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Amino Acid Sequence and Structural Properties of Protein p12, an African Swine Fever Virus Attachment Protein

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The gene encoding the African swine fever virus protein p12, which is involved in virus attachment to the host cell, has been mapped and sequenced in the genome of the Vero-adapted virus strain BA71V. The determination of the N-terminal amino acid sequence and the hybridization of oligonucleotide probes derived from this sequence to cloned restriction fragments allowed the mapping of the gene in fragment *Eco*RI-O, located in the central region of the viral genome. The DNA sequence of an *Eco*RI-*Xba*I fragment showed an open reading frame which is predicted to encode a polypeptide of 61 amino acids. The expression of this open reading frame in rabbit reticulocyte lysates and in *Escherichia coli* gave rise to a 12-kDa polypeptide that was immunoprecipitated with a monoclonal antibody specific for protein p12. The hydrophilicity profile indicated the existence of a stretch of 22 hydrophobic residues in the central part that may anchor the protein in the virus envelope. Three forms of the protein with apparent molecular masses of 17, 12, and 10 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been observed, depending on the presence of 2-mercaptoethanol and alkylation with 4-vinylpyridine, indicating that disulfide bonds are responsible for the multimerization of the protein. This result was in agreement with the existence of a cysteine-rich domain in the C-terminal region of the predicted amino acid sequence. The protein was synthesized at late times of infection, and no posttranslational modifications such as glycosylation, phosphorylation, or fatty acid acylation were detected.

African swine fever (ASF) virus is an icosahedral deoxyvirus with lipid envelopes that causes an important disease in domestic pigs (for reviews, see references 9, 15, 46, and 47). ASF virus is very specific of porcine species (46), in which it replicates mainly in peripheral monocytes and macrophages (31, 33). One of the most striking aspects of ASF virus is that sera from pigs and other virus-resistant animal species inoculated with ASF virus do not neutralize the virus, although they contain virus-specific antibodies (10). The Vero-adapted BA71 virus strain (BA71V) contains more than 30 proteins in the virus particle, with molecular masses ranging from 10 to 150 kDa (7). No structural glycoproteins have been found in purified virions (11), although the induction of several glycoproteins during infection has been reported (12, 44). Other modifications of viral proteins, such as phosphorylation (37, 44), protease processing (29, 30), and fatty acid acylation (1), have also been reported.

ASF virus binds to a cell surface receptor, and this interaction is required for a productive infection in Vero cells and swine macrophages (2, 3). Recently, the involvement of the structural protein p12 in the recognition of the cellular receptor, on the basis of the specific binding of the protein to susceptible Vero cells, has been reported (8). Protein p12 is externally located in the virus particle and forms homomultimers with an apparent molecular mass of 17 kDa in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the absence of reducing agents, indicating that disulfide bonds are involved in the multimerization of the protein (8).

We report here the mapping of the gene coding for protein p12 in the genome of the Vero-adapted virus strain BA71V. The nucleotide sequence showed the existence of an open reading frame (ORF) that is predicted to encode a protein of 61 amino acids and is characterized by the existence of a stretch of hydrophobic amino acids in the central part and a cysteine-rich domain in the C-terminal region. The identity of the ORF has been confirmed after expression in a cell-free system and in *Escherichia coli*. We also describe the synthesis and the posttranslational modifications of the protein in infected cells.

MATERIALS AND METHODS

Cells and viruses. Vero, L, and K562 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium supplemented with 10% calf serum. The Vero-adapted virus strain BA71V is described elsewhere (17) and was purified by Percoll equilibrium centrifugation (7).

Plasmids and bacterial strains. Plasmid p5RO (28) is a pBR325 recombinant containing the *Eco*RI O fragment of the BA71V genome. *E. coli* JM109 was used as host for plasmids. The host for the expression vector was *E. coli* BL21(DE3) (43), a lysogen of the lambda D69 derivative, which contains a single copy of the gene for T7 RNA polymerase in the chromosome under the control of the inducible *lacUV5* promoter.

Amino acid sequence analysis. Purified BA71V particles were incubated with 1% *n*-octyl-β-D-glycopyranoside (OG) as described previously (8). Subsequently, the proteins released from the virus particles were separated by SDS-PAGE in the absence or in the presence of 2-mercaptoethanol (2-ME). After transfer to Immobilon membranes, the amino-terminal sequence of the protein bands was determined by automated Edman degradation, using an Applied

