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Characterization of a Ubiquitinated Protein Which Is Externally Located in African Swine Fever Virions

PASCAL M. HINGAMP,[1] MARK L. LEYLAND,[2] JEANETTE WEBB,[1] SIMON TWIGGER,[2] R. JOHN MAYER,[2] AND LINDA K. DIXON[1]*

Pirbright Laboratory, Institute for Animal Health, Woking, Surrey GU24 0NF,[1] and Queens Medical Centre, University of Nottingham Medical School, Nottingham NG7 2UH,[2] United Kingdom

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An antiserum was raised against the African swine fever virus (ASFV)-encoded ubiquitin-conjugating enzyme (UBCv1) and used to demonstrate by Western blotting (immunoblotting) and immunofluorescence that the enzyme is present in purified extracellular virions, is expressed both early and late after infection of cells with ASFV, and is cytoplasmically located. Antiubiquitin serum was used to identify novel ubiquitin conjugates present during ASFV infections. This antiserum stained virus factories late after infection, suggesting that virion proteins may be ubiquitinated. This possibility was confirmed by Western blotting, which identified three major antiubiquitin-immunoreactive proteins with molecular masses of 5, 18, and 58 kDa in purified extracellular virions. The 18-kDa protein was solubilized from virions at relatively low concentrations of the detergent *n*-octyl-β-D-glucopyranoside, indicating that it is externally located and is possibly in the virus capsid. The 18-kDa protein was purified, and N-terminal amino acid sequencing confirmed that the protein was ubiquitinated and was ASFV encoded. The ASFV gene encoding this protein (PIG1) was sequenced, and the encoded protein expressed in an *Escherichia coli* expression vector. Recombinant PIG1 was ubiquitinated in the presence of *E. coli* expressed UBCv1 in vitro. These results suggest that PIG1 may be a substrate for UBCv1. The predicted molecular masses of the PIG1 protein and recombinant ubiquitinated protein were larger than the 18-kDa molecular mass of the ubiquinated protein present in virions. Therefore, during viral replication, a precursor protein may undergo limited proteolysis to generate the ubiquitinated 18-kDa protein.

African swine fever (ASF) is an economically important disease of pigs which is caused by a large cytoplasmically located icosahedral virus particle containing a long, linear, double-stranded DNA genome (~170 to 180 kbp) (see references 11, 13, 54, and 55 for reviews).

ASF virus (ASFV) has similarities in replication strategy and genome structure to poxviruses, although it is morphologically different and is not classified with them (3). Both ASFV and poxviruses replicate in the cytoplasm of infected cells and encode many enzymes required for virus replication and transcription. The target cells for ASFV replication in vivo are those of monocyte/macrophage lineage. ASFV early mRNA synthesis begins in the cytoplasm immediately following virus entry, using enzymes and factors packaged in virions. Replication of the virus genome begins in the cytoplasm 4 to 8 h postinfection. The shift to late gene expression is dependent on DNA replication and is accompanied by shutoff of early genes, although synthesis of some early genes continues throughout infection (see references 11 and 54 for reviews).

Sequencing of the ASFV genome identified a gene encoding a ubiquitin-conjugating (UBC) enzyme, which is the only such virus-encoded enzyme currently identified. This ASFV-encoded UBC enzyme (UBCv1) was shown to be functionally active when expressed in *Escherichia coli* (28, 46). Ubiquitin is a highly conserved 76-amino-acid protein (10) which is present in all eucaryotic cells. The posttranslational modification of proteins by covalent attachment of ubiquitin occurs in eucaryotes by a multistep process (see references 26, 27, 31, and 34 for reviews). During an initial ATP-dependent activation step, the C terminus of ubiquitin is attached via a thiolester bond to an internal cysteine residue of a ubiquitin-activating or E1 enzyme. Activated ubiquitin is then transferred to a specific cysteine residue of UBC or E2 enzymes. Finally, ubiquitin is covalently attached to lysine residues of protein substrates in a reaction which sometimes requires additional ubiquitin protein ligases (E3). A family of UBC enzymes which have pleiotropic functions has been described (see references 31 and 33 for reviews), and it is these enzymes, and in certain cases also E3 enzymes, which determine substrate specificity.

The ASFV-encoded UBC enzyme (UBCv1) contains a 56-amino-acid C-terminal extension, including a high proportion of acidic residues, in addition to the conserved 150-amino-acid N-terminal domain which is present in other UBC enzymes. The yeast UBC2 and UBC3 enzymes contain similar acidic C-terminal extensions, and this domain may be important for substrate specificity. UBC enzymes have very diverse roles ranging from bulk intracellular protein degradation (UBC1, UBC4, and UBC5) (15, 16, 50, 53, 58) to regulation of the transition from G_1 to S and G_2 to M phases of the cell cycle (UBC3 and UBC9) (17–19) or roles in DNA repair and sporulation (UBC2) (32, 38, 40). Although the first discovered role of protein ubiquitination was in targeting proteins for degradation, the presence of metabolically stable ubiquitin conjugates, such as histones, suggests alternative nonproteolytic roles for ubiquitin. The diversity of UBC enzyme functions suggest a variety of possible roles for UBCv1 in either regulating the virus replication cycle or modulating host cell function. Possible stages in virus replication when UBCv1 may function include during virus entry or uncoating, during the onset of DNA replication or early to late switch in gene expression, or during virus morphogenesis. Alternatively, UBCv1 may target host molecules for proteolysis and thus modulate host cell function.

Although UBCv1 is the only virus-encoded UBC enzyme

* Corresponding author. Phone: 483 232441. Fax: 483 232448. Electronic mail address: Linda.Dixon@BBSRC.AC.UK.

described, viruses use the ubiquitin pathway by other mechanisms. A striking example of virus-induced modulation of host cell function utilizing the ubiquitin pathway is by an E3-like enzyme encoded by human papillomavirus. The human papillomavirus E6 gene product, in cooperation with the cellular E6-associated protein, binds the host cell p53 tumor repressor and induces ubiquitin-dependent degradation of p53 (30, 47, 48). In contrast, the picornavirus encephalomyocarditis virus probably utilizes the ubiquitin system to regulate its replication cycle. The encephalomyocarditis virus-encoded 3C protease is rapidly degraded by an ATP-dependent proteolytic system present in reticulocyte lysates which is typical of the ubiquitin-dependent degradation pathway (42).

In this study, we have further investigated the function of the ASFV-encoded UBCv1 enzyme by studying its expression and localization during virus infection of cells. We have also identified novel ubiquitin conjugates present in virions. One of these conjugates was characterized by N-terminal amino acid sequencing, and the virus gene encoding it was sequenced.

