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Sequence and Characterization of the Major Early Phosphoprotein p32 of African Swine Fever Virus

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The gene encoding protein p32, the most abundant and immunogenic protein induced by African swine fever virus at early times of infection, has been mapped in the *Eco*RI C′ fragment of the genome of the Vero cell-adapted virus strain BA71V. Sequencing analysis has shown the existence of an open reading frame, named C′204L, encoding 204 amino acids. The protein is phosphorylated in serine residues located in the 115 N-terminal amino acids and was phosphorylated when expressed in cells infected with a vaccinia virus recombinant. Protein p32 is not glycosylated in spite of the presence of two putative N-glycosylation sites in the deduced amino acid sequence of the polypeptide. Immunofluorescence experiments have shown that the protein is localized in the cytoplasm of infected cells and not in the plasma membrane. In addition, the protein has been found in the soluble fraction and not in microsomes from BA71V-infected Vero cells. Low levels of the protein have been detected in the medium from infected swine macrophages, which probably corresponds to nonspecific release of cytoplasmic proteins. The protein encoded by other virus isolates shows different electrophoretic mobilities, indicating variability of p32.

African swine fever (ASF) is an important disease of domestic pigs and related species which is caused by an enveloped deoxyvirus with icosahedral symmetry (for reviews, see references 18, 24, 55, and 56). The classification of the virus is uncertain (12) because it has properties of both pox- and iridoviruses (55). The ASF virus genome is a DNA molecular of 170 to 190 kb which encodes nearly 100 polypeptides (25, 48); about 30 of them are incorporated into virions (13). Expression of viral proteins during the infection cycle is regulated in a temporal fashion, and proteins are classified as early or late on the basis of their requirement for viral DNA synthesis (48, 53). Some virus proteins undergo posttranslational modifications such as glycosylation (21, 52), phosphorylation (45, 52), proteolytic processing (37), or fatty acid acylation (2).

Since no significantly neutralizing antibodies against ASF virus have been found (19), it was of interest to identify immunogenic viral components synthesized at early times of infection that might be capable of eliciting other effective immune responses in natural infections. Previous reports have shown that antibodies to early proteins of 33 to 38 kDa are found in large amounts in sera from pigs (16) and rabbits (21) immunized with ASF virus. A comparative analysis of the time of synthesis, the molecular mass, and the antigenicity of the [^{14}C]glucosamine- and [^{35}S]methionine-labeled components in infected cells showed the existence of early virus-induced glycoproteins of 33 to 35 kDa that were highly antigenic (21). A protein of 32 kDa was detected in the membrane of infected Vero cells (48). Altogether, these data supported the existence of a 32- to 35-kDa glycoprotein induced at early times of infection that might be present in the membrane of infected cells and that induces a high humoral response. An early protein of the same molecular mass is induced in rabbit alveolar macrophages infected with ASF virus, which gives rise to an abortive infection in which the synthesis of only four virus-specific polypeptides has been detected (4).

In this paper we describe the use of an antiserum raised against ASF virus-infected rabbit macrophages to identify the gene coding for protein p32. The sequence of the gene is presented. We show that protein p32 is phosphorylated in serine residues located in the N-terminal half of the polypeptide and is not glycosylated. The cellular location and the presence of the protein in purified preparations of virions are discussed. The expression of the protein in vaccinia virus and the finding of differences in the size of p32 among virus isolates are also presented.

During the preparation of this report, a paper by Afonso et al. (1) reported the sequence of the gene encoding protein p32 in the ASF virus isolate E-75. Some of the characteristics reported in that paper for protein p32 are in opposition to the results presented here, such as the possible glycosylation, the membrane association, the secretion, and the relationship of protein mobility with virulence of the virus isolate.

MATERIALS AND METHODS

Cells and viruses. Vero, TK$^-$143B, and HeLa cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. The concentration of serum was reduced to 2% during virus infections. Porcine alveolar macrophages were obtained by broncho-alveolar lavage of miniature pigs as described elsewhere (14). The ASF virus natural isolate Badajoz 1971 (BA71), the BA71HP100 strain, and the Vero cell-adapted virus BA71V have been described previously (27). Other virus strains used were Cáceres 1983, Lisbon 1957, Uganda 1959 adapted to IBRS2 cells, Hinde 1954, Tengani 1961, Mozambique 1964, and Kirawira 1969 (KIR69), which are described elsewhere (10, 22, 27). Extracellular ASF virus particles of BA71V were purified by Percoll equilibrium centrifugation (13). Vaccinia virus strain

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Western Reserve (WR) was obtained from B. Moss (National Institutes of Health, Bethesda, Md.).

Plasmids and bacterial strains. Plasmid p5RC′ is a pBR325 derivative containing the *Eco*RI C′ fragment of the genome of BA71V (35). A *Hin*dIII fragment of 3.2 kb containing the open reading frame (ORF) C′204L was excised from p5RC′ and cloned into *Hin*dIII-cut pUC18 or M13mp18, and the resulting construct was named p18H3.2 or M18H3.2, respectively. DNA manipulations were performed as described elsewhere (46). *Escherichia coli* JM109 was used as the host for plasmids and bacteriophage M13. *E. coli* POP (50) and BL21(DE3) (51) were used for expression of pEX recombinants and C′204L cloned in pET3c vector, respectively.

Mapping of the gene. A rabbit serum raised against ASF virus-infected rabbit alveolar macrophages, kindly provided by Y. Revilla, was used for selection of clones expressing protein p32. A pEX expression library containing random DNA fragments of the ASF virus genome (5) was screened with the antiserum specific for p32 as described previously (50). The ASF virus DNA inserted into the pEX plasmid of the positive clones was used in hybridization experiments to group the clones. Clones encoding protein p32 were used to map the gene in the ASF virus genome by dot blot hybridization to viral DNA fragments covering the complete length of the genome (35).

In vitro translation of selected RNA. Early RNA synthesized in vitro by the viral RNA polymerase present in purified virus particles was selected by hybridization to plasmid pBR325 or pEX recombinants from the expression library immobilized on diazobenzyloxymethyl paper (44). The selected RNA was translated in vitro in a rabbit reticulocyte lysate (Amersham) in the presence of 4 mCi of [^{35}S]methionine (1,200 Ci/mmol; Amersham) per ml as described elsewhere (44).

DNA sequencing and computer analysis. DNA sequencing was carried out with single- and double-stranded DNA templates by the dideoxynucleotide chain termination method (47). The templates used were pEX recombinants, p5RC′, p18H3.2, and M18H3.2. The universal sequencing primer and specific oligonucleotides were used in the sequencing reactions. DNA and protein sequences were analyzed by using the University of Wisconsin Genetics Computer Group programs (23).

Construction of expression plasmids. The vector pET3c was used for the expression of ORF C′204L in *E. coli* under the control of the T7 RNA polymerase (51). Plasmid p18H3.2 was digested with *Pst*I, which cuts 73 nucleotides upstream of ORF C′204L. The 5′-flanking region was removed by BAL 31 exonuclease digestion, and the ORF was excised from the vector by *Hin*dIII digestion. The fragments obtained, some of which contained the initiator ATG of the gene at the 5′ end, were end repaired with the Klenow fragment and cloned into the Klenow fragment-filled *Nde*I site of pET3c. A recombinant containing the initiator ATG close to the Shine-Dalgarno sequence of pET3c, named pETC′204L, was selected by hybridization to a ^{32}P-labeled oligonucleotide, 5′-ATATACATAATGGATTT-3′, which contained nine nucleotides complementary to pET3c vector and eight nucleotides corresponding to the first nucleotides of ORF C′204L. In this construct, the C-terminal 8 amino acids of the ORF are replaced by 29 amino acids provided by pET3c.