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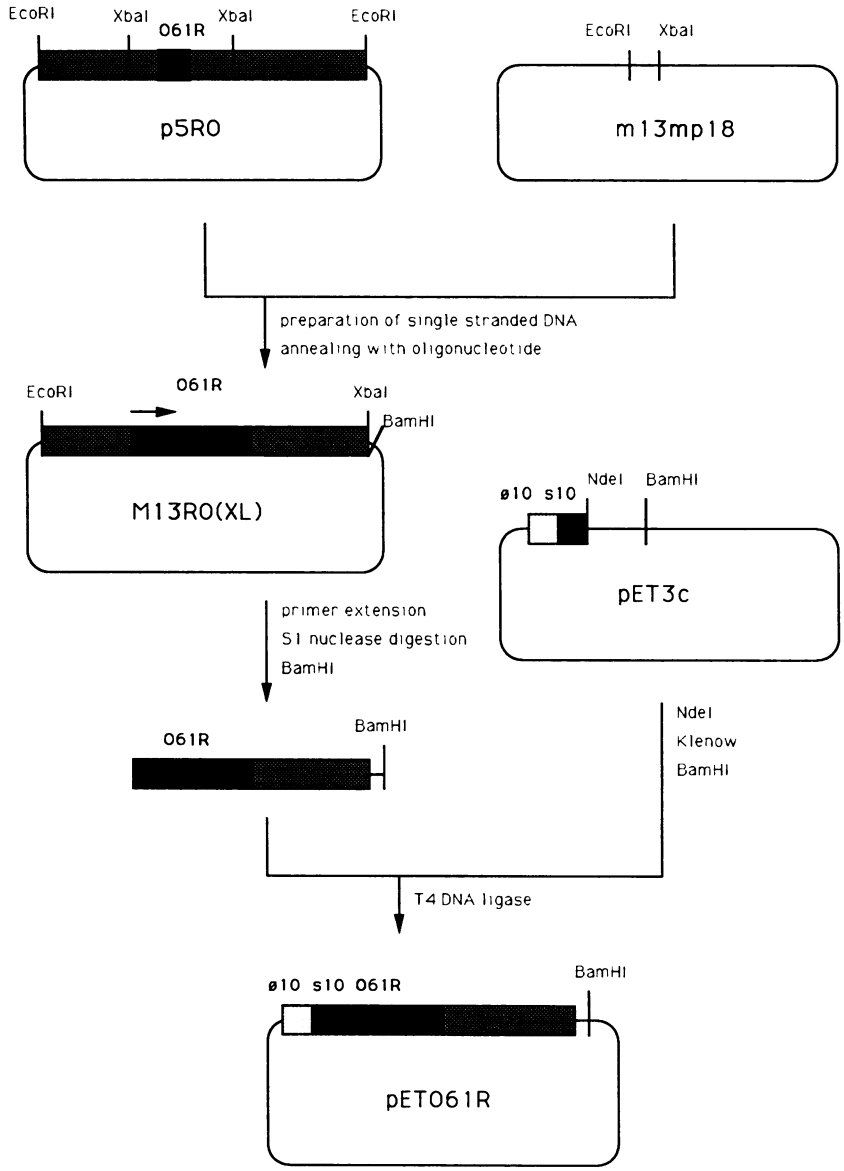


FIG. 1. Construction of plasmid pETO61R for the expression of protein p12. Experimental details are described in Materials and Methods.

Biosystems model 477A pulse-liquid sequenator equipped with a model 120A on-line phenylthiohydantoin analyzer (21, 22).

Gene mapping. Synthetic oligonucleotides, whose sequences were derived from the amino acid sequence determined for protein p12, were used to map the gene in the ASF virus genome. The oligonucleotides were synthesized by phosphoramidite method in an Applied Biosystems model 381A DNA synthesizer (32). The oligonucleotides were further purified by PAGE, and those used as probes were radiolabeled with [γ-^{32}P]ATP (500 Ci/mmol; Amersham UK) and T4 polynucleotide kinase according to standard procedures (38). The hybridization of labeled probes to dot blots and Southern blots of cloned ASF virus restriction fragments (28) were performed as described previously (29).

DNA sequencing and computer analysis. The 913-bp fragment (*Eco*RI-*Xba*I) from the left end of the *Eco*RI O fragment was inserted into the polylinker region of phage vector M13mp19 (34). DNA sequencing was carried out on single-stranded DNA templates by the dideoxynucleotide chain terminator method (39). The M13 sequencing primer and additional oligonucleotide primers corresponding to inner sequences were used. Nucleotide sequences were determined in both strands at least once. Computer analysis of DNA and protein sequences was performed with the software package of the University of Wisconsin Genetics Computer Group (13).

Construction of the expression plasmid pETO61R. The recombinant plasmid pETO61R, containing the gene coding for p12, was constructed as shown in Fig. 1. The *Eco*RI-*Xba*I fragment of 913 bp containing ORF O61R was subcloned into the polylinker region of the phage vector M13mp18, obtaining the recombinant phage vector M13RO (XL). ORF O61R was inserted immediately downstream of

the φ10 promoter and Shine-Dalgarno sequence of plasmid pET3c (43) by using the method described by Freije et al. (16) (Fig. 1). Briefly, single-stranded DNA was annealed with an oligonucleotide corresponding to the first 17 nucleotides of the gene (ATGGCACTTGATGGTTC). After primer extension with the Klenow fragment of *E. coli* DNA polymerase I, the remaining single-stranded DNA regions preceding the gene were removed by digestion with S1 nuclease. The blunt-ended fragment was excised from M13 DNA by digestion with *Bam*HI and inserted into the Klenow-repaired *Nde*I and the *Bam*HI sites of pET3c. Sequence of the recombinant plasmid showed that the gene had been correctly inserted into the vector.

In vitro translation of protein p12 in reticulocyte lysates. RNA was translated using a rabbit reticulocyte lysate (Amersham) in the presence of [^{35}S]methionine (1,200 Ci/mmol; Amersham) as described by Salas et al. (36). Two sources of RNA were used for the in vitro translation experiments. RNA extracted from infected Vero cells at 15 h of infection was selected by hybridization to plasmid pBR325 or p5RO immobilized on diazobenzyloxymethyl paper (36). On the other hand, specific transcripts containing ORF O61R were synthesized by the T7 RNA polymerase from plasmid pETO61R in an in vitro transcription system according to instructions provided by the manufacturer (Promega Biotec).

Expression of protein p12 in *E. coli*. *E. coli* BL21(DE3) transformed with pET3c or with the recombinant plasmid pETO61R was induced for expression by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and pulse-labeled for 5 min with 200 μCi of [^{35}S]methionine per ml at different times after induction, in the absence or in the presence of rifampin, as described previously (43).

