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Involvement of the Endoplasmic Reticulum in the Assembly and Envelopment of African Swine Fever Virus

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African swine fever (ASF) virus is a large enveloped DNA virus assembled in the cytoplasm of cells. In this study, the membrane compartments involved in the envelopment of ASF virus were investigated. A monoclonal antibody recognizing p73, the major structural protein of ASF virus, was generated to analyze the binding of p73 to membranes during the assembly of the virus. Approximately 50% of the intracellular pool of p73 associated with membranes as a peripheral membrane protein. Binding was rapid and complete within 15 min of synthesis. Subcellular membrane fractionation showed that newly synthesized p73 molecules cosedimented with endoplasmic reticulum (ER) membranes and remained associated with the ER during a 2-h chase. A similar distribution on gradients was recorded for p17, a structural membrane protein of ASF virus. The results suggested that the ER was involved in the assembly of ASF virus. A protease protection assay demonstrated a time-dependent envelopment of the membrane bound, but not cytosolic, pool of p73. Envelopment of p73 took place 1 h after binding to membranes and was completed 1 h before the first detection of p73 in virions secreted from cells. Envelopment was unaffected by brefeldin A and monensin, drugs that block membrane transport between the ER and Golgi. Taken together the results provide evidence for the binding of ASF virus structural proteins to a specific membrane compartment and implicate a role for the ER in the assembly and envelopment of ASF virus.

African swine fever (ASF) virus is a large enveloped DNA virus that causes a highly pathogenic hemorrhagic disease in domestic pigs. The virus is endemic in Africa and parts of southern Europe and presents a major economic problem for the development of pig industries in these countries. At present there are no vaccines or control measures other than irradication by slaughter (51). Ideally, the design of an effective vaccine against ASF virus will require a knowledge of the amino acid sequence and location of proteins within the virion, and also an understanding of how viral proteins are processed for presentation as antigens. Important information on both aspects can be obtained from studies on the intracellular site of assembly of ASF virus. This is because the membrane compartments that control the intracellular transport and assembly of viral structural proteins also regulate the processing of ASF virus proteins into peptides and their presentation to the immune system.

The recent sequencing of 170-kb double-stranded genome of ASF virus has revealed at least 150 reading frames (15, 52). These include coding sequences for 11 known structural proteins and enzymes involved in DNA replication and repair. Enzymes required for virus-mediated protein phosphorylation, prenylation, and ubiquitination have been identified (21, 22, 35, 52), as have proteins involved in host cell interactions such as hemadsorption and apoptosis (4, 11, 33, 36). While these data provide the primary amino acid sequences of several structural proteins and insights into the mechanisms of viral DNA replication and pathogenesis, they provide little information about the assembly of the virus.

Assembly is clearly a complex process. ASF virus particles contain the 170-kbp genome, the enzymes necessary for the production of early mRNA, and more than 50 structural proteins (8). When viewed by electron microscopy, virions appear as 200-nm-diameter hexagons in cross section, with several internal concentric layers surrounding an electron-dense nucleoprotein core. The concentric layers include an inner and outer membrane envelope and a capsid composed of capsomers arranged in a hexagonal lattice (1, 9, 10). Virus assembly takes place in discrete perinuclear plaques in the cytoplasm called viral factories. Factories contain fully assembled viruses and an ordered series of assembly intermediates with between one and six sides of a hexagon (31). Factories also contain large quantities of membranous material of unknown origin. In spite of these detailed morphological studies, the mechanism of assembly of ASF virus and the origins of the membrane envelopes of ASF virus are poorly understood.

Several viruses are enveloped by specific membrane compartments that have been determined by analyzing the intracellular trafficking of envelope glycoproteins (reviewed in reference 20). The major glycoproteins of herpesviruses, for example, are synthesized in the endoplasmic reticulum (ER), pass through the *cis* and medial Golgi, and accumulate in the *trans* Golgi and/or endosomes, where they direct the envelopment of viral nucleocapsids (18, 45, 49, 54). The budding of flavivirus and rotaviruses is driven by a concentration of envelope glycoproteins in the ER. Similarly, mouse hepatitis virus buds into the intermediate compartment between the ER and Golgi (26, 47), and the Golgi is a site of envelopment of coronavirus (2, 29, 46) and rubella virus (2, 23). One problem in conducting similar experiments on ASF virus has been the apparent absence of suitable glycosylated envelope proteins. Six structural proteins are derived from an ordered proteolysis of two polyproteins, pp20 and pp60, in the cytosol (41, 52). These do not enter the secretory pathway. Four structural proteins (6, 34, 42, 43, 52) have putative transmembrane domains and N-linked glycosylation sites, but these are not glycosylated in isolated extracellular virions (14).

In this study, an alternative approach to determining the origins of the membrane envelope of ASF virus was taken. The

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major structural protein of ASF virus, p73, is a capsid protein that is localized next to the inner membrane envelopes of ASF virus (10). Consequently, it can be argued that p73 associates with the membranes that form the envelope of the virus. We have generated a monoclonal antibody that recognizes p73 and have used subcellular membrane fractionation to monitor the association of p73 with individual intracellular membrane compartments. In addition, a novel protease protection assay was developed to demonstrate envelopment of membrane-associated p73 molecules. The data show the rapid binding of newly synthesized protease-sensitive p73 molecules to the ER. This is followed by a time-dependent envelopment of p73 associated with protease resistance. These data show the association of ASF virus structural proteins with a specific membrane compartment and implicate a role for the ER in the envelopment of ASF virus.

MATERIALS AND METHODS

Cells and viruses. Vero (ECACC 84113001) and HTK$^-$ (ECACC 88022409) cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, United Kingdom). RS cells are a continuous cell line generated from pig kidney maintained at the Institute for Animal Health, Pirbright Laboratories, Surrey, United Kingdom. The virulent Malawi (15) and tissue culture-adapted BA71 (8) strains of ASF virus have been described previously. Vesicular stomatitis virus (VSV; Indiana strain) was provided by Nigel Ferris (Institute for Animal Health, Pirbright Laboratories). Vaccinia virus (VTF7) expressing T7 polymerase (16) was obtained from Bernard Moss (National Institutes of Health, Bethesda, Md.).

Antibodies. Monoclonal antibodies recognizing p73 were generated from BALB/c mice immunized with a crude membrane fraction prepared from porcine alveolar macrophages infected with the Malawi strain of ASF virus. Hybridomas were generated by fusing popliteal glands with JK myeloma cells, using standard methods. P5D4 (25) was provided by T. Kries (Department of Cell Biology, University of Geneva, Geneva, Switzerland). Monoclonal antibodies 17KG12 and 18BB11 were purchased from Ingenasa (Madrid, Spain). A rabbit antibody raised against a peptide (C-KDDDQKAVKDEL) corresponding to the C terminus of protein disulfide isomerase (PDI) has been described previously (53).