MATERIALS AND METHODS

Viruses and cells. The Malawi L1L20/1 isolate (22), virulent ASFV isolate, was used to infect the adherent cell population, which contains monocytes and macrophages, from pig peripheral blood leukocytes. Cells were grown in Eagle's medium supplemented with 10% porcine serum. The tissue culture-adapted Uganda isolate was used to infect IBRS2 tissue culture cells which were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were infected at a multiplicity of infection of at least 3.

DNA sequencing. DNA sequencing was carried out by the chain termination method (2), using T7 DNA polymerase and [α-^{35}S]dATP. Primers used initially were from the sequence of PIG1 present in the EMBL database (accession numbers M88336 and M96345) and were designed to extend the sequence of the PIG1 open reading frame (ORF). Subsequently, primers used were based on the previously obtained sequence and were designed so that the sequences of both strands were obtained. The template for sequencing reactions was the RC′ plasmid clone, which contains the *Eco*RI C′ fragment of the Ba71V isolate (37). The complete PIG1 ORF was amplified by PCR from the Uganda ASFV isolate, cloned in the pGEMT vector (Promega), and sequenced by using the primers described above.

Construction of expression plasmids. The *PIG1* gene was PCR amplified from plasmid pRC′ (37), using the primers 5′-GGATCCATGCCCTCTAATATGAA ACAG-3′ and 5′-GGATCCTTAGGAACCGGCGGTCATT-3′, which incorporated *Bam*HI restriction sites at the 5′ ends. The PCR mixture contained 20 ng of pRC′ DNA, 100 pmol of each primer, 0.2 mM each dATP, dCTP, dTTP, and dGTP, 2 U of *Taq* DNA polymerase (Promega), 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris-HCl (pH 9.0) in 100 μl. Samples were cycled 35 times at 94°C for 60 s, 55°C for 60 s, and 72°C for 120 s. The final cycle was followed by a further incubation at 72°C for 10 min. The 507-bp product was purified from an agarose gel by using a QIAEX gel extraction kit (Qiagen, Teddington, United Kingdom) and subcloned into pT7Blue-T (Novagen, Madison, Wis.). Following digestion with *Bam*HI, the 500-bp fragment containing the *PIG*1 gene was inserted into the *Bam*HI site of pGEX-2T (Pharmacia, Uppsala, Sweden) to create plasmid pGPIG1L and used to transform *E. coli* DH5α.

The UBCv1 C-terminal deletion mutant uncv1ΔC was created by PCR amplification of the UBCv1 gene from plasmid pT1 (53a) with the primers 5′-GAC CACAACGGTTTCCCTCT-3′ and 5′-AGAGAGGGATCCCTAACTGGGTA CTGGTACAAC-3′, using the conditions described above. This produced a mutant UBCv1 gene with nucleotides 547 to 642 deleted. The PCR product was subcloned into pT7Blue-T, and after digestion with *Bam*HI, the 550-bp fragment containing the mutated UBCv1 gene was inserted into the *Bam*HI site of pGEX-2T to create pT2, which was then used to transform *E. coli* DH5α.

Expression and purification of UBCv1, ubcv1ΔC, and PIG1. *E. coli* DH5α harboring pGPIG1L (PIG1) or pT2 (ubcv1ΔC) was used to inoculate 200 ml of LB medium containing 50 μg of ampicillin per ml. *E. coli* TOPP2 harboring pH2 (UBCv1) was used to inoculate 200 ml of LB medium containing 50 μg of ampicillin per ml and 15 μg of tetracycline per ml. Cells were grown at 37°C to an A_{600} of 0.5, isopropylthiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the cells were incubated for a further 5 h. The bacteria were pelleted by centrifugation, resuspended in 2 ml of ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.2]), and lysed by sonication (four 15-s bursts). Triton X-100 (10%, vol/vol) in PBS was added to a final concentration of 1%, and the extract was cleared by centrifugation at 10,000 × *g* for 10 min. The supernatant was added to 1-ml bed volume of glutathione-Sepharose (Pharmacia) and rocked gently at 4°C for 1 h.

To isolate the glutathione *S*-transferase (GST)–PIG1 fusion protein, the beads were pelleted by centrifugation, washed five times with PBS and once with 1% (vol/vol) Triton X-100 in PBS, resuspended in 2 bed volumes of elution buffer (100 mM Tris-HCl, 20 mM reduced glutathione, 150 mM NaCl [pH 8.0]), and rocked gently at room temperature for 3 h. The beads were pelleted, and the supernatant containing the fusion protein was dialyzed extensively against TS buffer (50 mM Tris-HCl, 150 mM NaCl [pH 8.0]).

To isolate native UBCv1, the GST moiety was removed by thrombin cleavage. The GST-UBCv1 fusion protein was cleaved when bound to glutathione-Sepharose. The beads were pelleted by centrifugation and washed five times with PBS, once with 1% (vol/vol) Triton X-100 in PBS, once with wash buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.5]), and once with cleavage buffer (wash buffer plus 2.5 mM $CaCl_2$). The beads were resuspended in 1 bed volume of cleavage buffer containing 10 U of human thrombin (Sigma, Poole, United Kingdom) per ml and rocked gently at room temperature for 3 h. The beads were pelleted, and the supernatants containing the recombinant proteins were removed. The beads were washed twice with wash buffer, and the supernatants were combined.

Formation of ubiquitin-protein conjugates. The standard reaction mixture contained (in a final volume of 100 μl) 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM ATP, 1 U of inorganic pyrophosphatase, 2.5 mM phenylmethylsulfonyl fluoride, ^{125}I-ubiquitin (2 × 10^6 cpm), and 25 μg of GST-PIG1. UBCv1 (5 μg), ubcv1ΔC (5 μg), and rabbit reticulocyte lysate (15 μg of total protein) were added as appropriate. The ubiquitin-activating enzyme (E1) was added to the reaction mixture as a 13,000 × *g* supernatant of a human brain homogenate. The mixture was incubated for between 2 and 6 h at 37°C, diluted to 200 μl with PBS, added to a 50-μl bed volume of glutathione-Sepharose, and agitated gently for 30 min at room temperature. The beads were pelleted by centrifugation and washed five times with PBS. Ubiquitinated PIG1 was cleaved from the GST moiety by thrombin cleavage for 16 h in a total volume of 125 μl. After centrifugation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (35) was added to the supernatant, and the samples were subjected to SDS-PAGE followed by autoradiography.

Rabbit immunizations. Rabbits were immunized initially with purified UBCv1 (100 μg) in Freund's complete adjuvant by both intramuscular and subcutaneous routes. Rabbits were boosted with UBCv1 (100 μg) in Freund's incomplete adjuvant on days 21 and 42 and bled on day 63. Serum was stored at −20°C.