The transfer vector for construction of a vaccinia virus recombinant expressing p32 was pSC11, in which the cloned gene is transcribed under the control of the vaccinia virus p7.5 promoter and coexpresses *E. coli* β-galactosidase for selection of recombinants (17). ORF C′204L was excised from pETC′204L by digestion with *Nde*I and *Hin*dIII, end filled with the Klenow fragment to create blunt termini, and inserted into *Sma*I-cut pSC11. The resultant plasmid was named pSCC′204L and contained the ORF but lacked the 8 amino acids of the C terminus that were replaced by 3 amino acids provided by the vector.

Expression of protein p32 in *E. coli*. *E. coli* cells bearing pEX plasmids containing ORF C′204L, which were selected with antibodies against p32, were incubated at 42°C for 2 h to induce expression of the β-galactosidase–p32 fusion protein. The insoluble fusion protein was purified after sonication, repeated centrifugations, and washings with phosphate-buffered saline (PBS). For the expression of p32 under the control of the T7 RNA polymerase, *E. coli* BL21(DE3) transformed with pET3c or pETC′204L was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Cultures were pulse-labeled with 200 μCi of [^{35}S]methionine per ml at different times after induction and after addition of rifampin as described previously (51).

Antibodies. To prepare antigen for immunizations, cultures of *E. coli* transformed with pETC′204L were induced for 2 h with IPTG and the bacteria were lysed by sonication in PBS. In the expression of p32 in pEX plasmids, purified fusion proteins were used as antigens. In both cases, nonrecombinant-plasmid-transformed bacteria were used to raise negative control sera. Immunizations of rabbits and rats and preparation of antisera were performed according to standard procedures (28). Polyclonal antisera against ASF virus-induced proteins (21), purified virus particles (13), and structural proteins p72 and p12 (8) were used as controls, as were monoclonal antibodies specific for the membrane protein p24 (19E.H10 [49]) and for protein p12 (24BB7 [15]).

Metabolic labeling of protein p32 in ASF virus-infected cells. Metabolic labeling of protein p32 was performed in cultures of Vero cells or swine macrophages infected at 30 PFU per cell and pulse-labeled at different times of infection with 0.25 to 1 mCi of [^{35}S]methionine or Tran^{35}S-label (ICN Biomedicals; a mixture of about 80% [^{35}S]methionine and about 20% [^{35}S]cysteine, 1,200 Ci/mmol) per ml in methionine-free Dulbecco's modified Eagle medium. In pulse-chase experiments, the medium containing labeled methionine was replaced after the pulse period by Dulbecco's modified Eagle medium containing a 10-fold excess of the normal concentration of methionine. The medium was centrifuged for 1 min at 12,000 × *g* in a microcentrifuge before being used for immunoprecipitations. Dulbecco's modified Eagle medium containing 2 mM uridine instead of glucose was used throughout the infection and during the labeling period with 10 μCi of [^{14}C]glucosamine (200 mCi/mmol; Amersham) per ml (20). The protein was labeled in infected Vero cells or purified virus particles with 50 to 400 μCi of carrier-free [^{32}P]phosphate (200 mCi/mmol; Amersham) per ml or [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham), respectively, as described previously (45).

Immunoprecipitation, Western immunoblotting, and analysis of proteins by electrophoresis. Radioactive samples were dissociated in radioimmunoprecipitation assay buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin, 0.02% NaN_3, and 1 mM phenylmethylsulfonyl fluoride. The extracts were incubated with the different antisera diluted 1/20 to 1/50 for 90 min at 4°C, and the antigen-antibody complexes were precipitated with protein A-Sepharose as described elsewhere (28). Incubation of intact cells with sera was performed to

immunoprecipitate membrane proteins. Initial extracts or immune complexes were dissociated in sample buffer (40 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (33). Radioactive protein bands were detected by fluorography with either 2,5-diphenyloxazole (11) or Amplify (Amersham). For Western blotting assays, proteins separated by SDS-PAGE were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) and incubated with specific antisera according to standard procedures (28). Protein bands were quantitated by laser scanning densitometry, using preexposed films in the case of the radioactive bands (34). In the Western blotting performed to estimate the percentage of protein p32 secreted from the cell, medium and cells from infected cultures were harvested at the time indicated in the legend to Fig. 3 and 7 and mixed with equivalent amounts of cells or medium, respectively, from uninfected cultures before addition of sample buffer and boiling.

Phosphoamino acid analysis and treatment with NTCB. ASF virus-infected Vero cells labeled with [^{32}P]phosphate or [^{35}S]methionine from 2 to 6 h postinfection were immunoprecipitated by antiserum against p32 expressed in *E. coli*, and the proteins were separated by SDS-PAGE. ^{32}P-labeled p32 was visualized by autoradiography and eluted in 50 mM NH_4HCO_3–0.1% SDS. After addition of 50 μg of bovine serum albumin, the protein was precipitated with 3 volumes of ethanol. Samples were hydrolyzed in 5.8 M HCl for 2 h at 110°C and subjected to thin-layer electrophoretic and chromatographic analysis on cellulose plates as described previously (45). For the treatment with 2-nitro-5-thiocyanobenzoic acid (NTCB), ^{35}S- or ^{32}P-labeled p32 was identified by autoradiography and eluted in 0.2 M Tris-HCl (pH 8)–7.5 M urea–1 mM EDTA. Samples were incubated with 0.7 mM NTCB for 30 min at room temperature in the dark. At the end of this incubation, 3 M Tris was added to raise the pH of the samples to 9, and the samples were incubated for 12 h at 37°C (26). Proteins were dissociated with sample buffer and analyzed by SDS-PAGE.

Purification of p32-specific antibodies and immunofluorescence. Immunoglobulin G (IgG) specific for p32 was purified, from serum against the protein expressed in *E. coli* transformed with pETC'204L, by protein A-Sepharose chromatography according to standard procedures (28). Purified IgG was preadsorbed to *E. coli* proteins covalently bound to cyanogen bromide-activated Sepharose 4B, according to the instructions provided by the manufacturer (Pharmacia). Extracellular ASF virus was used as the inoculum for the immunofluorescence experiments. Vero cells were mock infected with UV-inactivated virus or infected with infectious virus at a multiplicity of infection of 0.1 PFU per cell. At the times indicated in Fig. 6, cells were fixed with methanol at −20°C for 5 min. Alternatively, cells were not fixed or were fixed with 4% *p*-formaldehyde in PBS (nonpermeable conditions for antibodies). Cells were incubated with 0.05 mg of purified IgG per ml and a 1/100 dilution of rhodaminated goat anti-rabbit IgG or fluoresceinated goat anti-mouse IgG (TAGO, Inc., Burlingame, Calif.). Fluorescence microscopy was performed in a Zeiss Axiovert microscope. A 1/50 dilution of a serum raised against *E. coli* BL21(DE3) transformed with pET3c was used as a negative control.