Screening of hybridoma supernatants. Subconfluent cultures of RS cells in 96-well plates were infected with the Uganda strain of ASF virus. The virus inoculum was adjusted to infect approximately 50% of the cells. Sixteen hours after infection, cells were fixed with methanol, blocked with 10% fetal calf serum in phosphate-buffered saline, and incubated with hybridoma supernatants. Washed plates were incubated with horseradish peroxidase (HRP)-coupled goat anti-mouse immunoglobulin G (IgG) (Southern Biotechnology Associates, Birmingham, Ala.), and positive supernatants were visualized by incubating washed plates with 3-amino-9-ethylcarbazole and H_2O_2. Visual inspection of wells with an inverted microscope was used to select supernatants that produced a brown intracellular precipitate in infected but not uninfected cells. Immune serum taken from pigs recovered from infection with ASF was used as a positive control.

Metabolic labeling and immunoprecipitation. Cells were labeled metabolically with [^{35}S]methionine and cysteine, using ^{35}S-Express label (New England Nuclear, Boston, Mass.). Cells infected with ASF virus were preincubated with methionine- and cysteine-free Eagle's medium for 15 min at 37°C, washed, and then pulse-labeled for the indicated time periods at 37°C in the same medium supplemented with 1 to 2 MBq of ^{35}S per ml. Cells were washed and chased in Dulbecco's modified Eagle's medium. At the appropriate time intervals, cells were washed once in phosphate-buffered saline and lysed in immunoprecipitation buffer (10 mM Tris [pH 7.8], 0.15 M NaCl, 10 mM iodoacetamide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg each of leupeptin, pepstatin, chymostatin, and antipain [Boehringer Mannheim, Lewes, United Kingdom] per ml). Lysates were precleared and immunoprecipitated at 4°C with antibodies immobilized on protein A- or protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden), and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography as described previously (50). Protein bands were quantitated with a Bio-Rad 620 video densitometer.

Expression of p73 and p17. HTK$^-$ cells were infected for 1 h at 37°C with the VTF7.3 strain of vaccinia virus encoding T7 polymerase. Cells were washed in serum-free medium and transfected by using Lipofectin (Gibco BRL, Life Technologies Ltd., Paisley, United Kingdom) with plasmid pGemAD62-18 (13), encoding p73, or the i1L reading frame subcloned into the pT7 vector (Invitrogen BV, NV Leek, The Netherlands); 24 h later, cells were analyzed for expression of antigen by immunoprecipitation.

Immunofluorescence. Vero cells grown to 80% confluence on glass coverslips were infected with ASF virus. Cells were fixed using 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with Tris buffer (100 mM, pH 7.8) containing 0.5% fish gelatin and 50% normal goat serum. Preparations were incubated with antibody diluted with the same buffer, washed, and then incubated with fluorescein-conjugated goat anti-mouse or rhodamine-conjugated rabbit IgG (Southern Biotechnology Associates). Cell preparations were viewed with a Leitz Diaplan microscope (Wild Leitz Co., Wetzler, Germany) fitted with epifluorescence optics and photographed with a Nikon UFX-DX camera system and Kodak EPL 400X film.

Subcellular fractionation. Vero cells (10^8/ml) were suspended in 0.25 M buffered sucrose (50 mM Tris, 1 mM EDTA [pH 7.4]) and homogenized by nitrogen cavitation (250 lb/in^2, 15 min, 4°C) in a cell disruption bomb (Parr Instrument Co., Moline, Ill.) or by 15 passages through a 25-gauge syringe needle. Whole cells and nuclei were removed by pelleting at 2,000 rpm for 10 min at 4°C in an Eppendorf 5402 centrifuge. Postnuclear supernatants were pelleted at 14,000 rpm for 20 min at 4°C in an Eppendorf 5402 centrifuge. Nycodenz (Nycomed Pharma AS, Oslo, Norway) solutions were prepared in 50 mM Tris (pH 7.4) containing 250 mM sucrose and 1 mM EDTA. Postnuclear membranes were resuspended in 1 ml of 30% Nycodenz and layered under a Nycodenz step gradient (1.5 ml of 25%, 4 ml of 15%, 3 ml of 10%, 2 ml of 5%, 2 ml of buffered sucrose). Gradients were centrifuged at 22,000 rpm in a Beckman SW40 Ti rotor for 90 min at 4°C. Fractions were collected from the bottom of the tube and assayed for the ER marker enzyme α-glucosidase by using 4-methylumbelliferyl-α-D-glucose, for the Golgi marker enzyme galactosyltransferase by monitoring the transfer of UDP-6-[^{3}H]galactose (New England Nuclear) to ovalbumin (5), and for the lysosomal enzyme β-hexosaminidase by using 4-methylumbelliferyl-*N*-acetylglucosamine. Endosomes were labeled by incubating cells with HRP (10 mg/ml) for 30 min and then chased for 10 min at 37°C. Fractions were assayed for HRP colorimetrically, using *o*-phenylenediamine and H_2O_2. Nycodenz concentrations were assessed by refractometry.

Triton X-114 extractions. Postnuclear membrane fractions prepared from 2×10^7 metabolically labeled cells infected with ASF virus were dissolved in 1% Triton X-114 (in 150 mM NaCl–10 mM Tris [pH 7.5]) and incubated on ice for 10 min. Lysates were then warmed to 30°C for 10 min and centrifuged at 2,000 rpm for 5 min (Eppendorf 5415 centrifuge). The detergent and aqueous phases were diluted to 1 ml with 150 mM NaCl–10 mM Tris (pH 7.5) and analyzed for the presence of antigen by immunoprecipitation followed by SDS-PAGE and autoradiography.

Alkali carbonate extractions. Membrane fractions prepared from metabolically labeled cells infected with ASF virus were suspended in 0.1 ml of Na_2CO_3 (100 mM [pH 11.5]) and incubated on ice for 20 min. The suspension was then diluted to 1 ml with phosphate-buffered saline, and membrane sheets were pelleted by centrifugation at 14,000 rpm for 30 min at 4°C (Eppendorf 5402 centrifuge). Supernatants and detergent-solubilized pellets were analyzed for the presence of antigen by immunoprecipitation as described above.

Trypsin protection assay. Vero cells infected with ASF virus strain BA71 were pulse-labeled with [^{35}S]methionine and -cysteine and homogenized as described above. Complete cell homogenates, or postnuclear membrane fractions, were incubated with trypsin (0.4 mg/ml) in acetate buffer (50 mM potassium acetate, 2.5 mM magnesium acetate, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.2], 20 mM $CaCl_2$) for 30 min at 37°C. Proteolysis was stopped by addition of a half volume of 10 mg of hen egg white trypsin inhibitor (Boehringer Mannheim) per ml. Reaction mixtures were diluted with 3 volumes of immunoprecipitation buffer supplemented with 5 mM phenylmethylsulfonyl fluoride, 3% fetal calf serum, and 10 mg of hen egg trypsin inhibitor per ml. The levels of p73 remaining were determined by immunoprecipitation and SDS-PAGE as described above.