Binding experiments to Vero and L cells. *E. coli* extracts containing protein p12 were obtained by sonication, centrifuged in a Beckman Airfuge at 100,000 × *g* for 1 h, and used in binding experiments. [^{35}S]methionine-labeled rabbit reticulocyte lysate and IPTG-induced *E. coli* extracts were diluted in Dulbecco's modified Eagle medium buffered at pH 7.4 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, supplemented with 2% calf serum and 50 mM methionine, and used in binding experiments to Vero and L cells at 4°C as described previously (8). As a control, the binding of ^{35}S-labeled protein p12 released from purified virus particles by incubation with 1% OG was carried out in parallel (8).

Immunoprecipitation and analysis of proteins by electrophoresis. The radioactive samples were dissociated in 10 mM Tris-HCl (pH 7.5)–150 mM NaCl–1% sodium deoxycholate–1% Nonidet P-40–0.1% SDS–1 mM phenylmethylsulfonyl fluoride and immunoprecipitated by monoclonal antibody (MAb) 24BB7 (8) and protein A-Sepharose (20). For the electrophoretic analysis, whole extracts or immune complexes were dissociated in sample buffer containing 0.4 M Tris-HCl (pH 6.3), 2.3% SDS, and 10% glycerol, in the absence or in the presence of 5% 2-ME. Samples were analyzed by SDS-PAGE in 7 to 20% acrylamide gels as described previously (25). Radioactive protein bands were detected by fluorography (6).

Reduction and alkylation of protein p12. ^{35}S-labeled protein p12, released from purified virus by treatment with 1% OG, or the products obtained after in vitro translation of RNAs synthesized by the T7 RNA polymerase from pETO61R were incubated with 4 volumes of denaturing solution (7.5 M urea, 4 mM EDTA, 0.25 M Tris-HCl [pH 8.5]) for 30 min at 37°C in a final volume of 60 μl. Six microliters of undiluted 2-ME was then added, and the samples were incubated in the dark under N_2 for 2 to 3 h. Alkylation was performed by incubation in the dark under N_2 for 30 min at room temperature with 2.5 μl of undiluted 4-vinylpyridine (Sigma), and the samples were analyzed by SDS-PAGE in sample buffer without 2-ME. Protein p12 released from purified virions was desalted by chromatography on Sephadex G-25 before analysis by electrophoresis.

Treatment of radioactively labeled proteins with glycosidases. Infected Vero cells labeled with [^{35}S]methionine or [^{14}C]glucosamine (200 mCi/mmol; Amersham) as described previously (11) were immunoprecipitated by an MAb specific for p12 (24BB7) or p72 (1BC11[40]) and incubated overnight at 37°C with 1 mU of endoglycosidase H, 0.2 mU of endoglycosidase F/N–glycosidase F, and 0.5 mU of endo-α-*N*-acetyl galactosaminidase (*O*-glycanase) in a buffer containing 0.1% SDS, 1% OG, 0.1 M sodium phosphate, 2 mM 2-ME, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (pH 6.1). All of the enzymes were purchased from Boehringer Mannheim. The activity of *N*-glycosidases was monitored by the decrease in the electrophoretic mobility of the glycoproteins of 62 and 49 kDa of Semliki Forest virus (18). For this purpose, whole extracts of radioactively labeled K562 cells infected with Semliki Forest virus were incubated in the buffer described previously, in the presence or in the absence of endoglycosidases. At the end of the incubation, samples were analyzed by SDS-PAGE in the presence of 2-ME.

Nucleotide sequence accession number. The GenBank accession number for the sequence of the *Eco*RI-*Xba*I fragment of 913 bp presented in this article is M84186.

RESULTS

Mapping and nucleotide sequence of the gene encoding protein p12. To design an oligonucleotide probe to map the gene in the viral genome, the NH_2-terminal amino acid sequence of protein p12 was determined. For this purpose, a mixture of structural proteins (p12, p14, p17, and p35) was obtained after treatment of purified BA71V particles with 1% OG. Proteins were subsequently separated by SDS-PAGE and transferred to Immobilon membranes, and the NH_2-terminal sequence of the band corresponding to protein p12 was determined by automatic Edman degradation on a pulse-liquid sequenator. A single amino acid residue was present in each degradation step, indicating the existence of a unique polypeptide chain in the band. The sequence of the first four amino-terminal amino acids, which did not contain the initiator methionine, was obtained (Fig. 2A). Assuming that the N-terminal methionine might have been removed in the mature protein, we included an ATG in the 5′ terminus of the probes (Fig. 2A), which were radioactively labeled and hybridized to selected DNA fragments, obtained by digestion with different restriction enzymes (Fig. 3A), that covered the complete length of the viral genome. The dot blot hybridization localized the gene coding for protein p12 in the *Eco*RI O fragment (Fig. 2B). Subsequent Southern hybridization experiments mapped the gene within an *Eco*RI-*Xba*I fragment of 913 bp located in the left end of fragment *Eco*RI-O (Fig. 3A). The nucleotide sequence showed an ORF that contained the N-terminal amino acid sequence established for protein p12 and was named O61R, since it is located in the *Eco*RI O fragment, is predicted to encode a polypeptide of 61 amino acids, and is transcribed rightward toward the genome terminus (Fig. 3B). The predicted molecular mass of the mature protein of 60 amino acids in length