Preparation of soluble and microsomal fractions from infected cells. The disruption and fractionation of the cells were done by a modification of the procedures described by Rome et al. (43). Radioactively labeled ASF virus-infected cells were resuspended in 2 mM EDTA–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4)–0.28 M sucrose–1 mM phenylmethylsulfonyl fluoride. All the subsequent procedures were performed at 4°C. The cell suspension was transferred to a nitrogen cavitation bomb (Parr Instruments Co.), and a pressure of 750 lb/in^2 was applied for 10 min. The disrupted cells were collected, and the breakage was monitored by optical microscopy. Large particulate material was removed by successive centrifugation steps at 800 × *g* for 5 min and 12,000 × *g* for 10 s. Microsomes were separated from the soluble fraction by ultracentrifugation at 210,000 × *g* for 30 min. Soluble and microsomal fractions were used in immunoprecipitation experiments.

Expression of protein p32 in vaccinia virus. The recombinant vaccinia virus was constructed by standard procedures (38). TK$^-$143B cells were used for selection of thymidine kinase-negative viruses in the presence of 5'-bromodeoxyuridine. The recombinant virus was identified in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), plaque purified three times, and named vC'204L. Virus stocks were grown in HeLa and Vero cells. Metabolic labeling of proteins in vaccinia virus-infected Vero cells was performed as described above for cultures infected with ASF virus.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article have been submitted to GenBank and assigned accession number M96354.

RESULTS

Mapping and nucleotide sequence of the gene encoding protein p32. ASF virus does not replicate in rabbit alveolar macrophages but induces the synthesis of some early, virus-specific proteins (4), among which is protein p32. In accordance with this, a rabbit antiserum raised against ASF virus-infected rabbit macrophages recognized mainly protein p32 in ASF virus-infected Vero cells by immunoprecipitation (Fig. 1b). This serum was used to screen an ASF virus expression library (pEX) in which viral proteins are synthesized as a fusion product to β-galactosidase (5). The selected clones were grouped by DNA hybridization, and representative members of each group were used to select by hybridization early RNA synthesized in vitro by the RNA polymerase present in virions. The in vitro translation of these RNAs showed that clones 1 and 25 of the main hybridization group selected RNA encoding a viral protein of 32 kDa (Fig. 1a). This result was corroborated by raising antisera in rats against the β-galactosidase fusion protein induced by clones 1 and 25. These antisera recognized by immunoprecipitation (Fig. 1b) and Western blotting (data not shown) a band of 32 kDa induced in ASF virus-infected Vero cells. The hybridization of DNA from clones of the main group to a genomic library of viral DNA (35) allowed mapping of the gene in the restriction fragment *Eco*RI C', located in the central region of the viral genome (Fig. 2a). Subsequent Southern blot hybridization mapped the gene in the *Hin*dIII subfragment of 3.2 kb of *Eco*RI-C'.

The first steps of sequencing the gene were performed by using the pEX recombinant clones 2, 15, 25, and 33 as templates. The sequence obtained served to design internal primers to be used to complete the sequencing of the gene, which was performed with p5RC', p18H3.2, and M18H3.2 as templates. Figure 2b shows the nucleotide sequence of the gene and the amino acid sequence predicted for protein p32.

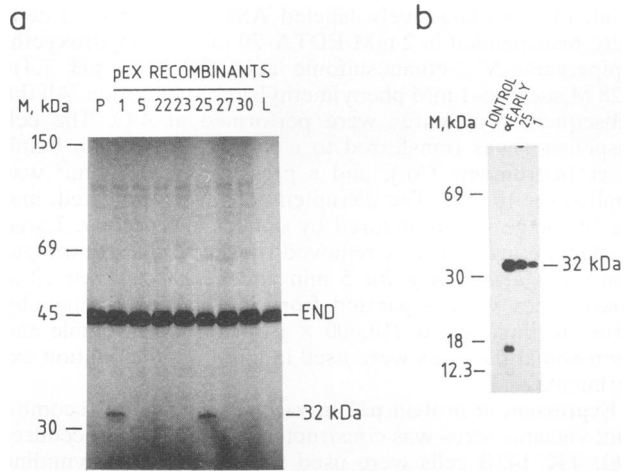

FIG. 1. Identification of proteins encoded by clones of the pEX library. (a) In vitro translation of selected RNA. Plasmid pBR325 (lane P) or the indicated recombinant pEX clones were used to select by hybridization early viral RNA. The selected RNA was translated in vitro in the presence of [^{35}S]methionine, and the translation products were analyzed by SDS-PAGE and fluorography. A control in the absence of RNA is included (lane L). The bands corresponding to an endogenous protein of the reticulocyte lysate (END) and the 32-kDa protein are indicated. (b) Immunoprecipitation of p32 at early times of infection. Vero cells infected with BA71V were pulse-labeled with [^{35}S]methionine from 2 to 6 h postinfection. Cell extracts were immunoprecipitated by rat serum raised to β-galactosidase protein expressed in *E. coli* (CONTROL), rabbit antiserum against ASF virus-infected rabbit macrophages (αEARLY), or rat antisera raised to the β-galactosidase fusion proteins expressed by clones 25 (lane 25) and 1 (lane 1). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. M, molecular mass.

The ORF was named C'204L because it is located at the *Eco*RI C' fragment, codes for 204 amino acids, and is transcribed leftwards. It is separated from other ORFs by 5'- and 3'-flanking intergenic regions of 31 and 72 nucleotides, respectively. A run of nine consecutive thymidylate residues (9T), which has been proposed to act as a transcriptional termination signal in ASF virus (6), is present in the intergenic region downstream the ORF. The predicted molecular mass (23,555 Da) is lower than the apparent molecular mass in SDS-PAGE of the protein synthesized in infected cells (32 kDa). Two N-glycosylation sites and 17 serine phosphorylation sites were predicted according to the method of Kemp and Pearson (29, 30) (Fig. 2b). The hydrophilicity profile indicated the absence of long hydrophobic sequences (Fig. 2c). Accordingly, no signal sequences or transmembrane domains were predicted (31, 57). The deduced amino acid sequence of ORF C'204L was compared with sequences in the protein data bases SWISSPROT (release 22.0) and PIR-Protein (release 32.0), but no significant matches were found.

Synthesis of p32 in ASF virus-infected cells. To obtain a serum specific for protein p32, ORF C'204L was inserted into pET3c for expression in *E. coli* under the control of the T7 RNA polymerase. The synthesis of two proteins of 32 and 34 kDa was induced by IPTG and occurred in the presence of rifampin, an inhibitor of the *E. coli* RNA polymerase (data not shown). *E. coli* lysates containing protein p32 were used to immunize rabbits, and the immune serum was used as a reagent specific for protein p32.

Figure 3a shows that protein p32 was synthesized in BA71V-infected Vero cells at early and late times postinfection, with a gap between 14 and 20 h postinfection in which the synthesis of the protein was dramatically reduced. Other pulse-labeling experiments showed that the protein could be detected as early as 1 h after the infection (data not shown). The protein was synthesized in the presence of the inhibitor of DNA replication cytosine arabinoside (Fig. 3a). Western blotting analysis showed that p32 accumulates in infected cells and can be detected at late times of infection (Fig. 3b). The stability of the protein in infected cells (Fig. 3c) accounts for the presence of p32 in cell extracts throughout the infection.