RESULTS

Monoclonal antibody 4H3 binds p73, the major capsid protein of ASF virus. Hybridoma supernatants were first screened for the presence of virus-specific antibodies by immunocytochemistry. Monoclonal antibodies recognizing the major capsid protein were then selected on the basis of immunoprecipitation analysis. Figure 1A shows the results obtained with one antibody, 4H3. Lane 1 shows that 4H3 immunoprecipitated a protein of approximately 70 kDa from lysates prepared from infected cells. In lane 2, the major antigens of ASF virus were visualized by immunoprecipitation of cell lysates with hyperimmune serum taken from infected pigs. The protein recognized by 4H3 comigrated with the major 70-kDa protein recognized by the pig serum. The molecular size and relative abundance of the 4H3 antigen suggested that it may be p73, the major capsid protein of ASF. The ASF virus genome has been sequenced (52), and p73 is encoded by reading frame B646L. To test the specificity of 4H3 directly, the B646L reading frame subcloned downstream of a T7 promoter (13) was

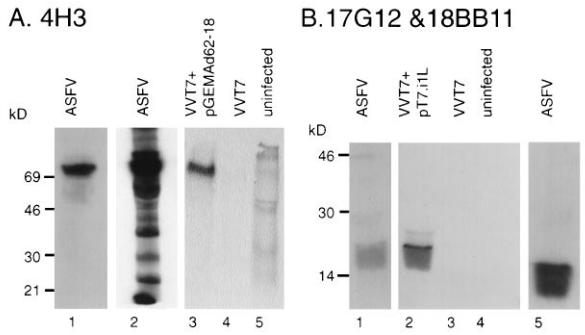

FIG. 1. Characterization of monoclonal antibodies. (A) Characterization of 4H3. Vero cells infected with ASF virus for 16 h were pulse-labeled with [^{35}S]methionine and -cysteine for 30 min at 37°C, lysed, and immunoprecipitated with 4H3 (lane 1) or hyperimmune pig serum (MI92) (lane 2). HTK$^-$ cells were pulse-labeled and immunoprecipitated with 4H3 after infection with vaccinia virus (VTF7.3) expressing T7 polymerase and transfection with the ASF virus reading frame B646L (lane 3), after infection with VT7 alone (lane 4), or without infection (lane 5). (B) Characterization of 17KG12 and 18BB11. Vero cells were infected with ASF virus and immunoprecipitated as described above, using 17KG12 (lane 1). HTK$^-$ cells were pulse-labeled and immunoprecipitated with 17KG12 after infection with vaccinia virus expressing T7 polymerase (VTF7.3) and transfection with the ASF virus i1L reading frame (lane 2), after infection with VTF7.3 alone (lane 3), or without infection (lane 4). Vero cells were infected with ASF virus and immunoprecipitated as described above, using 18BB11 (lane 5). Proteins were resolved by SDS-PAGE (12% gel) under reducing conditions and visualized by autoradiography. The positions of molecular mass markers are shown.

expressed transiently in HTK$^-$ cells infected with a recombinant vaccinia virus expressing T7 polymerase (16). Lane 3 of Fig. 1A shows that 4H3 immunoprecipitated a 70-kDa protein from cells expressing the B646L reading frame but not from mock-infected cells (lane 4) or from the parental cell line (lane 5). The experiment confirmed that 4H3 bound p73, the major capsid protein of ASF virus. 17KG12 was a second monoclonal antibody used in this study. The antibody binds a 17-kDa structural protein of ASF virus (37), but the amino acid sequence of the antigen has not been reported. ASF virus reading frame i1L encodes a 17-kDa protein (15, 42). By using the vaccinia virus expression method described above, the i1L gene was expressed in HTK$^-$ cells, and cell lysates were immunoprecipitated with 17KG12. Lane 2 of Fig. 1B shows that the antigen recognized by 17KG12 was expressed in the cells and appeared as a broad band between 15 and 18 kDa. A protein of the same molecular mass was immunoprecipitated from Vero cells infected with ASF virus (lane 1). The results showed that the 17-kDa protein precipitated by 17KG12 was the product of the i1L gene. 18BB11 was a third antibody used in this study. As described previously (7, 37), the antibody immunoprecipitated an abundant protein of 11 to 14 kDa from cell lysates prepared from cells infected with ASF virus (lane 5).

p73 is a late structural protein secreted from cells in virus particles. The 4H3 antibody was used to determine the onset of expression of p73 and the time course of packaging of capsid proteins into virions. Figure 2A shows that p73 was first detected in cells 8 h after infection. Expression of p73 increased fivefold between 8 and 10 h and reached a maximum between 12 and 16 h. The time course of packaging of a cohort of newly synthesized p73 molecules into virions secreted from cells was investigated 16 h after infection with ASF virus, a time when synthesis of p73 was maximal. At this time, cells were pulse-labeled with [^{35}S]methionine and -cysteine; virions were then pelleted from culture supernatants taken at increasing times and immunoprecipitated with 4H3. Figure 2B shows that labeled p73 first appeared in culture supernatants 3 h after