Western immunoblotting. Proteins were first separated by SDS-PAGE (35) and then transferred to either Hybond-C Super (0.45-μm pore size; Amersham) or polyvinylidene difluoride (PVDF) (Immobilon P; Millipore) membranes. Before immunoblotting, membranes were blocked by incubation in PBS containing 0.1% Tween 20 and 5% dried low-fat skim milk for 1 h at 37°C. Blots were then incubated in blocking solution containing primary antibody followed by secondary antibody (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G; Dako). Primary antibody was either rabbit antiubiquitin (Dako) or anti-UBCv1 prepared as described above. Blots were washed in PBS containing 0.1% Tween 20 before detection of horseradish peroxidase-conjugated antibodies by using enhanced chemiluminescence (ECL; Amersham). For N-terminal amino acid sequencing, SDS-polyacrylamide gels were run in cathode buffer containing 0.1 mM thioglycolate and transferred to PVDF membranes in buffer containing 10 mM CAPS (3-cyclohexylamino-1-propane-sulfonic acid; pH 11) in 10% methanol. Blotted proteins were briefly stained in Coomassie blue, and bands of interest excised and submitted to N-terminal sequencing.

Purification of ASF virions and preparation of infected cell extracts. Extracellular ASF virions were purified from the supernatant of IBRS2 cells infected with the Uganda ASFV isolate by Percoll gradient centrifugation (4). Cells were infected with ASFV at a multiplicity of infection of 5 to 10. At various times postinfection, cells were lysed in buffer containing 120 mM NaCl, 5 mM Tris-HCl (pH 7.5), and 2% Nonidet P-40. Extracts were centrifuged in a microcentrifuge, and supernatants were stored at −70°C.

Detergent extraction of ASFV structural proteins. Purified ASFV particles (250 μg of protein per ml) were incubated with 0.1 to 5% *n*-octyl-β-D-glucopyranoside (OG) in PBS for 2 h at 4°C. Subviral particles were pelleted through a 25% sucrose cushion at 20,000 rpm in a Beckman SW50.1 rotor, and supernatants were collected and concentrated in spin concentrators (Centricon-3; Amicon).

Immunofluorescence of ASFV-infected cells. ASFV-infected cells were fixed in ice-cold methanol-acetone (1:1) for 5 min at various times postinfection. Cells were rehydrated in PBS and incubated for 15 min at 37°C with 10% fetal calf serum. Cells were incubated with primary and then secondary antibody in PBS containing 0.01% Tween 20 and 10% fetal calf serum. Secondary antibodies were fluorescein isothiocyanate conjugated (Dako).

Electron microscopy and immunogold staining. Cells or virus pellets were washed with PBS and then fixed in 0.1 M cacodylate-buffered 2% paraformaldehyde–0.5% glutaraldehyde containing 1% sucrose and 3 mM $CaCl_2$. Cells or virus pellets were rinsed overnight, postfixed with 0.5% O_sO_4 en bloc, stained with 2% uranyl acetate, and then dehydrated with graded ethanol and embedded in araldite (TAAB Laboratories, Reading, United Kingdom). Immunogold labelling was performed by a three-step biotin-antibiotin-gold conjugate method. Ultrathin sections (80 to 100 nm) on nickel grids were etched with 5% H_2O_2, rehydrated with 2% bovine serum albumin (BSA) in PBS, and blocked with 10% preimmune swine serum and 2% BSA in PBS. Sections were incubated with primary antibody, either sheep antiubiquitin-protein conjugate (1:25; produced in R. J. Mayer's laboratory) or rabbit anti-UBCv1 (1:25) in PBS containing 5%

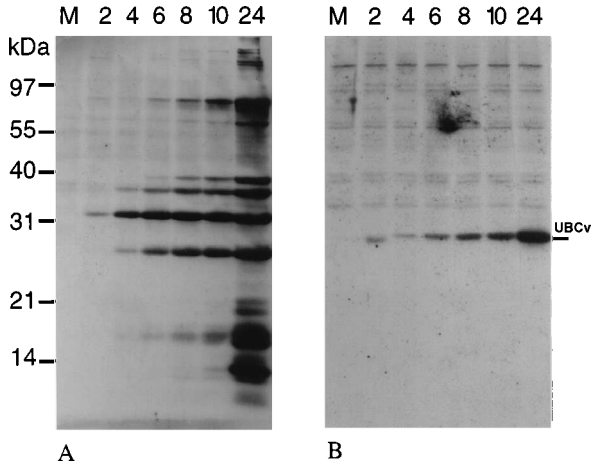

FIG. 1. Expression of UBCv1 during ASFV infection of cells. IBRS2 tissue culture cells were infected with the Uganda ASFV isolate. Mock-infected cell extracts (lane M) or extracts from cells harvested at 2, 4, 6, 8, 10, or 24 h postinfection (lanes 2, 4, 6, 8, 10, and 24) were separated by SDS-PAGE and blotted onto Hybond C membranes. Blots were probed with either pig hyperimmune ASFV antiserum (A) or UBCv1 antiserum (B), and bound antibodies were detected by ECL (Amersham). The positions of UBCv1 and of molecular weight markers are indicated.

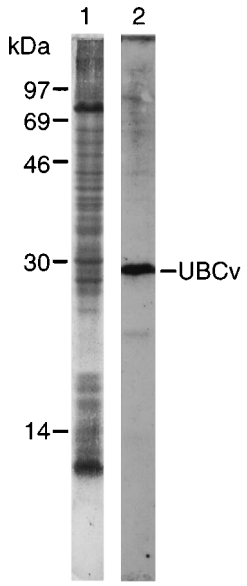

FIG. 2. Presence of UBCv1 in purified extracellular ASFV particles. Proteins from Percoll gradient-purified extracellular ASFV particles (Uganda isolate) were separated on SDS–15% polyacrylamide gels and either silver stained (lane 1) or blotted onto a Hybond C membrane (lane 2). The blot was then probed with rabbit anti-UBCv1 antiserum, and bound antibodies were detected by ECL (Amersham). The positions of molecular weight markers and UBCv1 are indicated.

swine serum and 1% BSA overnight at 4°C. Grids were then incubated for 1 h with secondary antibody, either biotinylated rabbit anti-sheep or biotinylated swine anti-rabbit (1:100) in PBS containing 1% BSA and 5% swine serum. Goat antibiotin-gold (10-nm particle size) diluted 1:100 in Tris-buffered saline containing 2% BSA and 0.25% Tween 20 was then added for 5 h at 4°C, and sections were counterstained with lead citrate before analysis using a Philips 410 microscope. Control sections were treated identically except that primary antibodies were omitted.