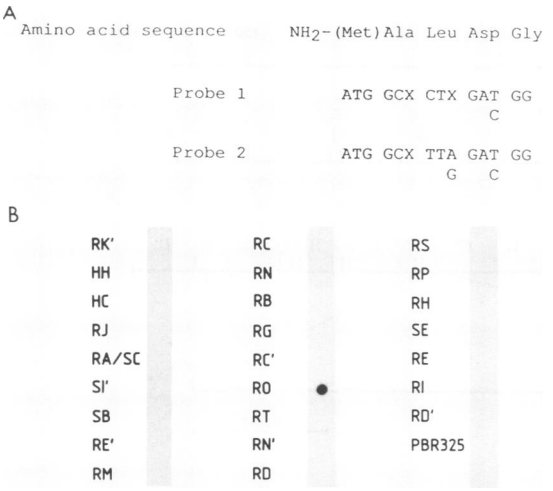


FIG. 2. Mapping of the gene encoding protein p12. (A) The NH_2-terminal sequence of protein p12 was determined by automated Edman degradation after transfer of the protein to Immobilon membranes. The oligonucleotide mixtures representing all the possible coding sequences that were used as probes for the hybridization are shown. X = A, T, C, or G. (B) Dot blot hybridization of the oligonucleotide probes to cloned ASF virus DNA restriction fragments *Eco*RI (R), *Sal*I (S), and *Hin*dIII (H), covering the complete length of the viral genome.

is 6,543 Da, lower than expected from its electrophoretic mobility in SDS-PAGE (12 kDa). The 49 nucleotides of the 5′ and 3′ flanking regions of ORF O61R constitute intergenic regions that separate the gene coding for protein p12 from other two genes transcribed toward the left end of the viral genome (not shown). Since protein p12 shows an apparent molecular mass of 17 kDa when analyzed in the absence of 2-ME (8), the N-terminal sequence of this 17-kDa band was also determined after transfer to Immobilon membranes; the same sequence as found for p12 was obtained (not shown).

Properties of the protein. Figure 4 summarizes some structural properties deduced from the predicted amino acid sequence of the protein. The hydrophilicity profile indicates that the protein does not possess an N-terminal hydrophobic signal sequence that might initiate the translocation across the endoplasmic reticulum membrane. However, a stretch of 22 hydrophobic residues in the central region most likely represents a transmembrane domain (19, 48) (boxed in Fig. 4) and contains at the right end a peculiar group of aromatic residues (YYFWW). On the right side of the hydrophobic segment, a group of amino acids (QQKK) is predicted to constitute the most hydrophilic region of the polypeptide. A sequence that may function as a substrate for protein kinase C (26) and a potential acceptor site for asparagine-linked carbohydrate are found in the predicted amino acid sequence. A striking feature of the C region is the existence of a cysteine-rich domain that may account for the multimerization of the protein through disulfide bonds.

A search of the GenBank national data base and the National Biochemical Research Foundation protein data base revealed no significant homology with the entire sequence of p12. However, the cysteine motif present in protein p12 (CX_5CXCX_4C) was found in metallothioneins (41) and in the von Willebrand factor (45), proteins that are very rich in cysteine residues. This motif was also found in the β chain of integrins (23), which contain in the extracellular domain four tandem repeats of an eight-cysteine motif. Four cysteines out of the eight that constitute each domain were found in the same positions in protein p12.

Expression of protein p12 in rabbit reticulocyte lysate and in *E. coli*. To confirm that ORF O61R codes for a polypeptide of 12 kDa, in vitro translation experiments were carried out. In vitro translation of RNA synthesized in Vero cells at late times of infection and selected by hybridization to fragment *Eco*RI-O showed that the most abundant transcript selected with this fragment codes for a polypeptide of 12 kDa (Fig. 5A). Since the *Eco*RI O fragment is 2.7 kb in length, there was still the possibility that the RNA selected would correspond to another gene encoded by this fragment. To ensure that ORF O61R codes for the polypeptide detected, specific transcripts were synthesized in vitro by T7 RNA polymerase, using the recombinant plasmid pETO61R as the template. Electrophoretic analysis of the cell-free translation products of these transcripts showed the same polypeptide of 12 kDa (Fig. 5A). The immunoprecipitation with a MAb that recognizes protein p12 in the virus particle confirmed the identity of the polypeptide. However, recognition of the protein expressed in reticulocyte lysates by the MAb was low compared with the amount of ^{35}S-labeled protein p12 immunoprecipitated from virions. The protein expressed in this system did not form multimeric structures of 17 kDa when analyzed in the absence of 2-ME (Fig. 6).