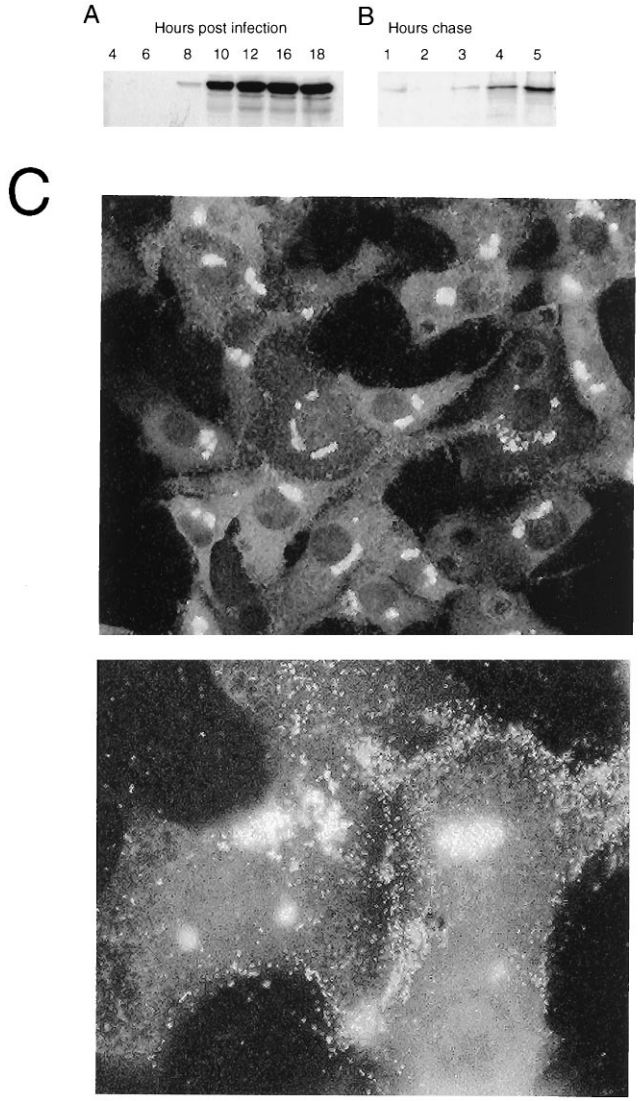

FIG. 2. p73 is incorporated into virions secreted from cells. (A) Time course of expression of p73. At the indicated time intervals after infection with ASF virus, cells were pulse-labeled with [^{35}S]methionine and -cysteine for 30 min at 37°C and lysed, and levels of p73 were determined by immunoprecipitation using 4H3. (B) Secretion of p73 in virions. At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 30 min at 37°C and then chased in complete medium. At the indicated time intervals, culture supernatants were collected from cell monolayers and secreted virions were recovered by centrifugation. Viral pellets were lysed and immunoprecipitated with 4H3 as described above. Proteins were resolved by SDS-PAGE (10% gel) under reducing conditions and visualized by autoradiography. (C) Subcellular distribution of p73. Vero cells infected with ASF virus were washed, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with 4H3. The antibody was visualized by using fluorescein-conjugated goat anti-mouse IgG. Top, distribution of p73 12 h after infection, viewed using a 40× objective; bottom, distribution of p73 16 hours after infection, viewed at higher magnification using a 100× objective.

synthesis. The levels of labeled virus secreted doubled approximately every 60 min, but even 5 h after synthesis, only 20% of the pulse-labeled p73 was recovered from cell supernatants. The results showed that p73 was synthesized in excess over the quantities incorporated into virions. Figure 2C shows the immunofluorescence staining pattern produced by 4H3 at 12 and 16 h after infection. At 12 h, 90% of the cells were infected with virus and staining was restricted to large perinuclear viral

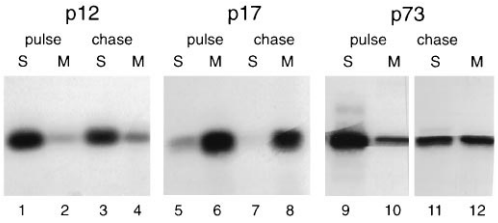

FIG. 3. Binding of ASF virus proteins to membranes. At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 20 min at 37°C and then chased in complete medium for 2 h. Cell samples from each time point were homogenized by nitrogen cavitation. Postnuclear supernatants were centrifuged at 14,000 × *g* for 30 min to recover a pellet of cellular membranes and a supernatant containing cytosol. Membranes were solubilized with 1% Brij 35, and membrane (M) and soluble (S) fractions were immunoprecipitated with 18BB11 to detect p12 (lanes 1 to 4), 17KG12 to detect p17 (lanes 5 to 8), and 4H3 to detect p73 (lanes 9 to 12). Proteins were resolved by SDS-PAGE (12% gel) under reducing conditions and visualized by autoradiography.

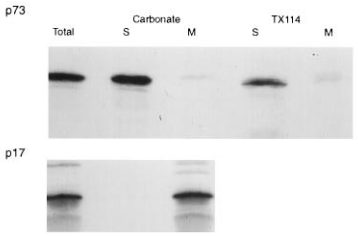

FIG. 4. p73 is a peripheral membrane protein. At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 60 min at 37°C. Postnuclear membrane fractions were extracted with Triton X-114 or sodium carbonate as indicated. Supernatants (S) and membrane (M) fractions were immunoprecipitated with 4H3 to detect p73 (top) or 17KG12 to detect p17 (bottom). Proteins were resolved by SDS-PAGE (12% gel) under reducing conditions and visualized by autoradiography.

factories. At 16 h after infection, punctate staining was observed in the factories, throughout the cytoplasm, and at the cell surface. The punctate staining indicated staining of individual virions by 4H3 and again demonstrated the incorporation of p73 into viral particles. At 16 h, there was also a diffuse cytoplasmic staining by 4H3. This may represent a pool of p73 molecules that are not incorporated into virions.

An intracellular pool of p73 associates with membranes. Having established that p73 was released from cells in secreted virions, experiments were designed to see if p73 associated with intracellular membrane compartments before release from cells. A specific association may indicate a membrane compartment involved in the envelopment of ASF virus. Cells infected with ASF virus were pulse-labeled, and half of the cells were chased in complete medium for 2 h. Postnuclear supernatants were prepared and centrifuged to produce a pellet of postnuclear membranes and a supernatant containing the cell cytosol. Figure 3 shows the distribution of proteins between membrane and soluble fractions and compares the distribution of p73 with that of p12 and p17, two other proteins encoded by ASF virus. p12 is an abundant nonstructural protein localized in the cytosol of infected cells (7), while p17 is a viral structural protein with a central hydrophobic membrane-spanning domain encoded by the i1L gene (reference 15 and Fig. 1). The majority of the p12 protein was found in the soluble fraction after pulse-labeling (lanes 1 and 2) and remained in the soluble fraction during the chase (lanes 3 and 4). In contrast, p17 localized predominantly to the membrane fraction during the pulse (lanes 5 and 6) and remained membrane associated during the chase (lanes 7 and 8). These results were consistent with the presence of a membrane-spanning domain in p17. Significantly, when the experiment was repeated for p73, the protein was recovered from both the soluble and membrane fractions (lanes 9 and 10). The relative intensity of the protein bands recovered from pulse-labeled cells showed that approximately 25% of the p73 protein distributed to the membrane fraction. After a 2-h chase, the total levels of p73 recovered from cells fell by approximately 25%; however, the proportion of p73 found in the membrane fraction at the end of the chase had increased to 50%.

The nature of the association of p73 with membranes was analyzed by using alkaline carbonate washes and Triton X-114 extraction. Figure 4 shows that p73 was quantitatively removed from membranes by carbonate washes and partitioned into the aqueous fraction after Triton X-114 extraction. In parallel experiments (lower panel), the membrane-anchored protein, p17, remained attached to membranes during carbonate washes. Taken together, these observations showed that p73 bound to the membrane fraction as a peripheral membrane protein.