RESULTS

Expression and localization of UBCv1 in ASFV-infected cells. Study of the expression and localization of UBCv1 during ASFV infection of cells may help define the protein's function. UBCv1 was expressed in *E. coli* as a fusion protein with GST. Following affinity purification on glutathione-Sepharose, UBCv1 was released from GST by cleavage with thrombin. This purified UBCv1 was used to raise antisera in rabbits, and the antisera were shown to react, by Western blotting, with the purified UBCv1, which migrated with an apparent mobility of 29 kDa (data not shown).

IBRS2 tissue culture cells were infected with the Uganda ASFV isolate, and cells were harvested at various times postinfection. Separation of cell extracts by SDS-PAGE followed by Western blotting showed that a 29-kDa protein was detected in extracts between 3 and 24 h after infection with anti-UBCv1 serum (Fig. 1B) but not with preimmune serum (data not shown). Greater amounts of this 29-kDa protein were detected late postinfection, indicating that it accumulates during infection. Blotting with pig hyperimmune ASFV serum confirmed that the normal early to late shift in ASFV gene expression had taken place in these cells (Fig. 1A). ASFV late gene expression is dependent on virus DNA replication and therefore does not occur in the presence of cytosine arabinoside (CAR), an inhibitor of ASFV DNA replication. In extracts prepared from cells infected with ASFV in the presence of CAR, the 29-kDa polypeptide was also detected between 3 and 24 h postinfection, confirming that UBCv1 is synthesized early postinfection. Control blots prepared by using pig hyperimmune anti-ASFV serum confirmed that late gene expression had been inhibited in the presence of CAR (data not shown). Cells were pulse-labelled with [^{35}S]methionine prior to harvesting at various times postinfection. Proteins were immunoprecipitated with anti-UBCv1 serum and then subjected to SDS-PAGE and autoradiography. The 29-kDa UBCv1 polypeptide was detected between 3 and 24 h postinfection, confirming that it is expressed both early and late postinfection (data not shown).

Indirect immunofluorescence using anti-UBCv1 serum was carried out on fixed and permeabilized IBRS2 cells infected with the Uganda isolate. A faint and diffuse staining above the background observed in mock-infected cells was detected late postinfection and was confined to the cytoplasm of infected cells, with no detectable staining of the plasma membrane, nucleus, or viral factories, although the latter were readily detectable in a parallel control experiment using anti-DNA serum (data not shown).

Presence of UBCv1 in purified virions. Extracellular ASF virions were purified from supernatants of Uganda-infected IBRS2 cells by two rounds of equilibrium centrifugation on Percoll gradients (4). These virions contained the typical protein profile of purified virus and lacked major contaminating proteins larger than 150 kDa which are present in vesicles that frequently copurify with virus preparations. A 29-kDa protein was detected by Western blotting with anti-UBCv1 serum following separation of proteins by SDS-PAGE (Fig. 2). The molecular weight of this protein was the same as that of the *E. coli*-expressed UBCv1, which indicates that it was not ubiquitinated in virions. This protein did not comigrate with any of the major virion proteins detected by silver staining, indicating that UBCv1 is a minor component of virions (Fig. 2).

Ubiquitin conjugates. Novel ubiquitin conjugates detected in ASFV-infected cells might indicate substrates for UBCv1, and characterization of these conjugates could lead to definition of the function of UBCv1. A commercially available antiserum which recognizes primarily ubiquitin-protein conjugates as well as free ubiquitin was used to identify the novel

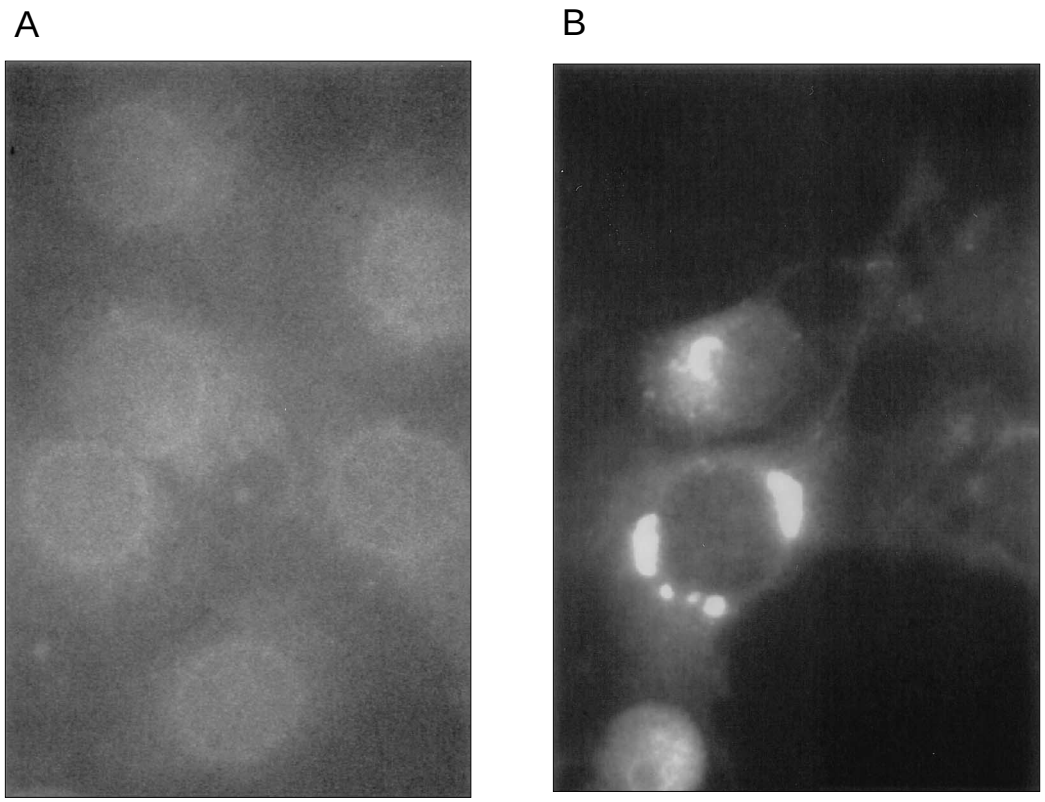

FIG. 3. Indirect antiubiquitin immunofluorescence of ASFV-infected cells. Tissue culture IBRS2 cells were either mock infected (A) or infected with the ASFV Uganda isolate (B). At 16 h postinfection, cells were fixed in ice-cold acetone-methanol and probed with rabbit antiubiquitin antiserum followed by FITC conjugated secondary antibodies and were photographed at a magnification of ×400.