Metabolic labeling with [^{35}S]methionine and immunoprecipitation of swine alveolar macrophages infected with different ASF virus strains showed variability in the apparent molecular mass (30 to 32.5 kDa) of the protein among viruses (Fig. 3d), which could be detected in total extracts and immunoprecipitates. A genetic difference between viruses, and not the cell type used in the infection, must account for this result, since the same electrophoretic mobility was observed in BA71V-infected Vero cells (Fig. 3a) and swine macrophages (Fig. 3d).

Presence of p32 in purified virus preparations. Western blotting (Fig. 4a) and immunoprecipitation experiments with ^{35}S-labeled purified virions (data not shown) detected protein p32 in the virus particle. However, quantitation of protein p32 in the different steps of the virus purification process revealed that the amount of p32 decreased throughout the purification steps (Fig. 4b), while an enrichment of structural proteins p72 and p12 (13) was detected. This suggested that p32 might represent a contaminant in the purified preparations of virions.

Posttranslational modifications of the polypeptide. The posttranslational modifications of the polypeptide were investigated by metabolic labeling with radioactive compounds followed by immunoprecipitation with specific antiserum raised against p32 expressed in *E. coli*. A component labeled with [^{14}C]glucosamine was immunoprecipitated from infected Vero cells (data not shown). However, the nonglycosylated viral protein p72 was also labeled with [^{14}C]glucosamine under these conditions, and the radioactivity incorporated into both p72 and p32 was resistant to a mixture of *N*- and *O*-glycosidases as described elsewhere (3), while the glycoproteins of Semliki Forest virus, which were used as a control, were sensitive to this glycosidase treatment (data not shown). These results supported the hypothesis that the label incorporated into p32 represented metabolic conversion of glucosamine to amino acids.

Labeling of ASF virus-infected Vero cells with [^{32}P]phosphate at early times of infection showed the existence of a 32-kDa component that was immunoprecipitated with antibodies specific for p32 (Fig. 5a). Similarly, a molecule of 32 kDa was phosphorylated with [γ-^{32}P]ATP in vitro, in the presence of Nonidet P-40, by the ASF virus protein kinase found in purified preparations of virus particles (Fig. 5a). To identify the amino acids that are phosphorylated in the polypeptide, infected Vero cells labeled with [^{32}P]phosphate were immunoprecipitated with specific antibodies and the 32-kDa band was isolated from polyacrylamide gels after SDS-PAGE. The protein was hydrolyzed and subjected to thin-layer electrophoresis and chromatography. As shown in Fig. 5b, the unique phosphoamino acid present in the protein was phosphoserine. The treatment of p32 with NTCB gave rise to an N-terminal fragment of 115 amino acids and a C-terminal fragment of 89 amino acids (Fig. 5c). The incu-

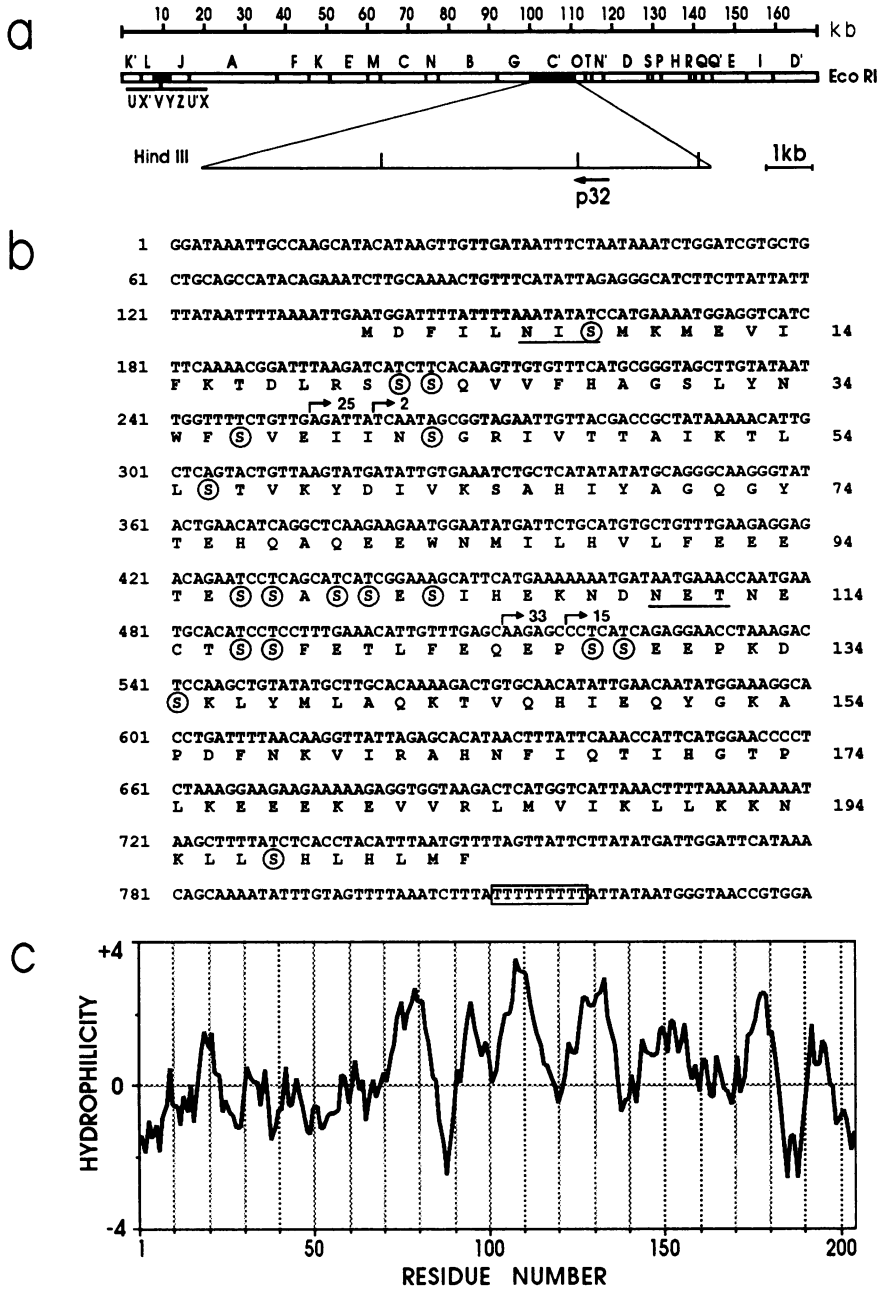

FIG. 2. Mapping and sequence of the gene encoding protein p32. (a) Position of ORF C'204L in the ASF virus genome and the *Eco*RI C' fragment. The *Hin*dIII sites within the *Eco*RI C' fragment are indicated. (b) Nucleotide sequence and deduced amino acid sequence of ORF C'204L and flanking regions. Arrows indicate the sites of insertion into the pEX vector for the indicated clones of the main hybridization group selected in the expression library. The serine residues at the putative phosphorylation sites are enclosed by circles. The N-glycosylation sites are underlined, and the putative motif for termination of ASF virus transcription is boxed. (c) Hydrophilicity profile of the predicted protein p32, obtained according to the method of Kyte and Doolittle (32).

bation of the immunoprecipitated, ^{32}P-labeled p32 with NTCB and subsequent SDS-PAGE showed that the phosphorylation sites are located in the N-terminal half of the polypeptide (Fig. 5c).