To determine the time course of association of p73 with the membrane fraction, cells were given a 2-min pulse-label and then chased in complete medium for increasing time periods. Membrane and cytosol fractions were separated and immunoprecipitated as described above. If polypeptide elongation proceeds at approximately 4 amino acids per s, then synthesis of p73 containing 646 amino acids would take 3 min. Consistent with this calculation, p73 molecules could not be precipitated after a 2-min pulse-label but were detected after a 2-min chase (Fig. 5A). Importantly, p73 was found exclusively in the soluble fraction, indicating synthesis on cytosolic ribosomes. p73 was first detected in the membrane fraction after a 5-min chase, and binding increased to a maximum within 15 min. Densitometric analysis of the autoradiographs (Fig. 5B) showed that a

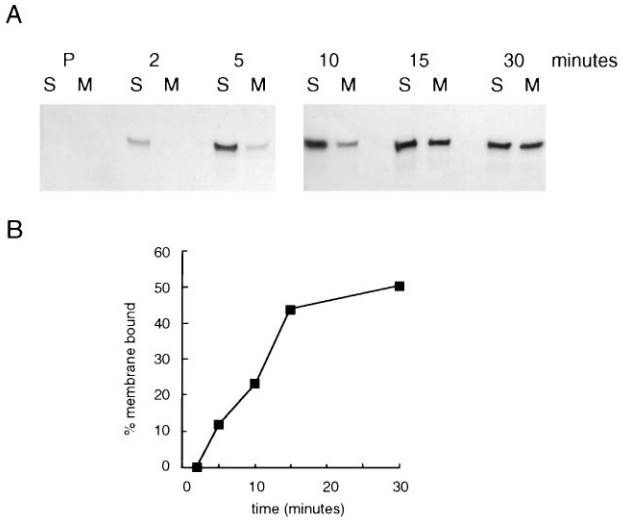

FIG. 5. p73 binds rapidly to membranes. At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 2 min at 37°C and then chased in complete medium for the indicated time intervals. Cells were homogenized, and soluble (S) and membrane (M) fractions were prepared and immunoprecipitated with 4H3 as described in the legend to Fig. 3. (A) Visualization of p73 by SDS-PAGE (10% gel) under reducing conditions followed by autoradiography. (B) Scanning densitometric analysis of autoradiographs was used to determine the distribution of p73. The results show the percentage of the total p73 present at each time point that was recovered from the membrane fraction.

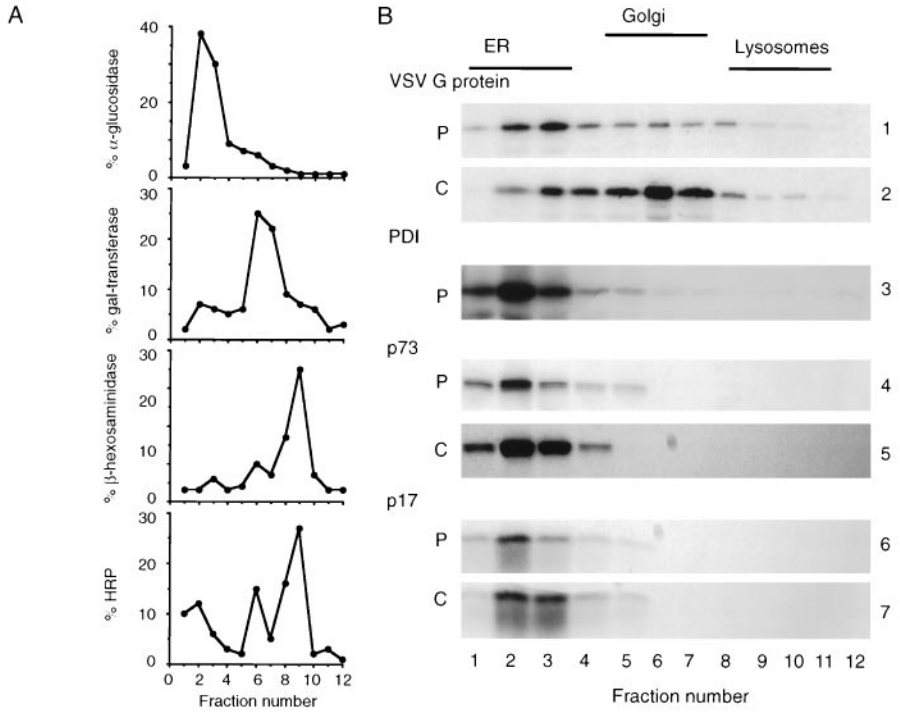

FIG. 6. p73 and p17 cosediment with the ER membrane fraction. (A) Distribution of membrane marker proteins. Vero cells were infected with ASF virus for 16 h, and a postnuclear membrane fraction was separated by using discontinuous Nycodenz gradients. Fractions were assayed as indicated for ER (α-glucosidase), Golgi apparatus (galactosyltransferase), lysosomes (β-hexosaminidase), and endosomes (endocytosed HRP). (B) Distribution of viral proteins. For gradients 1 and 2, Vero cells were infected with VSV for 12 h, labeled with [^{35}S]methionine and -cysteine for 15 min at 37°C, and then chased in complete medium for 2 h. Postnuclear membrane fractions were separated on discontinuous Nycodenz gradients. The distribution of the VSV G protein was determined by immunoprecipitating fractions with P5D4 (P, pulse-labeled cells; C, cells after a 2-h chase). For gradient 3, postnuclear membrane fractions were prepared from Vero cells pulse-labeled with [^{35}S]methionine and -cysteine for 2 h at 37°C. The distribution of PDI across the gradient was determined by immunoprecipitating fractions with an antibody recognizing the C terminus of PDI. For gradients 4 and 5, 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 15 min at 37°C (P) and then chased in complete medium for 2 h (C). The distribution of p73 was determined by immunoprecipitating fractions with 4H3. For gradients 6 and 7, infected cells were labeled and fractionated as described above, and the distribution of p17 was determined by using 17KG12. Proteins were resolved by SDS-PAGE (12% gel) under reducing conditions and visualized by autoradiography. P, pulse-labeled cells; C, cells after a 2-h chase.

maximum of 50% of the pulse-labeled p73 molecules bound to membranes. Binding was rapid, and the levels recovered from the membrane fraction doubled every 5 min.

p73 and p17 bind to an ER membrane fraction. The identity of the membranes binding p73 was determined by separating postnuclear membrane fractions prepared from Vero cells infected with ASF virus on Nycodenz gradients. Figure 6A shows the distribution of organelle marker enzyme activities. The ER migrated toward the bottom of the gradient, indicated by peak α-glucosidase activity in fractions 1 to 3. The majority of galactosyltransferase activity, characteristic of Golgi fractions, localized to the center of the gradient (fractions 5 to 7), and a small fraction (15%) of galactosyltransferase activity, possibly representing newly synthesized enzyme, was found in the ER fraction. The lysosomal enzyme β-hexosaminidase was found in three fractions. Minor peaks comigrated with ER and Golgi markers, possibly representing newly synthesized β-hexosaminidase in transit to lysosomes. The majority, however, sedimented in the upper part of the gradient (fractions 8 to 10). Endosomes in infected cells labeled with endocytosed HRP resolved as two peaks. The major peak comigrated with the lysosomal marker, and a heavier fraction comigrated with Golgi enzymes. The same distributions were obtained for each enzyme analyzed in uninfected cells except that the minor heavy endosome fraction was absent (data not shown).