ubiquitin conjugates. Western blotting of uninfected and infected cell extracts with antiubiquitin serum did not identify any novel ubiquitin conjugates (data not shown). However, when IBRS2 tissue culture cells were infected with the ASFV Uganda isolate and observed by immunofluorescence using antiubiquitin serum, the cytoplasm of mock-infected cells was weakly stained, whereas structures that resembled viral factories present in perinuclear areas late postinfection were intensely stained (Fig. 3). In control double-staining experiments, immunofluorescence of viral factories was observed by using a mouse monoclonal antibody against the ASFV structural protein VP72 and colocalized with the antiubiquitin staining (data not shown), confirming that the antiubiquitin staining was localized in virus factories. These results suggested that some ASFV structural proteins might be ubiquitinated. To investigate this possibility, proteins in purified extracellular ASFV particles were separated by SDS-PAGE and analyzed by Western blotting using antiubiquitin serum (Fig. 4). Three strongly immunoreactive proteins with molecular masses of 58, 18, and 5 kDa were detected. Four additional proteins with molecular masses of 51, 38, 30, and 23 kDa cross-reacted weakly with the antiubiquitin serum. The 38-kDa protein is of the molecular mass predicted for monoubiquitinated UBCv1 or a diubiquitinated form of the 30-kDa ubiquitin conjugate. Since extracts were run under denaturing conditions, if this 38-kDa protein is a ubiquitinated form of UBCv1, the ubiquitin moiety must be conjugated to UBCv1 rather than linked by a thioester bond. Similarly, CDC34 (UBC3) is autoubiquitinated in vivo (18). The 58- and 18-kDa proteins (UB58 and UB18) were relatively minor components of the total virion proteins as visualized by silver staining, whereas the 5-kDa

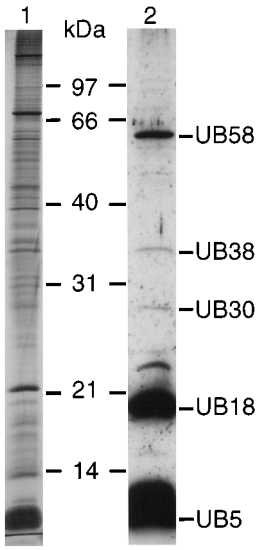

FIG. 4. Presence of ubiquitinated proteins in purified extracellular ASFV particles. Percoll-purified ASFV Uganda isolate particles were separated by SDS-PAGE on 12.5% gels. Separated proteins were either silver stained (lane 1) or blotted and probed with rabbit antiubiquitin serum (lane 2). Bound antibodies on the protein blot were detected by ECL (Amersham). The positions of molecular weight markers are indicated, as are the positions of ubiquitin conjugates referred to in the text.

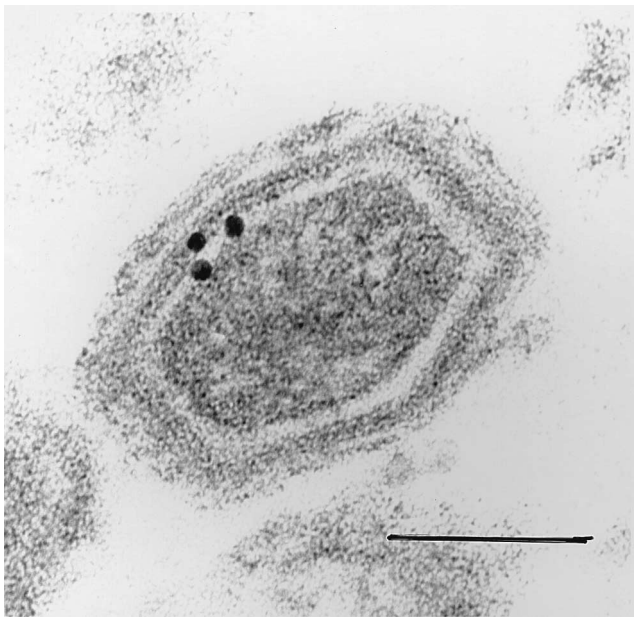

FIG. 5. Antiubiquitin immunogold labelling of ASFV-infected cells. IBRS2 tissue culture cells were infected with the ASFV Uganda isolate, and after 16 h, the cells were fixed and embedded in araldite. Thin sections were probed with antiubiquitin-protein conjugate followed by biotinylated secondary antibody and antibiotin-gold (10-nm particle size). Sections were analyzed with a Philips 410 microscope and photographed at a magnification of ×270,000. The bar indicates 100 nm.

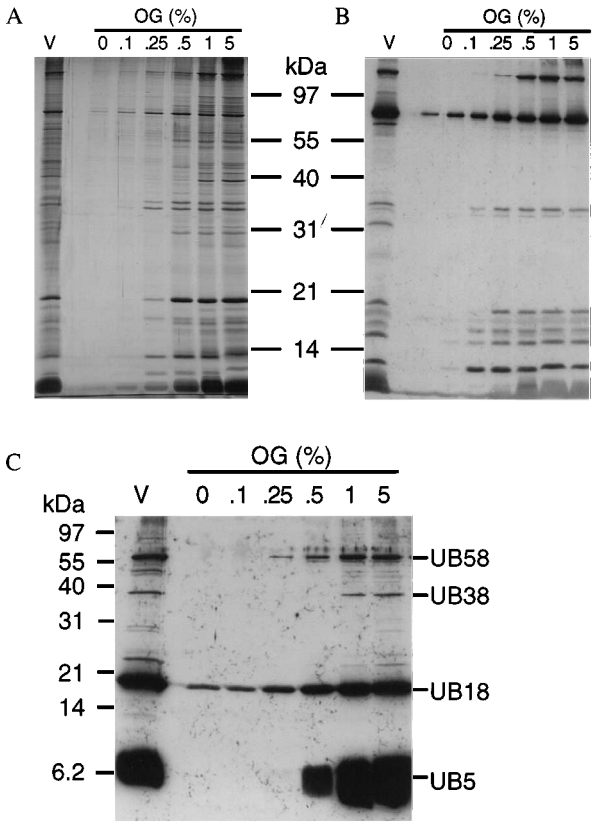

FIG. 6. Extraction of ASFV structural proteins with the nonionic detergent OG. Percoll gradient-purified extracellular ASFV Uganda isolate particles were either untreated (lanes V) or incubated with various concentrations (0, 0.1, 0.25, 0.5, 1, and 5%) of OG in PBS. Viral and subviral particles were pelleted by centrifugation, and solubilized proteins were analyzed by SDS-PAGE using a 12.5% (A and B) or 17.5% (C) polyacrylamide gel. Separated proteins were silver stained (A) or blotted and probed with either anti-ASFV hyperimmune serum (B) or antiubiquitin antiserum (C). The bound antibodies were detected by ECL (Amersham). The positions of molecular weight markers and, in panel C, of the ubiquitin conjugates mentioned in the text are indicated.

protein (UB5) was present in relatively large quantities. The 5-kDa protein migrated slightly more slowly than free ubiquitin on SDS-PAGE (data not shown) and might be either ubiquitin conjugated to a short protein fragment or a modified ubiquitin such as the form attached to phospholipid described in baculovirus particles (21). The failure to detect novel ubiquitin conjugates in total cell extracts by Western blotting may be because virion proteins represent a relatively small proportion of total cell proteins. Virion ubiquitin conjugates therefore represent a relatively minor proportion of the total ubiquitin conjugates in infected cells. The intense staining observed by immunofluorescence in virus factories indicates that either ubiquitin or ubiquitin conjugates are concentrated in these sites.