Localization of protein p32 in ASF virus-infected Vero cells. Immunofluorescence experiments were carried out to determine the subcellular localization of protein p32 during virus infection. Figure 6a shows that when cells were permeabilized to antibodies, protein p32 was detected at 7 h of infection as a diffused immunofluorescence distributed throughout the whole cytoplasm. From 14 h (Fig. 6a) and up to 24 h (data not shown) postinfection, a cytoplasmic but more speckled pattern, which excluded viral factories labeled with antibodies against the structural protein p12 (Fig. 6b) and areas that might correspond to Golgi-derived membranes, was observed. No immunofluorescence was observed in the plasma membrane of the infected cell. Experiments performed under nonpermeable conditions for the

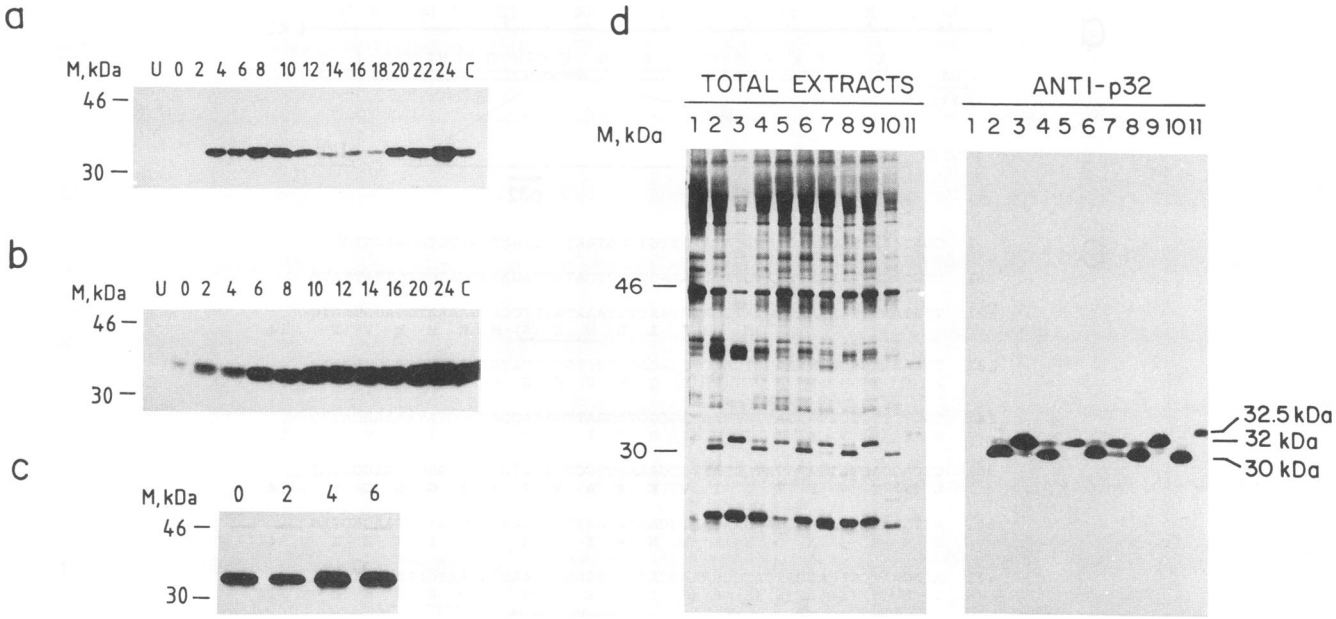

FIG. 3. Synthesis of protein p32 in ASF virus-infected cells. (a) Kinetics of synthesis of p32 in infected Vero cells. Mock- (lane U) or BA17V-infected Vero cells were pulse-labeled with Tran^{35}S-label for 1 h at the indicated times of infection, and cell extracts were immunoprecipitated by an antiserum raised to p32 expressed in *E. coli*. Samples were analyzed by SDS-PAGE and fluorography. The results obtained with cells infected in the presence of 40 μg of cytosine arabinoside per ml and labeled from 14 to 15 h are shown (lane C). (b) Accumulation of protein p32 in infected cells. Western blotting analysis with serum specific for p32 of mock- (lane U) or BA71V-infected Vero cells harvested at the indicated times of infection. Results obtained with cells infected in the presence of 40 μg of cytosine arabinoside per ml and harvested at 14 h postinfection are shown (lane C). (c) Stability of protein p32 in infected cells. BA71V-infected Vero cells were labeled with Tran^{35}S-label from 3 to 5 h postinfection and chased for the indicated hours with medium containing an excess of unlabeled methionine, and cell extracts were immunoprecipitated by a p32-specific antiserum. A fluorograph of the SDS-PAGE analysis is shown. (d) Synthesis of p32 in infected swine macrophages. Alveolar swine macrophages were infected with different virus isolates, pulse-labeled with [^{35}S]methionine from 1 to 5 h postinfection, and immunoprecipitated by antiserum specific for p32. A fluorograph of the electrophoretic analysis of total extracts or immunoprecipitates is shown. Lanes 1, uninfected cells; lanes 2 to 11, cells infected as follows: 2, BA71; 3, BA71V; 4, BA71HP100; 5, Cáceres 1983; 6, Lisbon 1957; 7, Uganda 1959 adapted to IBRS2 cells; 8, Hinde 1954; 9, Tengani 1961; 10, Mozambique 1964; 11, KIR69. M, molecular mass.

antibodies, in which cells were fixed with *p*-formaldehyde, detected a very weak immunofluorescence in the plasma membrane after an adsorption period of 2 h at 4°C and after 8 or 16 h postinfection (data not shown). In control experiments, an intense fluorescence in the plasma membrane was detected with a monoclonal antibody against the membrane protein p24 (49) while no signal was detected with a monoclonal antibody specific for the structural, nonmembrane protein p12 (15) (data not shown). Experiments performed with cells not fixed showed that the weak signal detected in the cell surface with antibodies to p32 was also found in cells mock infected with UV-inactivated virus (Fig. 6c), indicating that this is not due to newly synthesized p32 protein. In a control experiment for the proper inactivation of the virus, no immunofluorescence was detected in cells infected with UV-inactivated virus after permeabilization (data not shown). The degree of purification of the inoculum also affected the background since this surface signal decreased when purified extracellular virus was used as the inoculum (data not shown).

The presence of protein p32 in the plasma membrane was also investigated by incubation of intact infected cells [^{35}S]methionine labeled with antiserum specific for p32, extensive washing by centrifugation, and immunoprecipitation with protein A-Sepharose in the presence of dissociating buffer. As shown in Fig. 7a, p32 was not immunoprecipitated from infected cells unless lysed previously. As a control, a serum against ASF virus proteins selectively precipitated

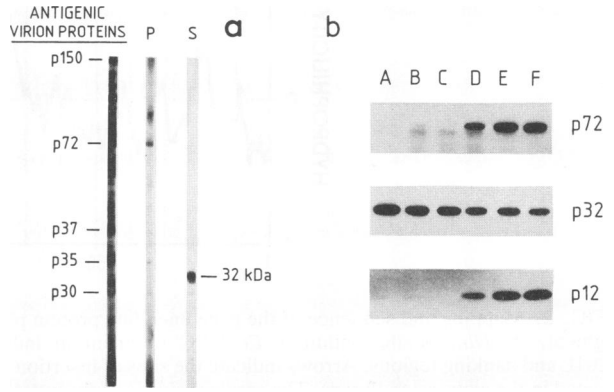

FIG. 4. Presence of p32 in purified virions. (a) Detection of p32 in BA71V particles. Western blotting analysis of purified extracellular virus particles using serum against ASF virus structural proteins, serum against pET3c-transformed bacteria (lane P), or a p32-specific antiserum (lane S). The positions of the major virus structural proteins are indicated. (b) Analysis of the purification steps. The same amount of protein from the different steps of the purification procedure was subjected to SDS-PAGE and transferred to Immobilon-P membranes. The membrane was divided in three parts corresponding to high, medium, and low molecular masses and incubated with antisera specific for proteins p72, p32, and p12, respectively. Lanes: A, infected cells; B, extracellular virus concentrated by high-speed centrifugation; C, vesicle fraction; D, virus fraction in the first Percoll gradient; E, virus fraction in the second Percoll gradient; F, purified virus after Sephacryl column purification.

