-infected cells with MAb 19B.A2, specific for protein p72, a major component of the virion, labeled a region near the external part of the virus particles (Fig. 4). Similar results were obtained by using other MAb specific for other protein p72 epitopes (data not shown). The
FIG. 3. Preembedding immunolabeling of ASF virus particles with either MAb specific for protein p14 or MAb specific for p24. ASF virus-infected Vero cells were treated as described in the legend to Fig. 2 except that they were labeled with either MAb specific for protein p14 (a) or MAb specific for protein p24 (b). Bar, 200 nm.
radial distribution value of protein p72, obtained from 1,202 gold granules counted in 88 virions, was 0.80 ± 0.18 (mean value ± standard deviation).

Labeling of this area of the virion was also obtained with MAbs specific for other ASF structural proteins. The labeling patterns obtained with the MAbs specific for proteins p12, p17, and p37 presented in the electron microscope a general aspect indistinguishable from that shown for p72 in Fig. 4 (data not shown). The MAb 18B.B11, specific for protein p12, presented a radial distribution value of 0.82 ± 0.17 (from 509 gold granules counted in 100 virions). For MAb 17K.G12, specific for protein p17, the radial distribution value of the gold granules was 0.72 ± 0.21 (from 910 gold granules in 90 virions). A similar value was obtained from MAb 17F.E10, specific for protein p37 (0.71 ± 0.19 from 1,300 gold granules in 140 virions).

(iii) Protein p150. Protein p150, one of the major virus structural proteins, is antigenically related to the virus-induced, nonstructural protein p220 (17). Figure 5 shows that MAbs 17A.H2 and 17K.G6, which recognize both proteins p220 and p150 (Fig. 5a and b) and only protein p150 (Fig. 5c and d), respectively, labeled the nucleoid and one vertex of the virion. From 187 virions, 58 contained gold granules in the nucleoid, 101 contained granules in one vertex, and 28 contained granules in both.

The MAb that recognized both proteins p220 and p150 labeled cell nucleus from infected (Fig. 6a and b) and from uninfected (data not shown) cells. In contrast, the MAb that recognized only protein p150 did not label the nucleus from either cell (Fig. 6c).

DISCUSSION
A study of ASF virus-infected cells by immunoelectron microscopy, using MAbs that bound protein A-gold complexes, allowed us to localize seven structural proteins and to define three regions in the virions: the external region, the intermediate layers, and the nucleoid. Both the nucleoid and the intermediate layers correspond to regions defined previously, but it is uncertain whether the external region defined here corresponds to the most external virion membrane (4).

A serum against purified virions (Fig. 1) and MAbs specific for either protein p14 or p24 (Fig. 3) labeled the external region in the virion in nonsectioned cells permeable to the antibodies. It is likely that protein p24 is a host protein since a MAb specific for protein p24 binds to uninfected and ASF virus-infected cells and immunoprecipitates a protein of relative molecular weight of 24,000 from both kinds of cells (17). The location of protein p24 in the external region of ASF virus particles is consistent with the finding that a MAb specific for that protein reduces virus infectivity about 100-fold (L. Enjuanes, B. García-Barreno, A. Sanz, M. L. Nogal, and E. Víñuela, unpublished data).

The virus antiserum labeled the external region, the intermediate layers, and the nucleoid in thin-sectioned cells (Fig. 2). The labeling pattern of the external region showed gold granules closely following the external virus shape at distances ranging from direct contact to 13 nm apart or even more. The labeling of the most external region was less intense in virions labeled after the infected cells were thin sectioned than in those labeled before thin sectioning. This could be due to a partial denaturation of the outer epitopes during the process of fixation and embedding.

The intermediate layers of ASF virus particles contained proteins p12, p17, p37, and p72. Antibodies against these proteins labeled the areas corresponding to the virus capsid and the membrane layers situated below (4). The values obtained for the radial distribution of these proteins ranged from 0.70 to 0.83 with standard deviations around 20 to 30%.
FIG. 5. Postembedding immunolabeling of ASF virus particles with either MAb 17A.H2, specific for proteins p220 and p150, or MAb 17K.G6, specific for protein p150. ASF virus-infected Vero cells were treated as described in the legend to Fig. 2, except that they were labeled with either MAb 17A.H2 (a and b) or MAb 17K.G6 (c and d). The insert in panel a shows a detail of the labeling of a virion corner with MAb 17A.H2. Bar, 200 nm.

of those values. Taking into account the actual dimensions of ASF virus particles (4), the values of the standard deviations ranged between 16 and 20 nm. These values were similar to the average value for the probe (about 18 nm), which consisted of an immunoglobulin G molecule tagged with protein A and gold granules with a size of around 5 nm (14). This meant that the deviation of the experimental data was due mainly to the size of the probe, indicating that the resolution was around 20 nm or less.

The inner nucleoid and one vertex of the virion were labeled by two MAbs, 17A.H2 and 17K.G6; MAb 17A.H2 immunoprecipitates the nonstructural, virus-induced protein p220 and the structural protein p150 (17) and labeled the nucleus from both uninfected and virus-infected cells (Fig. 6). This result indicated the existence of a structural relationship between protein p150 and a nuclear component in the host-cell nucleus. That is not surprising since ASF virus, like the poxviruses, contains in the virion most, if not all, of the enzymes required for the synthesis of mRNA (11, 12, 15, 16). The second MAb (17K.G6), which recognizes only protein p150, did not label the host-cell nucleus from either uninfected or virus-infected cells. This indicated that the virus and the cell component recognized by MAb 17A.H2 were different.

The labeling of two different regions (nucleoid and one vertex) with two MAbs specific for p150 raises a question about the location of this protein. Either p150 is located at both sites or there is a common epitope in two different structural proteins located in those positions. The first hypothesis is consistent with the results obtained by treatment of ASF virus particles with nonionic detergents, in which p150 seems to be released under two different conditions, suggesting two different locations for this polypeptide (A. L. Carrascosa, J. F. Santarén, and E. Viñuela, unpublished data). The second possibility has not been supported by immunoprecipitation experiments, since MAbs that recognize both proteins p150 and p220 only precipitated protein p150 from dissociated virus (17).

Figure 7 summarizes the localization in ASF virus particles of the proteins detected by immunolabeling with MAbs
FIG. 6. Postembedding immunolabeling of the nucleus of ASF virus-infected Vero cells by MAb 17A.H2, specific for proteins p220 and p150. ASF virus-infected Vero cells were treated as described Fig. 2, except that they were labeled with either MAb 17A.H2, specific for proteins p220 and p150 (a and b), or MAb 17K.G6, specific for protein p150 (c). Bar, 200 nm.
and protein A-gold complexes. The relative position of these proteins has been confirmed by immunolabeling with pairs of MAbS tagged with gold granules of different size (data not shown).

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LITERATURE CITED