Localization of ubiquitin conjugates in purified virions. Thin sections of ASFV-infected cells were probed with antiubiquitin antibodies and gold-labelled secondary antibodies and observed by transmission electron microscopy. These antibodies were associated with ASF virions in viral factories, in virions migrating to the plasma membrane, and in enveloped extracellular virions (Fig. 5). The position of 115 gold particles relative to the virus capsid was measured in 52 virions which had a clear hexagonal outline. This measurement showed that 70% of the ubiquitin-specific antibodies were situated in or internal to the virus capsid, most frequently either between the virion nucleoprotein core and the internal membrane or in the virus capsid. This result was consistent with the results described above which identified several antiubiquitin-immunoreactive proteins in purified virions. Furthermore, the electron microscopy results suggested that these immunoreactive proteins may have different locations within the virion.

The location of ubiquitin conjugates was also investigated by detergent extraction of purified extracellular virus as described previously (6). Purified virions were incubated with various concentrations of OG and centrifuged through a 25% sucrose cushion to pellet viral and subviral particles. Solubilized proteins were recovered from the supernatant and analyzed by SDS-PAGE followed by either silver staining or Western blotting with antiubiquitin serum (Fig. 6). The supernatant obtained after treatment with 0.25% OG contained only nine major proteins (Fig. 6A). Of these, five cross-reacted with pig anti-ASFV serum (Fig. 6B), including a major protein with a molecular mass about 72 kDa, which is a major virion capsid protein (5). When the concentration of OG was increased to 0.5%, many more proteins (~20) were solubilized, and when the concentration of OG was increased to 1%, most (~30) virion proteins were solubilized. Thus, the proteins solubilized by 0.25% OG were probably located either external to or in the virus capsid, and those solubilized by 1% OG were probably located inside the virus capsid in either the nucleoprotein core or inner lipid membrane. Western blotting with antiubiquitin serum (Fig. 6C) showed that the UB18 protein was solubilized at OG concentrations of less than 0.25% and had OG solubility characteristics similar to those of the 72-kDa protein. The UB58 protein was partially solubilized at 0.25% OG, but most was solubilized at OG concentrations of 0.5% or greater. UB5 was solubilized at OG concentrations of 0.5% or greater, and other minor antiubiquitin reactive proteins were solubilized at

TABLE 1. Sequence of the N-terminal amino acid residues of UB18[a]

Cycle	Primary[b]		Secondary[c]	
	Residue	Raw yield (pmol)[d]	Residue	Raw yield (pmol)
1	Pro	32.5	Met	26
2	Ser	22.6	Gln	6.3
3	Asp	15.2	Ile	2.4
4	Met	8.8	Phe	3.1
5	Lys	7.6	Val	0.84
6	Gln	10.6		
7	Phe	8.3		

[a] The UB18 protein was blotted on a PVDF membrane and subjected to 10 cycles of Edman degradation (model 473A; Applied Biosystems). The amino acids generated were identified by their retention factor during reverse-phase high-pressure liquid chromatography.

[b] The most abundant amino acid for each cycle.

[c] Amino acids detected in smaller quantity that correspond to the sequence of ubiquitin.

[d] Amount (adjusted by subtracting the background reading for the previous residue) estimated by the UV absorbance.

OG concentrations of 1% or greater. These results suggest that UB18 may be located external to or within the virion capsid, whereas the other antiubiquitin-immunoreactive proteins may be closer to the virion core.

Characterization of UB18 present in virions. Purified extracellular virions were extracted with 0.25% OG, and solubilized proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. UB18 cross-reacted with antiubiquitin serum and comigrated with a protein that was stained with Coomassie blue. This protein band was excised and subjected to automated N-terminal protein sequencing. If this protein consisted of a ubiquitin polypeptide covalently linked via its C terminus to an internal lysine of an unknown protein, then the N termini of both ubiquitin and the conjugated UB18 protein should be available for sequencing. The first seven cycles of Edman degradation each generated a predominant amino acid, corresponding to the UB18 protein sequence, and for the first five cycles, a secondary residue that corresponded to the known N-terminal sequence of mammalian ubiquitin was also detected. This finding suggested that UB18 was a ubiquitin conjugate (Table 1).

The observed molar ratio between the amino acid raw yields of ubiquitin and UB18 was less than the 1:1 molar ratio expected of a monoubiquitinated protein. Similar results were obtained when the ubiquitinated lymphocyte homing receptor (51) was sequenced and could be attributed to incomplete blocking of the ubiquitin N terminus either prior to its integration into the ubiquitin conjugate or during purification.