The density gradient was used to compare the membrane distribution of p73, the envelope G protein of VSV and the ASF structural membrane protein, p17. VSV was chosen for comparison because the VSV envelope protein is synthesized in the ER and moves through the Golgi apparatus to the plasma membrane (25). Analysis of the location of the G protein at increasing times after labeling can be used to monitor the movement of proteins through membrane fractions derived from the secretory pathway. Cells infected with VSV or ASF virus were homogenized and fractionated on Nycodenz gradients. The subcellular distribution of VSV G protein is shown in gradients 1 and 2 of Fig. 6B. The third gradient shows the distribution of the resident ER protein, PDI. As expected the newly synthesized G protein localized with PDI in the ER fraction at the bottom of the gradient. Two hours after synthesis, the bulk of the G protein had moved from the ER and cosedimented with the Golgi marker enzyme galactosyltransferase in fractions 6 and 7. These results are consistent with the transport of the G protein from the ER to the Golgi apparatus. The next gradients show the experiment repeated for ASF virus proteins. Pulse-labeled p73 localized specifically with the ER membrane fractions (gradient 4). Significantly, p73 remained associated with the ER fraction during a 2-h chase (gradient 5). The localization of p17, a membrane-anchored structural protein of ASF virus, was similarly tested. p17 showed the same ER localization during the pulse and the chase as p73 (gradients 6 and 7). The results show that p73 and p17 associate with ER membranes and, unlike the VSV G

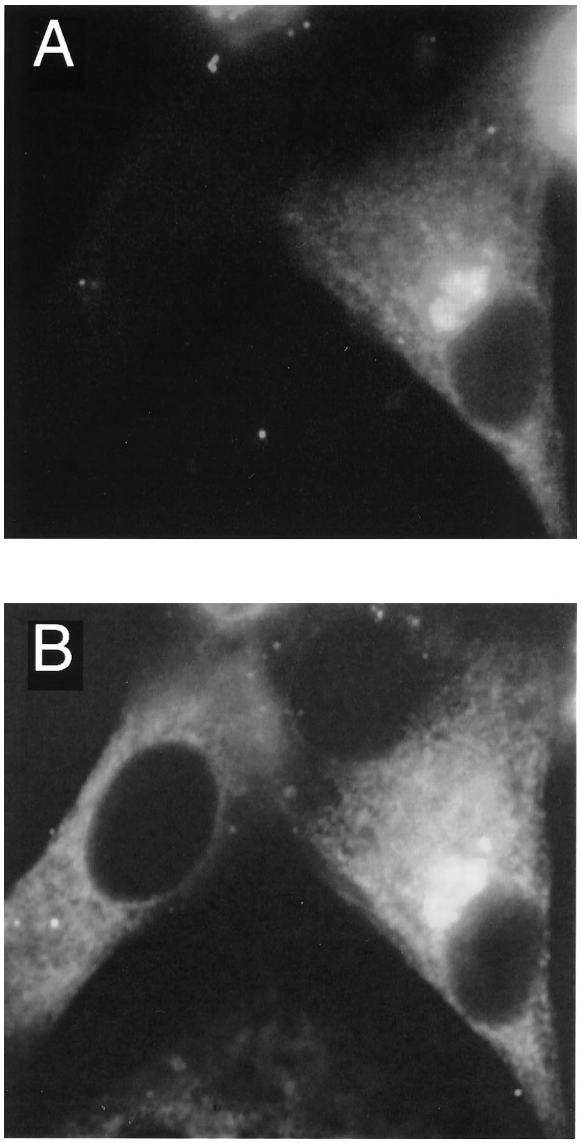

FIG. 7. p73 colocalizes with PDI by immunofluorescence. At 16 h after infection with ASF virus, Vero cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with mouse monoclonal antibody 4H3, specific for p73, and a rabbit antibody recognizing the C terminus of the resident ER protein PDI. (A) Distribution of p73 visualized by using fluorescein-conjugated goat anti-mouse IgG; (B) distribution of PDI in the same cells visualized by using rhodamine-conjugated goat anti-rabbit IgG. Cells were viewed with a 100× objective. Note that uninfected cells in the figure stained for PDI but not for p73.

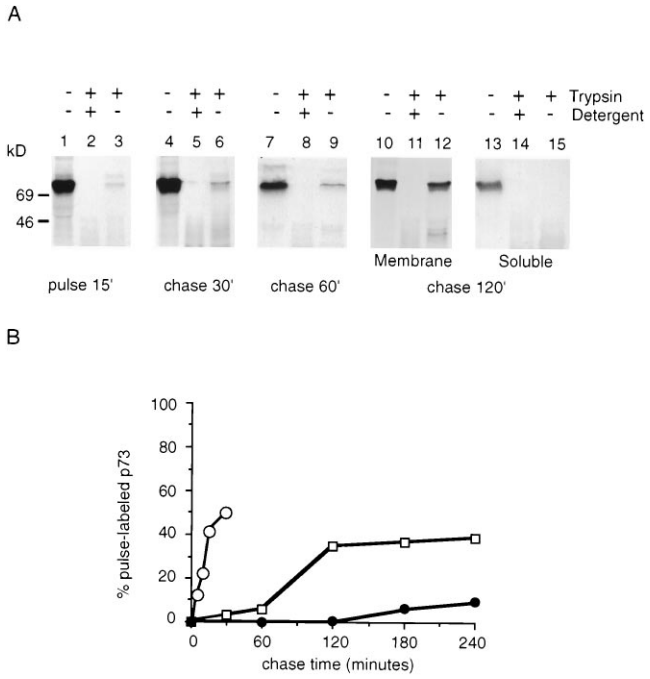

FIG. 8. A protease protection assay demonstrates time-dependent envelopment of p73. (A) At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 15 min at 37°C and then chased in complete medium for the indicated time intervals. Cells were homogenized, and postnuclear membranes (lanes 1 to 12) were incubated in the absence or presence of trypsin or 1% Triton X-100 as indicated. For cells chased for 120 min, cytosol fractions (soluble) were also tested (lanes 13 to 15). The levels of p73 remaining after incubation were determined by immunoprecipitation with 4H3 followed by SDS-PAGE and autoradiography. (B) The experiment was repeated, and the chase time was increased to 240 min. The relative levels of p73 present on autoradiographs were determined by densitometry. The levels of p73 resistant to trypsin (□) were plotted as percentage of total p73 recovered from a representative sample of pulse-labeled cells. The levels of p73 protected from trypsin at each time point are compared with the levels of pulse-labeled p73 that bound to membranes (○) and the levels of p73 recovered from virions secreted from cells (●).

protein, do not move to the Golgi but remain associated with the ER during the chase.