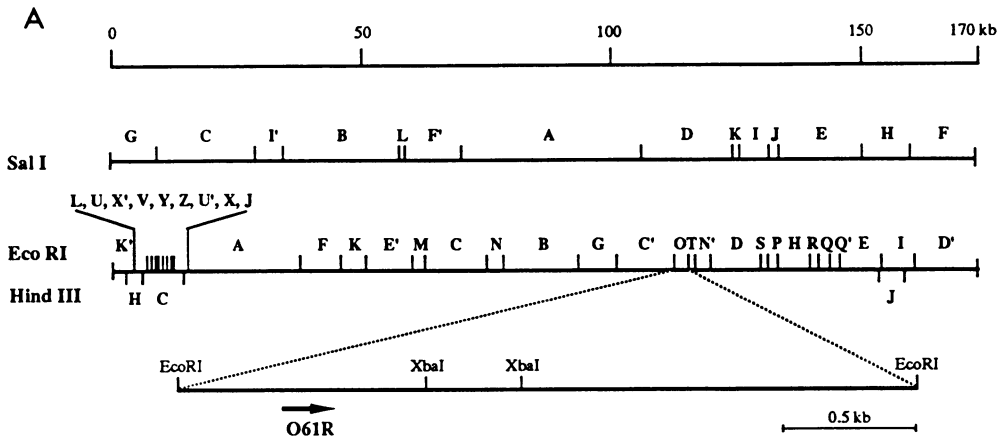
The recombinant plasmid pETO61R was also used for expression in *E. coli* under the control of T7 RNA polymerase. After transformation of *E. coli* BL21(DE3) with the recombinant plasmid pETO61R, the cultures were induced for expression of the viral polypeptide by addition of IPTG. Although the expression level of the protein was low, the induction of a 12-kDa polypeptide in *E. coli* transformed with pETO61R was detected after labeling with [^{35}S]methionine (Fig. 5B). The induced protein was weakly recognized by MAb 24BB7, and no multimeric forms of 17 kDa were observed in polyacrylamide gels in the absence of 2-ME (not shown).

^{35}S-labeled IPTG-induced *E. coli* extracts or reticulocyte lysates containing the specific polypeptide encoded by O61R were used in experiments to test binding to Vero or L cells as described in Materials and Methods. However, no specific binding of the protein expressed in both systems to virus-sensitive Vero cells was obtained (not shown).

Alkylation of protein p12. To investigate the possibility that the 12-kDa band corresponds to dimers that could be mediated through disulfide bonds resistant to the standard reducing conditions, protein p12 released from purified virions with OG or translated in vitro was reduced with 2-ME and alkylated with 4-vinylpyridine as described in Materials and Methods. Figure 6 shows that a band of 10 kDa was obtained when both the protein present in the virus particle and the in vitro translation product were alkylated.

Synthesis of protein p12 in infected cells. To define the time course of synthesis of protein p12, BA71V-infected Vero cells were pulse-labeled with [^{35}S]methionine at different times of infection and immunoprecipitated by MAb 24BB7. An immunoprecipitated band was detected in Vero cells after 15 h of infection and not in the presence of an inhibitor of DNA synthesis (cytosine arabinoside) (not shown). Protein p12 was not secreted, as indicated by the immunoprecipitation of the culture medium, and showed an apparent molecular mass of 17 kDa in the absence of reducing agents (not shown).

Since the amino acid sequence of the polypeptide showed the existence of sites for phosphorylation and N glycosylation, labeling experiments in infected cells and subsequent



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361  CTTGAATAAGCGTTAACATTTTATATTTAATATTAAAATCTTTTCATTTTATATATTATA  420

421  TACGCAAAATGGCACTTGATGGTTCAAGTGGTGGAGGCTCTAATGTAGAAACATTACTTA  480
             M  A  L  D  G  S  S  G  G  G  S  N  V  E  T  L  L  I

481  TCGTAGCAATCATTGTGGTTATTATGGCAATCATGCTTTACTATTTTTGGTGGATGCCCC  540
       V  A  I  I  V  V  I  M  A  I  M  L  Y  Y  F  W  W  M  P  R

541  GCCAGCAAAAAAAAATGTAGCAAGGCTGAAGAATGCACATGTAATAACGGAAGCTGTTCCC  600
       Q  Q  K  K  C  S  K  A  E  E  C  T  C  N  N  G  S  C  S  L

601  TAAAAACAAGTTAAAAAATGCAATTATATGTATGCATGTAAAACGCGTAAATACCACATA  660
       K  T  S

661  AAACTATAACATGTCAATCATGGAATCAACACTTTTATAATTTTCCGTAATATATTTTTC  720
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FIG. 3. Sequence of the gene encoding protein p12. (A) Schematic representation of the mapping of the gene in the ASF virus genome by dot blot and Southern blot hybridization, using oligonucleotide probes derived from the NH_2-terminal amino acid sequence. The localization of ORF O61R within the *Eco*RI-*Xba*I fragment of 913 bp is shown. (B) Nucleotide sequence and deduced amino acid sequence of ORF O61R and flanking regions. Numbers correspond to the positions in the complete nucleotide sequence of the *Eco*RI-*Xba*I fragment of 913 bp.

immunoprecipitations with the MAb 24BB7 were carried out. No protein was immunoprecipitated from infected Vero cells labeled with [^{32}P]phosphate or from purified virus particles labeled with [γ-^{32}P]ATP by the endogenous protein kinase (37) (not shown). A band of 12 kDa was detected, after long exposures of the fluorographs, by immunoprecipitation with the MAb in both infected Vero cells (Fig. 7) and extracellular purified virus particles (not shown) metabolically labeled with [^{14}C]glucosamine. The possibility that this band corresponds to metabolic conversion of the radioactive sugar to amino acids was supported by the finding that the structural protein p72, which is not glycosylated (11), was also labeled with [^{14}C]glucosamine in infected cells (Fig. 7) and virions (not shown). In agreement with this finding, the [^{14}C]glucosamine incorporated into proteins p12 and p72 was resistant to treatment with *O*- and *N*-glycosidases (Fig. 7). The activity of the glycosidases was tested in glycoproteins of Semliki Forest virus as described in Materials and Methods. As an internal control, the label found in the cellular glycoprotein gp126 (12), which is coimmunoprecipitated with p72 by MAb 1BC11 for unknown reasons, was degraded by glycosidases (Fig. 7).