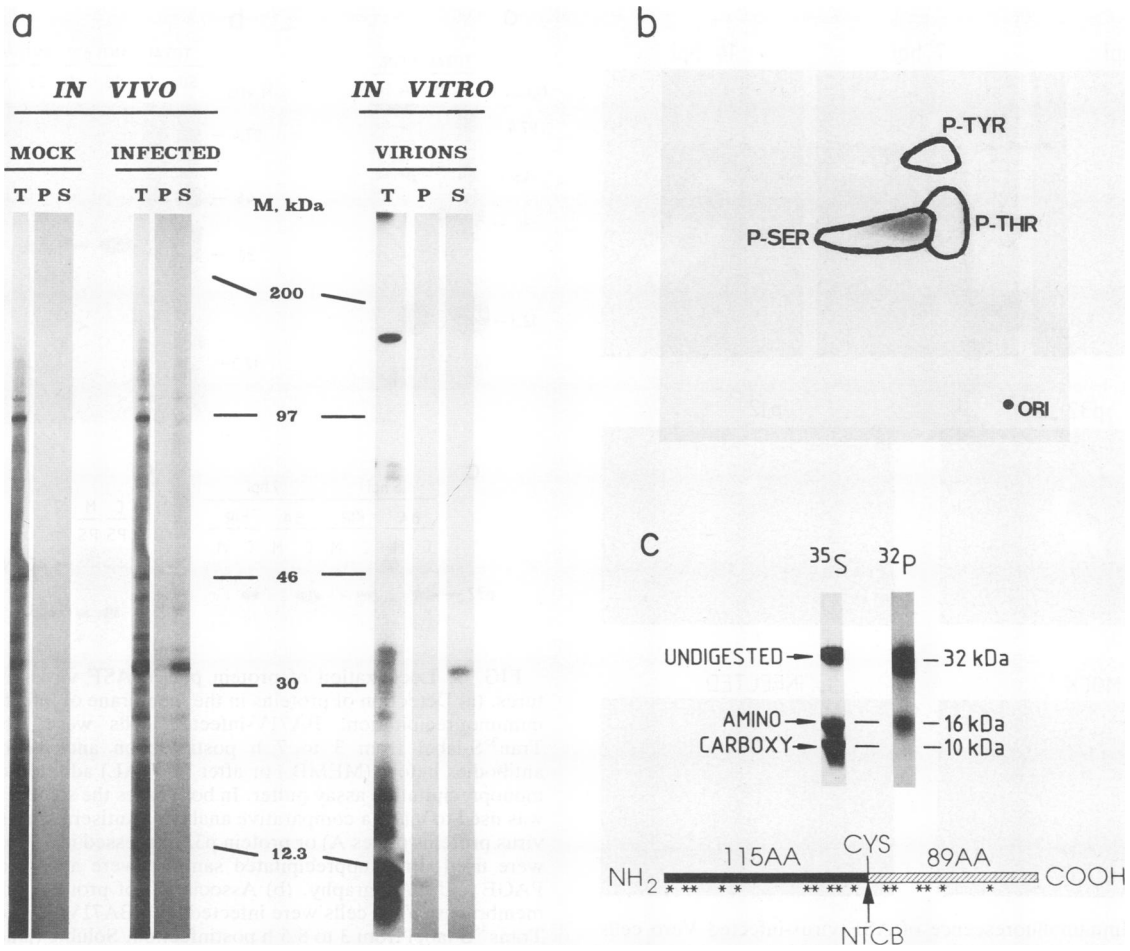

FIG. 5. Phosphorylation of protein p32. (a) Phosphorylation of protein p32 in ASF virus-infected cells. Mock- or BA71V-infected cells were labeled with [^{32}P]phosphate from 2 to 6 h postinfection (IN VIVO). Viral proteins were also phosphorylated in purified preparations of virus particles incubated in protein kinase reaction mixture in the presence of [γ-^{32}P]ATP (IN VITRO). Samples were analyzed by SDS-PAGE and autoradiography before (lanes T) or after immunoprecipitation by serum against pET3c transformed bacteria (lanes P) or antiserum against p32 expressed in *E. coli* (lanes S). M, molecular mass. (b) Phosphoamino acid analysis. BA71V-infected Vero cells labeled with [^{32}P]phosphate from 15 to 19 h postinfection were immunoprecipitated by antiserum against p32 expressed in *E. coli*. Protein p32 was isolated from polyacrylamide gels and treated with acid, and the hydrolysis products were subjected to two-dimensional thin-layer electrophoretic (horizontal axis) and chromatographic (vertical axis) analysis. *O*-Phospho-L-serine (P-SER), *O*-phospho-L-threonine (P-THR), and *O*-phospho-L-tyrosine (P-TYR), detected by ninhydrin staining, were used as internal markers. The place where samples were applied is indicated (ORI). An autoradiograph is shown. (c) Treatment with NTCB. BA71V-infected Vero cells were labeled with Tran^{35}S-label or [^{32}P]phosphate for 24 h or from 2.5 to 9 h postinfection, respectively. Protein p32 was isolated as in panel b and incubated with NTCB, and the resultant fragments were resolved by SDS-PAGE. Below the autoradiograph, the positions of the cleavage by NTCB and the putative phosphorylation sites are indicated by an arrow and asterisks.

from intact infected cells a 12-kDa polypeptide which might correspond to the protein detected by the monoclonal antibody 18B.B11 in the plasma membrane of infected Vero cells (41).

To investigate the possibility that protein p32 is associated with intracellular membranes, infected cells labeled with [^{35}S]methionine were disrupted by nitrogen cavitation and the soluble and microsomal fractions were analyzed by SDS-PAGE. As shown in Fig. 7b, protein p32 was found in the soluble fraction. The small amount of p32 detected in microsomes, about 5%, is not significant since the contamination of this fraction with soluble proteins was estimated to be about 13% for different proteins in the total extracts. As a control, the membrane protein p24 (49) was detected mainly in the microsomal fraction.

Protein p32 was hardly detected in supernatants of BA71V- or KIR69-infected swine macrophages by Western blotting (Fig. 7c). The amount of p32 in the medium after 9 h, compared with that found in cell extracts, was estimated to be 6.7 and 9.5% for BA71V and KIR69, respectively. Labeling experiments with BA71V-infected cells with [^{35}S]methionine from 2 to 5 h postinfection followed by a 13-h chase showed that 0.9% of p32 is secreted from infected Vero cells (data not shown). In swine macrophages this value was 5% (Fig. 7d). However, a similar level of a cellular protein of 45 kDa, presumably actin, was detected in supernatants from infected macrophages (Fig. 7d), suggesting that the protein p32 found in the medium might represent nonspecific release of viral proteins from infected cells, which is higher in infected macrophages.