p73 colocalizes with PDI by immunofluorescence. To define the association of p73 with the ER more closely, the subcellular distribution of p73 and the ER marker protein PDI was investigated by immunofluorescence microscopy. Vero cells infected with ASF virus for 16 h were fixed and permeabilized and incubated with the 4H3 mouse monoclonal antibody, specific for p73, and a rabbit antipeptide antibody specific for PDI. Figure 7 compares the distribution of the two antigens in the same cell. In Fig. 7A, a strong labeling of p73 was seen in the viral factory just above the nucleus of the infected cell. An immunofluorescence signal for p73 was also observed in the cytoplasm of the cell. The granular/reticular cytoplasmic staining of p73 extended from the nucleus to the cell periphery and was suggestive of ER membrane localization. The distribution of ER marker enzyme, PDI, in the same cell is shown in Fig. 7B. Significantly, a comparison of the two panels showed that the peripheral cytoplasmic staining produced from PDI colocalized with the signal observed for p73. The colocalized staining patterns observed for PDI and p73 suggested that p73 binds to the ER membrane. Interestingly, the antibody specific for PDI also produced a strong immunofluorescence signal from the viral factory. The presence of PDI in the viral factory suggested that the ER may be recruited into the viral factory during virus morphogenesis. Importantly, both observations are consistent with the subcellular fractionation results showing localization of membrane-bound p73 with the ER membrane fraction.

p73 is enveloped between 1 and 2 h after synthesis. A protease protection assay was designed to test whether p73 bound to membranes was enveloped. The assay was based on the prediction that p73 molecules enveloped by a membrane would be protected from proteolysis by trypsin. Figure 8A shows the results of adding trypsin to postnuclear membrane fractions prepared from pulse-labeled cells (lanes 1 to 3) and cells chased for increasing times at 37°C (lanes 4 to 12). Intact p73 was recovered from membranes incubated in the absence

of trypsin (lane 1); however, when trypsin was added to membranes isolated from pulse-labeled cells, the 73-kDa band was lost from gels (lanes 2 and 3). The results showed that the pulse-labeled protein was not protected from proteolysis and suggested that it was not enveloped.

The same experiment was carried out on membranes isolated from cells chased for 30, 60, and 120 min after labeling. The quantity of p73 associated with the membrane fractions (lanes 4, 7, and 10) was comparable to that seen in pulse-labeled cells (lane 1). After 30 min of chase, a small quantity of p73 was detected after addition of trypsin; however, the bulk of p73 was still susceptible to proteolysis and was lost from gels (lane 6). A similar profile was observed at 60 min (lanes 7 to 9). Significantly, the quantity of p73 resistant to proteolysis increased markedly between the 60- and 120-min time points (lane 12). Importantly, the trypsin-resistant p73 protein seen in the presence of intact membranes was not present when membranes were lysed by addition of Triton X-100 (lanes 8 and 11). This control showed that the protease protection observed in lane 12 occurred because of envelopment by a membrane and not because of steric protection resulting from assembly of p73 with other viral structural proteins. The soluble pool of p73 isolated from cells chased for 120 min was also incubated with trypsin. Lanes 13 to 15 show that, unlike the membrane associated pool of p73, the soluble pool was not protected from proteolysis. The results suggested that binding to the membrane was required for protection against trypsin.

Envelopment occurs 1 h before release of virions from cells. It was argued that if protease protection took place before appearance of virions in culture supernatants, then this would indicate an intracellular site for envelopment. Alternatively, if protease protection occurred at the same time as release of virions into culture supernatants, then this may indicate envelopment during budding from the plasma membrane. The envelopment assay was therefore repeated at different times, and the levels of p73 protected from trypsin were compared with the time taken for newly synthesized p73 to be secreted from cells in virions (Fig. 2B) and the kinetics of membrane binding of p73 (Fig. 5). Figure 8B shows that envelopment of p73 commenced 1 h after the protein bound to the ER membrane. Importantly, envelopment was completed at 2 h, 1 h before virions were released from cells. The data argued that p73 was enveloped by intracellular membranes and not during budding from the plasma membrane.

Envelopment of p73 does not require ER-to-Golgi transport. Monensin and brefeldin A inhibit the transport of proteins from the ER to the Golgi apparatus (27, 44) and disrupt the assembly of viruses that are enveloped by the Golgi or by more distal membrane compartments (12, 24, 49). To test whether inhibitors of ER-to-Golgi transport would block membrane envelopment of ASF virus, the protease protection experiment was repeated for cells incubated with monensin or brefeldin A. The first six lanes of Figure 9 show that under control conditions, approximately 70% of the membrane-associated p73 protein was resistant to trypsin after a 2-h chase. The next six lanes compare the effects of brefeldin A and monensin. The levels of p73 protected during the chase were identical to those seen in control cells. The same results were obtained when cells were pretreated with brefeldin A or monensin for 30 min before metabolic labeling (not shown). The last three lanes show that cycloheximide, an inhibitor of protein synthesis that has been shown to inhibit replication of ASF virus (1), markedly reduced the levels of p73 protected from trypsin during the chase. Taken together, the results showed that brefeldin A and monensin had no effect on envelopment and again suggest

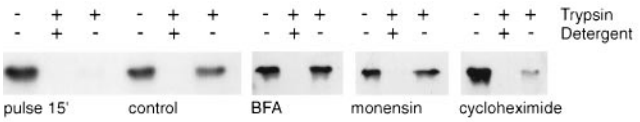

FIG. 9. Envelopment of p73 does not require ER-to-Golgi transport. At 16 h after infection with ASF virus, Vero cells were pulse-labeled with [^{35}S]methionine and -cysteine for 15 min at 37°C and then chased in complete medium for 2 h under control conditions or in the presence of brefeldin A (BFA; 10 μg/ml), monensin (10 μM), or cycloheximide (10 pg/ml). Cells were homogenized, and postnuclear membranes were incubated in the absence or presence of trypsin and 1% Triton as indicated. The levels of p73 remaining after incubation were determined by immunoprecipitation with 4H3 followed by SDS-PAGE and autoradiography.

that p73 was enveloped by the ER and not by the Golgi or more distal membrane compartments.

DISCUSSION

The objective of this study was to determine the membrane compartment involved in the envelopment of ASF virus. A monoclonal antibody recognizing p73, the major capsid protein of ASF virus, was generated from mice immunized with macrophages infected with ASF virus. A quantitative immunoprecipitation analysis performed with the antibody showed that p73 molecules bound rapidly to membranes and that 50% of the intracellular pool of newly synthesized p73 molecules were recovered from a crude cellular membrane fraction within 15 min of synthesis. Subcellular membrane fractionation demonstrated that the membrane-bound p73 cosedimented specifically with three ER marker proteins: α-glucosidase, pulse-labeled VSV G protein, and PDI. Furthermore, immunofluorescence experiments demonstrated the colocalization of p73 with PDI in the cytoplasm and in viral factories. Taken together, the results showed that p73 bound specifically to the ER and implicated the ER as the site of envelopment of ASF virus.