The seven-amino-acid N-terminal sequence of UB18 was used to screen protein sequence databases and showed a single 100% match with residues 2 to 9 from the N terminus of a predicted ORF in the center of the ASFV genome. The sequence of this ORF in the database (accession numbers M88336 and M96354) was incomplete, and it was located upstream and read from the opposite strand of a previously characterized ASFV gene identified on the Ba71V and E70 isolate genomes (1, 44). Since cloned DNA from the Ba71V isolate was available (37), the remainder of this incomplete ORF was sequenced by using synthetic oligonucleotides from the RC′ clone of the Ba71V isolate as primers and RC′ double-stranded DNA as the template. The experiments described in previous sections were carried out with the Uganda isolate; the ORF was therefore also sequenced from the Uganda isolate genome. Primers flanking the ORF were used to amplify

```
          10                  30                  50
GATGACCTCCATTTTCATGGATATATTTAAAATAAAATCCATTCAATTTTAAAATTATAA
          70                  90                 110
AATAATAAGAAGATGCCCTCTAACATGAAACAGTTTTGCAAGATTTCTGTATGGCTACAT
              M   P   S   N   M   K   Q   F   C   K   I   S   V   W   L   H
         130                 150                 170
CAGCACGATCCGGATCTATTAGAAATTATCAACAACCTATGTATGCTTGGCAATTTATCC
Q   H   D   P   D   L   L   E   I   I   N   N   L   C   M   L   G   N   L   S
         190                 210                 230
GCCGGCAAAGTACAAACACGGAGTGACCTTCATTTATCCAAACAGGCAAAGATCCGTGAT
A   G   K   V   Q   T   R   S   D   L   H   L   S   K   Q   A   K   I   R   D
         250                 270                 290
GAAATAAAAAAAACATGCCTATTCCAATGACCCCTCACAGGCCATAAAGACCCTAGAATCA
E   I   K   K   H   A   Y   S   N   D   P   S   Q   A   I   K   T   L   E   S
         310                 330                 350
CTCATCCTTCCATTTTACATTCCCACTCCAATGGAGTTCACCGGGGAAATCGGCTCCTAC
L   I   L   P   F   Y   I   P   T   P   M   E   F   T   G   E   I   G   S   Y
         370                 390                 410
ACCGGAGTGAAATTAGAGGTCGAAAAAAAAAGAAGCGAATAAAGTTATTTTGAAAAACGGG
T   G   V   K   L   E   V   E   K   K   E   A   N   K   V   I   L   K   N   G
         430                 450                 470
GAAGCAGTCCTAATACCAGCGGCCGATTTTAAACCCTTTCCTAATCGCCGGCTAACGGTC
E   A   V   L   I   P   A   A   D   F   K   P   F   P   N   R   R   L   T   V
         490                 510                 530
TGGATCATGGAGGCAGGCTCTATGCCCCTAGAGGGGCCTCCCTATAAGCGGAAAAAGGAG
W   I   M   E   A   G   S   M   P   L   E   G   P   P   Y   K   R   K   K   E
         550                 570                 590
GGTGGAGGGAATGACCCGCCGGTTTCTAAGCATATCTCGCCGTATACTCCGCGCACGCGT
G   G   G   N   D   P   P   V   S   K   H   I   S   P   Y   T   P   R   T   R
         610                 630                 650
ATTGCCATTGAGGTAGAAAAGGCCTTCGATGAAGTATGCGTCAAAACTGGTGTAGTGTCA
I   A   I   E   V   E   K   A   F   D   E   V   C   V   K   T   G   V   V   S
         670                 690                 710
ATAATCCCTATCTTGCCAACTCGGTCTCCTTGCTGTCTTTCTTGTCGCTCAACCATCCAC
I   I   P   I   L   P   T   R   S   P   C   C   L   S   C   R   S   T   I   H
         730                 750                 770
CCGAGTTTATTAAGGTACTGCCGCTTCTAGACTTTGACCCCTTGGTGACCTTTATCTACT
P   S   L   L   R   Y   C   R   F   *
         790
TCTGAGCCTATAAACGCAT
```

FIG. 7. Nucleotide sequence of the PIG1 ORF and amino acid sequence of the encoded protein. The nucleotide sequence of the PIG1 ORF was obtained for the Uganda ASFV isolate as described in Materials and Methods. The amino acid sequence of the encoded polypeptide was obtained by using the programs Map and Translate (12) and is displayed underneath the nucleotide sequence.

the same genome region from Uganda isolate virus DNA by PCR, and this product was cloned in the pGEMT vector and sequenced by using the primers used in sequencing the Ba71V ORF. This ORF was named PIG1; its nucleotide sequence and the predicted amino acid sequence of the encoded protein are shown in Fig. 7. The ORF is 681 nucleotides long and encodes a predicted protein of 25 kDa. Secondary structure predictions showed that the protein did not contain long hydrophobic regions typical of transmembrane domains. The predicted amino acid sequence was used to screen the Swissprot version 28 protein sequence database, but no significant homologies were found. There are 20 lysine residues in the PIG1 protein sequence to which ubiquitin could be conjugated. The short (30-bp) intergenic distance upstream of the PIG1 ORF is, in common with other ASFV promoter regions, relatively AT rich.

In vitro ubiquitination of PIG1 by UBCv1. The PIG1 ORF from the Ba71V isolate was expressed in *E. coli* as a GST fusion protein (GST-PIG1) and purified to apparent homogeneity with glutathione-Sepharose. Digestion with thrombin resulted in the cleavage of the GST moiety and produced PIG1 with an apparent molecular mass of 18 kDa.

To clearly demonstrate that PIG1 was ubiquitinated in vitro, we developed an assay which involved incubation of the GST-PIG1 fusion protein in a ubiquitination reaction mixture. The fusion protein was subsequently separated from other components of the mixture with glutathione-Sepharose, and the PIG1 protein was isolated by thrombin cleavage.

Incubation of GST-PIG1 in the presence of UBCv1, E1, ATP, and ubiquitin produced a ubiquitinated protein with an apparent molecular mass of 26 kDa (Fig. 8, lane 1). This protein corresponded to a monoubiquitinated conjugate of

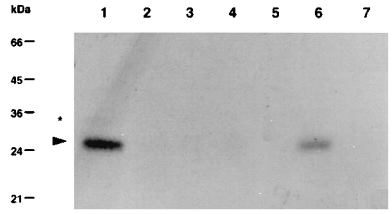

FIG. 8. In vitro ubiquitination of PIG1 by UBCv1. Ubiquitin conjugation reactions were performed as described in Materials and Methods. The products were analyzed by electrophoresis in an SDS–15% polyacrylamide gel followed by autoradiography. The reaction mixtures contained the following components: lane 1, UBCv1, E1, and GST-PIG1; lane 2, as lane 1 but lacking UBCv1; lane 3, as lane 1 but lacking GST-PIG1; lane 4, rabbit reticulocyte lysate and GST-PIG1; lane 5, as lane 4 but lacking GST-PIG1; lane 6, ubcv1ΔC, E1, and GST-PIG1; lane 7, as lane 6 but lacking GST-PIG1. The arrowhead indicates the location of the 26-kDa monoubiquitinated PIG1, and the asterisk (*) indicates the faint band corresponding to the 35-kDa diubiquitinated PIG1.