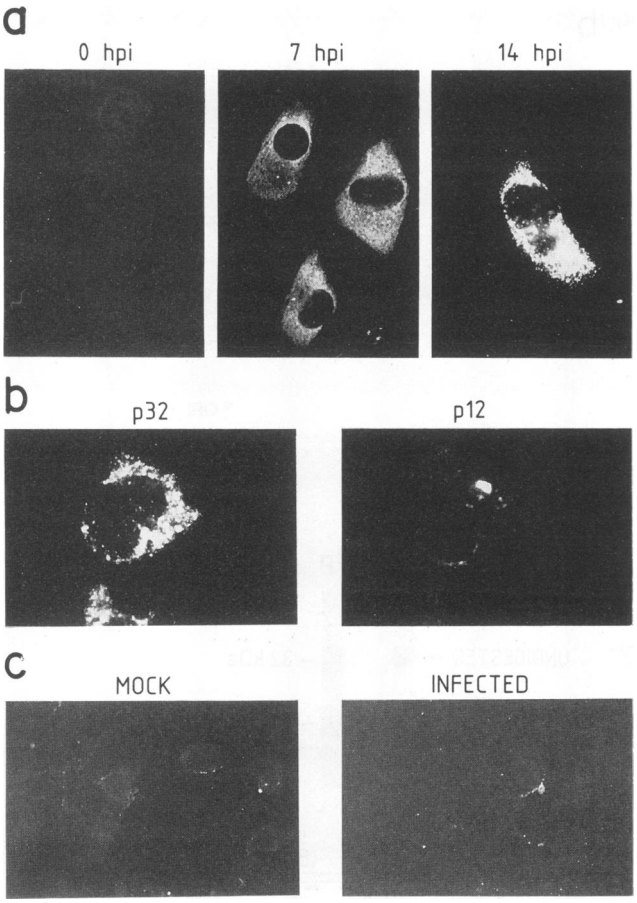

FIG. 6. Immunofluorescence of ASF virus-infected Vero cells. (a) BA71V-infected Vero cells were fixed with methanol and used for immunofluorescence with antibodies specific for p32 at the indicated times (hours) postinfection. In the case of 0 hpi the exposure of the photograph was longer to detect the low background. (b) Methanol-fixed infected Vero cells were double labeled with antibodies against p32 (p32) and the structural protein p12 (p12) and subsequently incubated with rhodaminated (p32) or fluoresceinated (p12) antibody conjugates. The fluorescence micrographs correspond to the same field. (c) Vero cells were mock infected with UV-inactivated BA71V (MOCK) or infected with infectious virus (INFECTED). The unfixed cells were transferred to ice at 7 h postinfection and used for membrane immunofluorescence with antibodies specific for p32.

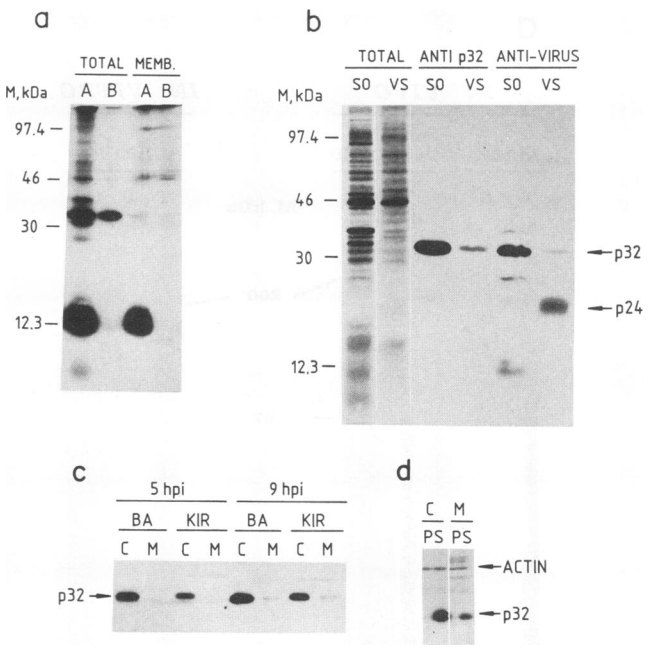

FIG. 7. Localization of protein p32 in ASF virus-infected cultures. (a) Detection of proteins in the membrane of infected cells by immunoprecipitation. BA71V-infected cells were labeled with Tran^{35}S-label from 3 to 7 h postinfection and incubated with antibodies before (MEMB.) or after (TOTAL) addition of radioimmunoprecipitation assay buffer. In both cases the same amount of Ig was used to make a comparative analysis. Antisera specific for ASF virus proteins (lanes A) or protein p32 expressed in *E. coli* (lanes B) were used. Immunoprecipitated samples were analyzed by SDS-PAGE and fluorography. (b) Association of proteins with cellular membranes. Vero cells were infected with BA71V and labeled with Trans^{35}S-label from 3 to 8.5 h postinfection. Soluble (lanes SO) and membrane (lanes VS) fractions were obtained by nitrogen cavitation as described in Materials and Methods. Samples were analyzed by SDS-PAGE before (TOTAL) or after immunoprecipitation by serum specific for protein p32 (ANTI p32) or a mixture of serum against ASF virus proteins and monoclonal antibody 19E.H10 against p24 (ANTI-VIRUS). A fluorograph is shown. M, molecular mass. (c) Secretion of protein p32 from infected macrophages. Cells (lanes C) and medium (lanes M) from cultures of swine alveolar macrophages infected with BA71V (BA) or KIR69 (KIR) were harvested at 5 or 9 h postinfection. Samples were analyzed by Western blotting using antiserum specific for p32 expressed in *E. coli*. (d) Analysis of the secretion of protein p32 by pulse-chase labeling of BA71-infected macrophages. Cells were labeled from 3 to 5 h postinfection and chased from 5 to 16 h postinfection. Cells (C) and medium (M) were immunoprecipitated by serum against pET3c-transformed *E. coli* (lanes P) or serum against p32 (lanes S). The fluorograph of the lanes corresponding to the medium was exposed 12 times longer than that of the cells.

Expression of protein p32 in vaccinia virus. To express the protein in the vaccinia virus system, ORF C'204L was cloned under the control of the vaccinia virus p7.5 promoter and inserted into the thymidine kinase locus of the vaccinia virus genome to produce the recombinant virus named vC'204L. The molecular mass of the protein was predicted to be 22,858 Da, since the C-terminal 8 amino acids were replaced by 3 provided by the vector. Labeling experiments with [^{35}S]methionine and [^{32}P]phosphate in cells infected with vC'204L and immunoprecipitation showed the synthesis of a 32-kDa polypeptide that was also phosphorylated during the vaccinia virus replication cycle (Fig. 8).

DISCUSSION

In this paper we report the mapping and sequence of the gene encoding the highly immunogenic ASF virus protein p32. Although the ORF, designated C'204L, is predicted to encode a polypeptide of 204 amino acids with a molecular mass of 23,551 Da, the size estimated in SDS-PAGE when expressed in rabbit reticulocyte lysates, *E. coli*, and mammalian cells is higher (32 kDa). This discrepancy might be explained as a result of specific exclusion of SDS molecules due to the negative charges of this acidic protein, enhanced by the charges provided by the phosphate residues (54).