To analyze this finding further, a protease protection assay was designed to test for the presence of an envelope surrounding membrane-associated p73. Importantly, the assay demonstrated a time-dependent protection of p73 from proteolysis. Evidence that protease protection reflected envelopment by a membrane was provided by the following observations. First, p73 molecules in the cytosolic fractions were not protected from trypsin, indicating that membrane association was required for protease protection. Second, resistance to trypsin was abolished on addition of mild nonionic detergent, arguing that protease protection resulted from protection by a lipid membrane and not through steric hindrance arising from assembly of p73 with other viral proteins. Two further experiments provided evidence for envelopment of p73 by the ER and not other compartments of the secretory pathway. First, the capsid protein remained associated exclusively with the ER during the time required for envelopment, and second, envelopment was unaffected by brefeldin A or monensin, two drugs that block ER- to -Golgi transport. Delivery of p73 to Golgi or more distal membrane compartments was not therefore required for envelopment. The involvement of the ER in the assembly of ASF virus was further implicated by the specific localization of pulse-labeled p17, a structural membrane protein of ASF virus, to the ER membrane fraction and the retention of p17 in the ER during the time course of envelopment. An alternative interpretation of the results could be that p73 bound to the ER membrane but was enveloped by the plasma membrane, as virions bud from cells. The kinetics of protease protection argued against this interpretation because

p73 molecules were protected from trypsin 1 h before the first detection of p73 molecules in virions secreted into culture supernatants.

Interestingly, the deduced amino acid sequence of p73 (13, 28) does not reveal membrane targeting sequences such as hydrophobic stretches characteristic of leader sequences or transmembrane domains, suggesting that p73 associated with the ER as a peripheral membrane protein. The nature of the interaction was demonstrated by the removal of p73 from membranes by using alkaline bicarbonate washes and the observation that p73 partitioned into the aqueous fraction during Triton X-114 extractions. Furthermore, p73 molecules detected after a 2-min pulse-labeling localized to the cytosolic fraction, indicating synthesis on cytosolic ribosomes, and not ribosomes bound to the ER. It remains to be determined how p73 binds to the membrane. The protein may have an inherent affinity for an endogenous component of the ER, or alternatively, proteins encoded by ASF virus may be targeted to the ER to recruit p73 to the membrane. Figure 2 showed that recovery of newly synthesized p73 in secreted virions was relatively low. These results can be explained from the observation that only 50% of newly synthesized p73 molecules bound to membranes and that only 70% of the membrane-bound pool was enveloped. At maximum, therefore, only 30% of p73 molecules would be packaged into virions. Given that association with membranes is required for envelopment, the proteins required for recruitment of p73 to the ER membrane may control the quantity of virus packaged by cells. Recent work by Sodiek et al. (40) shows that vaccinia virus cores are wrapped by the intermediate membrane compartment that lies between the ER and the Golgi apparatus. The intermediate compartment is functionally linked to the ER and is involved in trafficking proteins to and from the Golgi (38). The resolution of the Nycodenz gradients and immunofluorescence methods used in this study did not allow discrimination between the ER and the intermediate compartment. We cannot therefore exclude the possibility that the intermediate compartment is also required for envelopment of ASF virus.

It has been reported that p73-specific monoclonal antibodies neutralize the infectivity of ASF virus (3) and that gold-labeled antibodies recognizing p73 bind to electron-dense layers outside the inner membrane of ASF virus (10). At first inspection, our results showing that p73 is protected from trypsin by a membrane, and not therefore accessible to antibodies, would appear to be in conflict with these reports. Recent studies, however, propose that the concentric membrane layers of large enveloped DNA viruses, such as poxviruses and herpesviruses, arise when nucleoprotein cores are wrapped by membrane compartments of the secretory pathway (39, 40, 45, 49, 54). The present study does not distinguish between envelopment of p73 by budding into the ER and wrapping by the ER. ASF virus does, however, have concentric membrane layers suggestive of wrapping, and a wrapping model for ASF virus would reconcile the results of the protease protection assay reported here with the antibody neutralization and localization studies cited above. In the model shown in Fig. 10, p73 molecules bind to both faces of the ER cisternae. After intracellular envelopment by the ER, p73 molecules on the inside of the membrane cisternae are protected from trypsin, but those exposed on the outside are still accessible to the protease. In this study, most (70%) of the membrane-associated p73 molecules were protected from trypsin during envelopment. Even so, 30% of the membrane-associated pool of p73 was susceptible to trypsin at the end of a 4-h chase. These molecules may be nonproductive p73 intermediates that bind to ER membrane but do not become incorporated into virions. Alternatively, they may represent p73 molecules exposed on the outer membrane of the immature virus after the first stage of ER envelopment. It is thought that the subsequent budding of ASF virus from the plasma membrane adds a loose outer membrane. The outer membrane is often lost during purification and is not required for cell entry because virions lacking the membrane are infectious. In the model, a loss of the outer envelope from mature virions during purification would uncover the p73 molecules that remained on the outside of the virion during the ER phase of envelopment. This would allow p73-specific antibodies to neutralize infectivity (3) and explain the immunogold labeling pattern seen with antibodies raised against p73 (10).

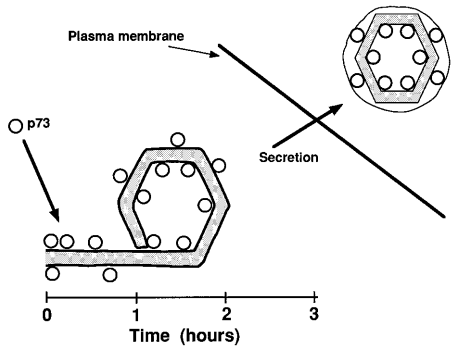

FIG. 10. A model describing the envelopment of p73 during the assembly of ASF virus. Newly synthesized p73 molecules bind rapidly to cisternae of the ER. The lumen of the ER is shown in grey, and the time course of subsequent envelopment and secretion is shown at the bottom. After a 1-h lag period, progressive bending of the ER results in the envelopment of p73 by two membranes. Envelopment takes a further hour, and a proportion of the p73 molecules originally bound to the membrane may remain outside the ER cisternae. An outer envelope is thought to be added when the virus is secreted from cells by budding through the plasma membrane. This envelope is unstable and is often lost during purification of the virus. Loss of the outer envelope may expose p73 molecules at the surface of the virus.

The classification of ASF virus remains controversial (48, 52). Genetically, ASF virus resembles the poxviruses because the double-stranded ASF virus genome has inverted repeats and hairpin loops, and the enzymes required for early RNA synthesis are packaged in the virion. Structurally, however, ASF virus more closely resembles the iridoviridae. Both viruses have striking icosahedral symmetry, and there are significant sequence homologies between p73 and the major capsid proteins of iridoviruses (30). In addition, unlike poxviruses, both ASF virus and iridoviruses require a nuclear phase of DNA replication, before virus assembly in the cytoplasmic factories (17, 19, 32). Given these similarities between ASF and iridoviruses, it will be interesting to see if iridoviruses also utilize the ER as a site of membrane envelopment. An ordered assembly of capsid proteins on the ER membrane may be required for the production of icosahedral enveloped particles.

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