To investigate the possibility that the protein would be covalently bound to fatty acids, labeling experiments with [^{3}H]myristic and [^{3}H]palmitic acids, followed by immunoprecipitation with the MAb, were performed as described previously (1). No band of 12 kDa was detected in, or immunoprecipitated from, infected-cell extracts or purified virus particles labeled with radioactive fatty acids (not shown).

DISCUSSION

The results presented in this report establish the amino acid sequence of protein p12, an ASF virus protein involved in cell attachment. The gene coding for protein p12 was mapped in fragment *Eco*RI-O of the ASF virus DNA and encodes a polypeptide that is predicted to consist of 61 amino acids, but the mature protein is devoid of the initiator methionine, as deduced from the N-terminal amino acid sequence determined. The predicted molecular mass of the polypeptide (6,543 Da) is different from the one found by SDS-PAGE (12 kDa). However, the following results demonstrate that ORF O61R codes for protein p12: (i) the NH_2-terminal amino acid sequence of the protein after electrophoresis in the absence (17 kDa) or in the presence (12 kDa) of reducing agents was identical to that of ORF O61R; (ii) the products obtained after expression of ORF O61R in a cell-free translation system or in *E. coli* showed a molecular mass of 12 kDa; (iii) both the protein present in the virion and the in vitro translation product showed an electrophoretic mobility of 10 kDa after alkylation; and (iv) the

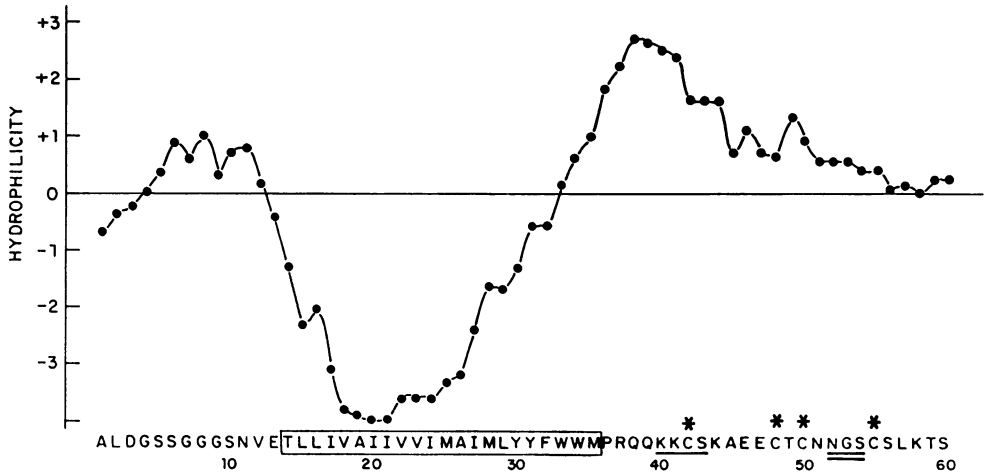

FIG. 4. Hydrophilicity profile and properties of protein p12. The hydrophilicity profile of the predicted protein p12 was obtained according to the method of Kyte and Doolittle (24). The boxed region is the putative hydrophobic transmembrane segment. The phosphorylation site for protein kinase C is underlined. The potential N-linked glycosylation site is doubly underlined. Cysteines are identified by asterisks.

protein encoded by ORF O61R was immunoprecipitated by a MAb that recognizes the protein in the virus particle (8). The possibility that the affinity of the MAb 24BB7 would be higher for the 17-kDa form of the protein than for the 12-kDa form could explain the weak immunoprecipitation of the protein expressed in reticulocyte lysates and in *E. coli*, systems in which multimerization of the protein does not take place.

Previous results indicated that protein p12 is located in the outer envelope of the virion (8). The deduced amino acid sequence of the polypeptide shows a stretch of 22 hydrophobic residues that could function as a membrane-spanning domain to anchor the protein in the external virus envelope. Alternative mechanisms to anchor the polypeptide in the membrane, such as covalent binding to fatty acids, have not been found. The absence of cleavable N-terminal signal sequences (19, 48) in the predicted amino acid sequence of protein p12 suggests that the polypeptide is inserted into the membrane through an alternative mechanism, as has been proposed for other proteins of low molecular mass (27, 49).

The existence of a cysteine-rich domain in the polypeptide is of interest since the electrophoretic mobility of the protein is highly influenced by the presence of reducing agents. Three forms of the protein, with apparent molecular masses of 17, 12, and 10 kDa in SDS-PAGE, have been found in the protein present in the virus particle; however, the multimers of 17 kDa were not detected in the expression in reticulocyte lysates and in *E. coli*. The finding that the protein showed an apparent molecular mass of 10 kDa after alkylation strongly suggests that this band corresponds to the monomeric form of the protein. The anomalous migration of the polypeptide in SDS-PAGE could be explained by a peculiar secondary structure of the protein in the presence of SDS, probably influenced by the long stretch of hydrophobic residues that accounts for one-third of the polypeptide. The 12-kDa mobility could be explained by the formation of intrachain disulfide bonds, resistant to the standard reducing conditions, that would promote a conformational change and alter the apparent molecular mass in polyacrylamide gels. Finally, the 17-kDa form could probably correspond to dimers or trimers that are covalently bound through interchain disulfide bonds sensitive to the standard reducing conditions.

Cell-free translation of late viral RNA selected by the *Eco*RI O fragment indicated that ORF O61R is transcribed late during infection. This finding was in agreement with labeling experiments with [^{35}S]methionine in infected Vero cells and immunoprecipitation with MAb 24BB7, which showed that protein p12 belongs to the late class of viral genes. Protein p12 was not secreted from the cells and, as previously shown in purified virus particles (8), was synthesized in infected cells as a disulfide-linked multimer of 17 kDa.