The kinetics of synthesis of p32 showed that the protein is translated early during infection, which is in agreement with the selection of specific RNA encoding the protein from

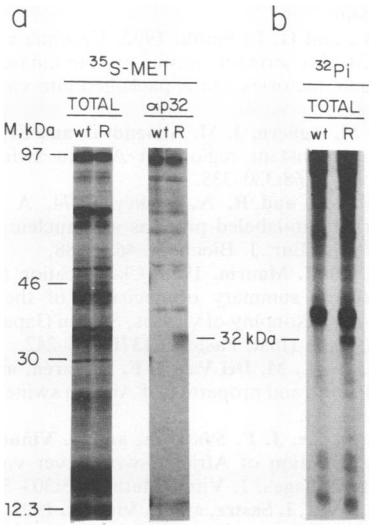

FIG. 8. Synthesis and phosphorylation of protein p32 in vaccinia virus-infected cells. Vero cells were infected with vaccinia virus wild type (lanes wt) or recombinant vC'204L (lanes R) and labeled with [^{35}S]methionine or [^{32}P]phosphate from 9 to 24 h postinfection. (a) Analysis of ^{35}S-labeled proteins by SDS-PAGE and fluorography before (TOTAL) or after (α-p32) immunoprecipitation by serum against p32 expressed in *E. coli*; (b) SDS-PAGE and autoradiography of ^{32}P-labeled cell extracts. M, molecular mass.

early RNA synthesized in vitro in purified ASF virus particles and with the fact that p32 is one of the few early viral proteins expressed in infected rabbit macrophages (4). As expected, the synthesis of the polypeptide falls after 12 h postinfection, coincidental with the onset of late protein synthesis, but newly synthesized p32 is also detected at very late times of infection. The kinetics of synthesis of the protein parallels that of p32-specific RNA, in which an increase in specific transcripts after 20 h postinfection is detected (42). This phenomenon has been observed in other ASF virus early genes and has been interpreted as a basal transcriptional activity of early promoters after viral DNA replication (6). Therefore, the properties of the gene agree with those of early genes that are transcribed before the onset of DNA replication, and the stability of the protein accounts for the increased detection of p32 throughout the infection.

Although we expected that p32 was a glycoprotein, according to previous data obtained by metabolic labeling with radioactive precursors of glycoproteins (21), our experiments indicate that the incorporation of labeled sugar into p32 takes place after the conversion of these compounds into amino acids by the cellular metabolism, as has been described for other ASF virus proteins (3, 20). The absence of glycosylation of protein p32 was supported by the failure to detect the protein in the Golgi apparatus by immunofluorescence and the fact that the protein is not retained in lectin columns (39).

Addition of [^{32}P]phosphate to infected cells labeled specifically protein p32 as a result of the action of a serine protein kinase. This protein may correspond to the previously identified cytoplasmic phosphoproteins of 30 kDa (52) or 35 kDa (45). The phosphorylation of serine residues, which were localized at the N-terminal half of the polypeptide, is in accordance with the properties of the viral protein kinase (45), which is one of the enzymes implicated in the phosphorylation of p32, as demonstrates the kinase labeling reaction carried out by the viral enzyme incorporated into virions (Fig. 5). Although we cannot rule out the possibility that a cellular enzyme also phosphorylates p32, a phenomenon described for vaccinia virus (36), the phosphorylation of p32 when expressed from a vaccinia virus recombinant suggests that the vaccinia virus protein kinase recognizes the ASF virus protein as a substrate, which is in agreement with previous data showing that both viruses encode a serine-threonine protein kinase (9, 36, 40, 45).

Although protein p32 was detected and phosphorylated in virus preparations, quantitative analysis of the different steps of the purification procedure supported the view that the final destiny of p32 is not incorporation into virus particles, since the majority of the polypeptide remains in the cell even after the release of the extracellular virions. The protein present in virion preparations might be a contaminant that, because of the abundance and highly antigenic properties of the protein, is detected in preparations of virus. However, the possibility that very small amounts of protein p32 are incorporated into virions cannot be ruled out. The fact that virus factories in infected cells were not strongly stained by immunofluorescence supported the hypothesis that p32 is not a true structural protein.

The results presented in this report indicate that protein p32 is found in the cytoplasm of infected cells. According to the speckled immunofluorescence pattern, it might be associated with intracellular vesicles at late times of infection. However, the finding that the protein is present in the soluble fraction of the cytoplasm suggests that the immunofluorescence pattern observed might correspond to aggregates of the protein possibly due to its accumulation in the cytoplasm during the infection. In accordance with this hypothesis, the protein is found in infected cells as an oligomer with an apparent molecular mass of 220 kDa (7). The small amount of protein detected in the supernatants might be attributed to nonspecific release as a result of cell damage and not to specific secretion of the polypeptide.

The previous report describing this protein (1) reported the sequence of the gene in the virus strain E-75; the protein was named p30 because of its smaller apparent size in this isolate. The predicted amino acid sequences of p30 and p32 are almost identical, but remarkably, the protein is 10 amino acids longer in the BA71V strain because of a frameshift close to the end of the protein caused by the deletion of an adenylate residue from a 10-adenylate array. Although Afonso et al. claimed that protein p30 is located in the plasma membrane, we have been unable to detect p32 in the cell surface above the background attributable to the virus inoculum, either in BA71V or in KIR69V strains, by immunofluorescence or by immunoprecipitation. Consistent with this result are the nonprediction of transmembrane domains according to the algorithm of Klein et al. (31) or of a signal peptide according to the method of Von Heijne (57) and the nondetection of the protein in the endoplasmic reticulum, Golgi apparatus, or other intracellular membrane systems by immunofluorescence or immunoprecipitation of fractionated cell extracts.

Similarly, the secretion of the polypeptide reported in the previous paper, which was based on the detection of a weak band of 30 kDa in supernatants from infected cells, seems to be, according to the data presented here, the result of a nonspecific release of cytoplasmic soluble proteins, probably due to cell damage. In addition, the absence of an immunofluorescence pattern consistent with trafficking of the protein through the secretory pathway, together with the

finding of the protein in the soluble fraction of the cytoplasm, makes it very difficult to imagine a process by which p32 is actively secreted.

In conclusion, we propose that the cellular localization of this protein is the cytoplasm and that its properties do not agree with those of secreted or plasma membrane proteins.

The difference in electrophoretic mobility of the protein among virus isolates suggests variability in this protein. Different degrees of phosphorylation might explain size variation; however, attempts to reduce the apparent molecular mass of the protein in SDS-PAGE by treatment with acid or alkaline phosphatase have been unsuccessful (39). Alternatively, a different length of the polypeptide chain may account for this difference, as appears to be the case for the isolate E-75 (1) and BA71V. Interestingly, a similar difference in the size of the protein between the Vero cell-adapted virus BA71V and the natural isolate BA71, from which it derives, has been found. Although a correlation between the electrophoretic mobility and virus virulence was previously suggested on the basis of the analysis of five non-African virus strains (1), we have not found this correlation when comparing 10 strains which included 5 African isolates that show more genetic variation (10).

In summary, we describe here the initial characterization and the cellular localization of protein p32, the major ASF virus phosphoprotein synthesized at early times of infection. The phosphorylation of the polypeptide might play a role in the regulation of p32 function during the virus replication cycle. The availability of specific antisera and protein expressed in heterologous systems will provide tools to study the immune response against this abundant and highly immunogenic ASF virus phosphoprotein induced early during infection.

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