PIG1. We also observed a faint band of 35 kDa which may be a diubiquitinated conjugate of PIG1. No ubiquitination of PIG1 was detected in the absence of UBCv1 or if GST-PIG1 was omitted from the reaction (Fig. 8, lanes 2 and 3). Similar results were obtained with recombinant E1 from *Arabidopsis thaliana* (23) (data not shown). These results confirm that the ubiquitination of PIG1 was dependent on the presence of UBCv1 but did not require additional factors such as E3s which might have been present in the E1 preparation from brain. To determine whether the conjugation of ubiquitin to PIG1 was specifically catalyzed by UBCv1, the enzyme was replaced in the reaction mixture by a rabbit reticulocyte lysate. The rabbit reticulocyte lysate contained UBC enzymes which ^{125}I-ubiquitinated multiple cellular proteins in the lysate as visualized by autoradiography of the total reaction mixture (data not shown). However, no ubiquitination of PIG1 was observed in reticulocyte lysate following separation of the GST-PIG1 fusion protein from the reaction mixture and release of PIG1 by cleavage with thrombin (Fig. 8, lane 4). Ubiquitination of PIG1 was, however, observed when UBCv1 was included with the reticulocyte lysate (data not shown). This finding demonstrates that failure to ubiquitinate PIG1 in the reticulocyte lysate in the absence of UBCv1 was not due to degradation or deubiquitination of PIG1 by enzymes present in the reticulocyte lysate. These results suggest that PIG1 is a specific substrate of UBCv1 and not of cellular enzymes. However, it remains possible that this in vitro ubiquitination is not representative of what happens in vivo. UBCv1 could be successfully replaced in the reaction mixture by ubcv1ΔC, a mutant form of the enzyme which lacks all 31 amino acids of the acidic carboxyl terminus of the enzyme (Fig. 8, lane 6). This finding demonstrates that the acidic C-terminal extension of UBCv1 is not essential for ubiquitination of PIG1 in vitro.

DISCUSSION

The identification of an ASFV gene (UBCv1) encoding an enzyme with homology to UBC enzymes and demonstration that this gene was functionally active when expressed in *E. coli* suggested that UBCv1 may function to either regulate the ASFV replication cycle or modulate host cell function. The pleiotropic functions of UBC enzymes indicate that UBCv1 may have a variety of possible functions. Here we have demonstrated, by Western blotting and immunoprecipitation using antisera raised against *E. coli* expressed UBCv1, that UBCv1 is expressed throughout ASFV infection and accumulates late in infection. The enzyme is also present in purified extracellular virions. Immunofluorescence demonstrated that UBCv1 is located throughout the cytoplasm of infected cells late postinfection. These results argue against a role for UBCv1 in the temporal regulation of the virus replication cycle by, for example, regulating the onset of virus DNA replication or early to late shift in gene expression. They also argue against a nuclear role for UBCv1 such as mediating the degradation of host transcription factors. The presence of UBCv1 in virions and its accumulation late during infection are consistent with a function very early in infection, immediately following virus entry and before viral transcription. However, expression of UBCv1 both early and late postinfection and distribution throughout the cytoplasm late during infection are not consistent with only a very early role for UBCv1. Possibly UBCv1 has more than one function at different stages of replication, and the availability of substrates may regulate the different activities.

Another approach to understanding the function of UBCv1 is to identify its substrate(s). We have initially approached this by characterizing novel ubiquitin conjugates produced during ASFV infection by using a commercially available antiserum, which recognizes both conjugated and free ubiquitin, in Western blotting and immunofluorescence experiments. An inherent problem with this approach is that if ubiquitin conjugates are targeted for degradation, they are short-lived and difficult to detect. Any conjugates detected are therefore probably relatively stable. Viral factories were intensely stained by immunofluorescence using this antiserum, indicating that ubiquitin conjugates are located in the factories. Of the three main virion proteins which reacted on Western blots with this antiubiquitin serum, the 5-kDa protein may be ubiquitin conjugated to a short protein fragment or a modified form of ubiquitin. Baculovirus particles contain ubiquitin attached to phospholipid (21). Specific packaging of free ubiquitin in avian leukosis virions has been reported (45). The absence of a ladder of bands, differing in molecular mass by about 8 kDa, above the other two major immunoreactive bands (UB18 and UB58) suggests they are monoubiquitinated. Since these proteins were detected by Western blotting, they are probably relatively stable. This is expected for monoubiquitinated conjugates, which are known to be poor substrates for ubiquitin mediated proteolysis (8).

Mild detergent (OG) extraction of virions showed that UB18 was solubilized at relatively low concentrations and was therefore probably externally located, whereas UB5 and UB58 were solubilized at higher OG concentrations and were therefore probably located closer to the virus nucleoprotein core. Immunogold labelling suggested that most conjugates were located either in the virion capsid or between the virion nucleoprotein core and internal membrane. Thus, UB18 may be located in or close to the virion capsid, and UB5 and UB58 may be located close to the nucleoprotein core. Structural proteins of several plant viruses are ubiquitinated, but the role of this ubiquitination is unknown (14, 24).

The ORF encoding the UB18 protein (PIG1) encodes a predicted protein of 25 kDa, while the recombinant form of the N-terminal 165 amino acids of PIG1 has an apparent molecular mass of 18 kDa. The in vitro ubiquitination of PIG1 by UBCv1 produced a monoubiquitinated conjugate which ran on SDS-PAGE with an apparent molecular mass of 26 kDa. However, the ubiquitin-protein conjugate originally isolated from virions (UB18) had a molecular mass of 18 kDa, which suggests that the nonubiquitinated PIG1 protein has a molecular mass of approximately 10 kDa. This discrepancy in the size of the predicted protein encoded by the PIG1 ORF and of the recombinant PIG1-ubiquitin conjugate to that found in virions suggests that the PIG1 protein may normally be proteolytically

processed in vivo. Since the N-terminal sequence obtained from UB18 corresponds to that coded for by the *PIG1* gene (Fig. 7), the putative cleavage of this protein in vivo must occur at the C terminus. Proteolytic cleavage of structural proteins is an important feature in the maturation and assembly of DNA viruses (25), and recently a protease has been implicated in the processing of a 220-kDa polyprotein in ASFV (52). Further experiments will need to be performed to determine whether PIG1 is processed in vivo and whether this occurs before or after conjugation to ubiquitin.

The role of ubiquitin conjugation in virus infection is still unknown. It is, however, known that monoubiquitinated conjugates are poor substrates for ubiquitin-dependent degradation (8). It is possible that monoubiquitinated UB18 could act upon virus entry as a primer for the formation of a polyubiquitin chain. This could target multiubiquitinated UB18 for degradation and participate in virus uncoating. If so, a mechanism must exist to prevent degradation of UB18 during the previous replication cycle. Alternatively, ubiquitination of UB18 may play a role in virus morphogenesis late during infection. Although a variety of physiological substrates for ubiquitin-dependent degradation are known (7, 9, 17, 20, 29, 36, 38, 39, 41, 43, 47, 51, 56, 57), the UBC enzymes responsible for their specific ubiquitination are mostly unidentified. Consequently, the mechanism of substrate recognition by the UBC and E3 enzymes is still poorly defined.

Our results have identified a possible substrate for UBCv1 in vivo, and this will allow further investigation in vitro of the amino acid residues important for recognition and ubiquitination of PIG1. In future, a deletion or temperature-sensitive mutation will be constructed in the UBCv1 gene in the ASFV genome to investigate the function of UBCv1 and confirm that PIG1 is a substrate in vivo for UBCv1.

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