The phosphorylation site for a protein kinase C found in the amino acid sequence of the polypeptide does not seem to be used, as deduced from labeling of infected Vero cells or purified virions with [^{32}P]phosphate or [γ-^{32}P]ATP, respectively, and subsequent immunoprecipitation with the MAb. Although no glycosylated proteins have been detected in extracellular ASF virus particles (11), the glycosylation of p12 was investigated since a putative N-glycosylation site was found in the predicted amino acid sequence. The detection of protein p12 in virions labeled with radioactive glucosamine was probably due to metabolic conversion of the sugar to amino acids, a phenomenon that has been previously described for [^{14}C]glucosamine-labeled purified preparations of ASF virus (11) and was supported by the labeling of the major capsid protein p72 with the radioactive sugar. The resistance of the [^{14}C]glucosamine label in p12 immunoprecipitated from infected cells to *O*- and *N*-glycosidases and the fact that ^{35}S-labeled p12 released from purified virions with OG is not retained in lectin columns (concanavalin A and wheat germ) (4) support the absence of glycosylation of the polypeptide.

The similarity of the cysteine motif of p12 with the one present in the β chain of integrins is interesting, since the latter corresponds to a repeated, disulfide-bonded cysteine motif found in the extracellular domain of the β chain. The detailed folding and structure of this cysteine-containing segment is not defined, but it is tempting to speculate that p12 and integrins, both involved in cellular recognition (8, 42), share a common structural motif. However, the functional implications of these similarities are presently unknown.

A reasonable model for the topology of the protein is that

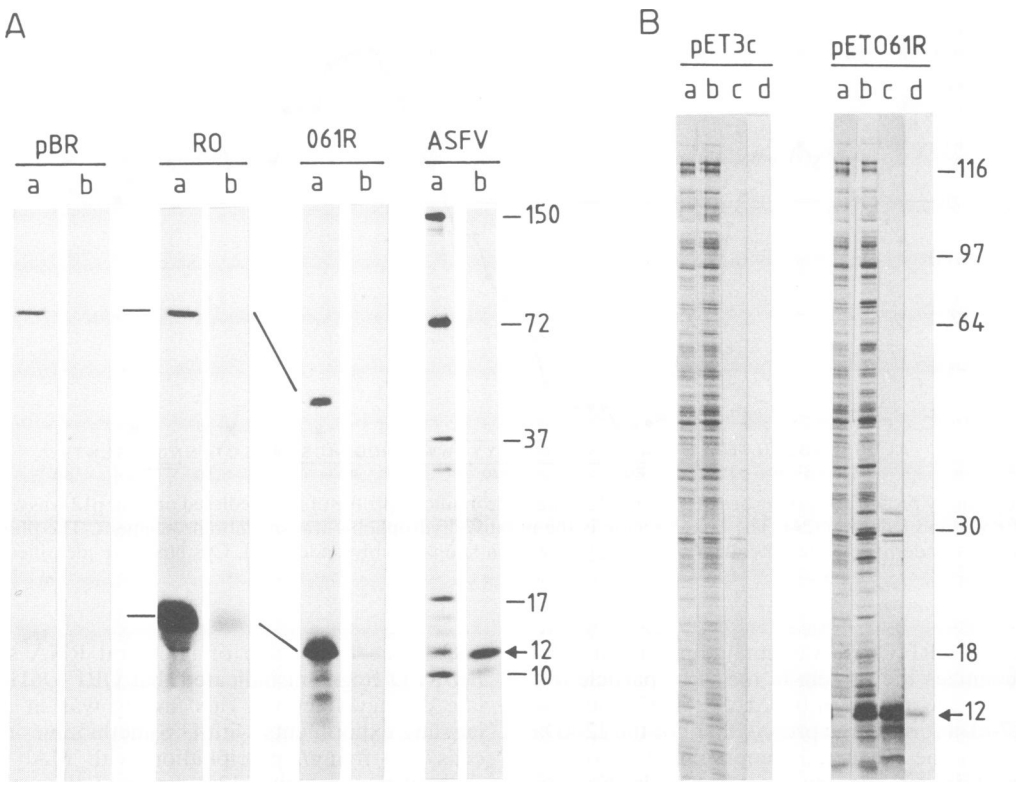

FIG. 5. Expression of p12 in reticulocyte lysates and in *E. coli*. (A) In vitro translation of ORF O61R. Translation products of RNA were obtained from BA71V-infected Vero cells at late times of infection, selected by hybridization to pBR325 (pBR) or p5RO (RO), which contains fragment *Eco*RI-O, and RNA transcribed in vitro by T7 RNA polymerase from recombinant plasmid pETO61R (O61R), which contains the gene encoding protein p12. The [^{35}S]methionine-labeled polypeptides were resolved before (lanes a) or after immunoprecipitation (lanes b) with MAb 24BB7 by SDS-PAGE in the presence of 2-ME. The results obtained with ^{35}S-labeled purified virus are shown (ASFV). (B) Expression in *E. coli* of ORF O61R. *E. coli* BL21(DE3), transformed with pET3c or pETO61R, was pulsed-labeled with [^{35}S]methionine for 5 min before (lanes a) or after (lanes b) induction for 45 min with 1 mM IPTG, which induces the synthesis of T7 RNA polymerase. IPTG-induced cultures were incubated with rifampin (80 μg/ml) for 45 min to inhibit the synthesis of endogenous RNAs by the host RNA polymerase and were pulsed with radioactive methionine (lanes c). Cultures grown in the presence of IPTG and rifampin (lanes c) were immunoprecipitated by MAb 24BB7 (lanes d). Samples were analyzed by SDS-PAGE in the presence of 2-ME. Molecular masses in kilodaltons are indicated.

the cysteine-rich carboxy-terminal region is located in the extracellular space, and the hydrophobic transmembrane domain functions as an anchor to the viral envelope. Consistent with this model, the finding that a 12-kDa protein was produced in the cell-free system and in *E. coli* could be explained by the absence of translocation of the carboxy-terminal region of p12 across the membrane, conditions in which dimers of 17 kDa would not be obtained because interchain disulfide bonds are probably not formed under the reducing conditions of the cytosol. The detection of the 17-kDa multimeric form when the protein is expressed in eukaryotic systems, such as vaccinia virus or baculovirus, that would allow the protein to be inserted properly into competent membranes supports this hypothesis (4). According to this model, the carboxy-terminal region would contain the determinants involved in the recognition of the cellular receptor.

The formation of 17-kDa multimers might be required for the binding capacity of protein p12, explaining that the 12-kDa protein expressed in reticulocyte lysates and in *E. coli* did not bind to Vero cells. Alternatively, posttranslational modifications or a correct folding of the monomer, which do not take place in the expression systems described here, might be necessary for binding.

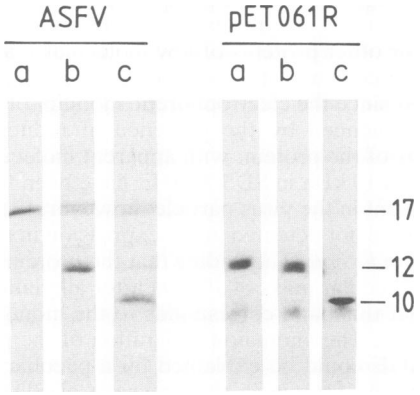

FIG. 6. Alkylation of protein p12 released from purified virus particles with OG or expressed in a cell-free system. ^{35}S-labeled protein p12 obtained from purified virus particles after incubation with 1% OG or transcribed and translated in vitro from ORF O61R (pETO61R) were analyzed by SDS-PAGE in sample buffer without (lanes a) or with (lanes b) 5% 2-ME. Samples were reduced with 2-ME and subsequently alkylated with 4-vinylpyridine before analysis in sample buffer without 2-ME (lanes c) as described in Materials and Methods. Molecular masses in kilodaltons are indicated.

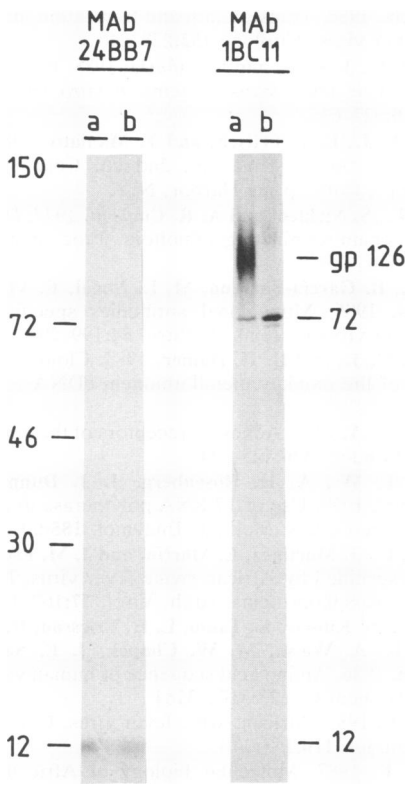

FIG. 7. Treatment of [^{14}C]glucosamine-labeled proteins p12 and p72 with glycosidases. Vero cells were infected at a high multiplicity of infection and labeled with [^{14}C]glucosamine from 14 to 20 h of infection. Cell extracts were immunoprecipitated with MAb 24BB7 or 1BC11, which recognize protein p12 or p72, respectively. Immunoprecipitated proteins were analyzed by SDS-PAGE in the presence of 2-ME before (lanes a) or after (lanes b) treatment with *O*- and *N*-glycosidases as described in Materials and Methods. Molecular masses in kilodaltons are shown. The bands corresponding to viral proteins p12 and p72 and to the cellular glycoprotein gp126 are indicated.

The sequence of the gene coding for protein p12 has been obtained from the Vero-adapted virus strain BA71V, and possible mutations in the protein might have been selected during the adaptation process to Vero cells, which were initially resistant to the infection. We have found that this sequence is identical in the nonadapted virus strain BA71, and a few conservative mutations have been detected in other field virus isolates that are restricted to grow in swine macrophages (5).

In conclusion, we have sequenced the gene and defined structural properties of the ASF virus protein p12, an ASF virus attachment protein. This will allow the identification of the binding domain involved in the recognition of the cellular receptor and determination of this interaction at the molecular level. Since one of the most straightforward methods of neutralization is to prevent virus binding to the target cell (14), experiments are in progress in an attempt to induce antibodies against protein p12 that are able to block virus attachment to the host cell, with the hope of finding neutralizing antibodies. ASF virus may have developed mechanisms to avoid an efficient immune response against the binding determinants of protein p12, as has been described for the virus attachment protein of influenza virus and rhinovirus (35, 50). This possibility could explain the absence of neutralizing antibodies in ASF virus-infected pigs (10); therefore, the immune response against p12 in infected pigs is presently under investigation.

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