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African Swine Fever Virus Structural Protein p72 Contains a Conformational Neutralizing Epitope

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We have previously described a monoclonal antibody (mAb 135D4) to an unidentified 70- to 72-kDa African swine fever virus (ASFV) protein that exhibited high levels of neutralizing activity against various virulent ASFV isolates. Here, we identify the reactive ASFV protein as the major virus structural protein p72. *In vitro*-translated products of the p72 protein gene were specifically immunoprecipitated by mAb 135D4. Immunoprecipitation of a nested set of truncated p72 *in vitro* translation products defined the region between amino acid residues 400 and 404 as necessary for mAb 135D4 reactivity. Five partially overlapping peptides (15mers) covering residues 388–446 failed to react with mAb 135D4, suggesting the conformational dependence of the epitope. Supporting this interpretation, larger *in vitro* translation products representing residues 56–282, 159–361, 360–508, and 507–646 also failed to react with mAb 135D4. Consistent with its involvement in virus neutralization, immunoelectromicroscopy, using a rabbit antiserum against mAb 135D4-purified p72, located the protein on the surface of unenveloped virus particles. © 1994 Academic Press, Inc.

African swine fever virus (ASFV) is a large, enveloped DNA virus that is the causative agent of ASF, a highly lethal disease of domestic pigs (*2, 3*). Because there is no available vaccine, control of ASFV is achieved solely by animal slaughter and strictly enforced sanitation procedures. Though poorly understood, homologous protective immunity does develop in pigs surviving viral infection; pigs surviving acute infection with moderately virulent or attenuated variants of ASFV develop long-term resistance to homologous virulent virus (*4–7*). Anti-ASFV antibodies are a significant component of the protective immune response in that they alone will protect pigs against lethal ASFV infection (*8*); however, the antibody-mediated mechanism(s) responsible for this protective effect has not as yet been identified. Although early attempts to demonstrate ASFV neutralizing antibodies in sera from recovered animals were unsuccessful (*9–12*), reports by Ruiz Gonzalvo *et al.* (*13*) and more recently by Zsak *et al.* (*1*) have described neutralization of virulent ASFV isolates in both Vero cell cultures and swine macrophages using convalescent swine serum. Recently, we have described a mouse monoclonal antibody (mAb 135D4) to a 70- to 72-kDa ASFV protein that exhibited high levels of neutralizing activity against several virulent ASFV isolates (*1*). Here, we identify the ASFV protein recognized by mAb 135D4 as the major virus structural protein p72 and present evidence that this neutralizing epitope is conformational dependent.

We have previously shown that mAb 135D4 specifically immunoprecipitated a viral protein of approximately 70–72 kDa from ASFV-infected cell lysates (*1*). ASFV-infected cells contain at least four viral proteins in this size range (*14–17*), including a major structural protein, p72 (*18*). The p72 structural protein has been shown to be highly antigenic (*19*), and one report has localized the protein at the surface of unenveloped virions (*20*). To determine if p72 was the viral protein reacting with mAb 135D4, immunoprecipitation experiments using *in vitro* translation products of p72 were conducted. *In vitro* translation of T7 polymerase-generated p72 gene transcripts from the plasmid pGEM Ad 62-18 (*21*) yields three products of different molecular weights, depending on the initiation codon used: a 72-kDa (full-length protein with 646 amino acid residues), a 60-kDa (residues 128 to 646), and a 47-kDa (residues 221 to 646) product mAb 135D4 immunoprecipitated all three products indicating that this antibody was directed against the structural protein p72 and that the amino-terminal 221 amino acids were not necessary for antibody recognition (Fig. 1A).

In order to characterize regions of p72 associated with mAb 135D4 reactivity, truncated p72 protein products varying at their carboxy terminus were produced using *in vitro* transcription/translation and tested for reactivity with mAb 135D4. Truncated p72 transcripts were obtained by linearization of pGEM Ad 62-18 at unique restriction sites within the p72 coding sequences. Truncated p72 proteins of 312 (*Pvu*II site), 393 (*Dra*I site), 442 (*Bst*EII site), and 550 (*Cla*I site) amino acids were all immunoprecipitated by ASFV convalescent serum; however, only the two longer products of 442 and 550 amino acid residues were immunoprecipitated by mAb 135D4

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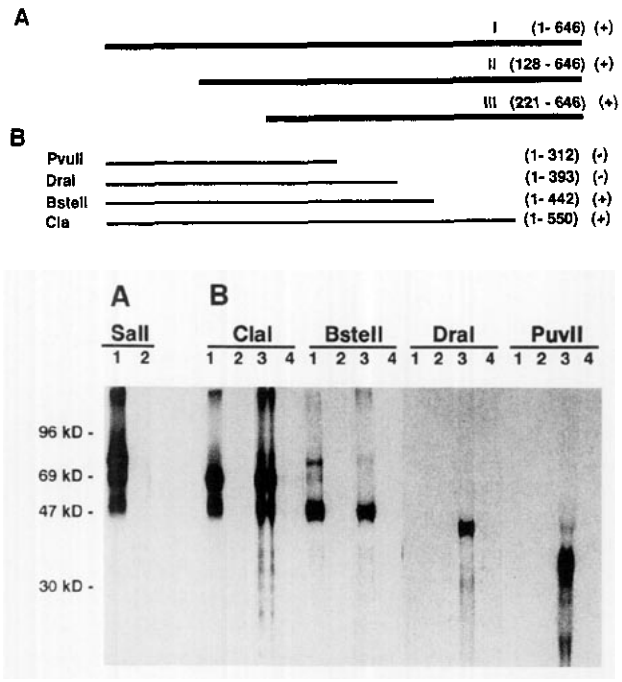


FIG. 1. Reactivity of mAb 135D4 with full-length and truncated p72 protein. Plasmid pGEM Ad 62-18 (*21*) containing the complete p72 gene of ASFV isolate Badajoz was purified by CsCl gradient and linearized by restriction enzyme digestion at unique restriction sites (*Sal*I, *Cla*I, *Bst*EII, *Dra*I, and *Pvu*II). Digested DNAs were treated with proteinase K (1 mg/ml) for 30 min at 37°, ethanol precipitated, and resuspended in RNAse-free TE (10 m*M* Tris–HCl, pH 8, 1 m*M* EDTA) at a concentration of 1 μg/μl. Twenty micrograms of plasmid DNA was then *in vitro* transcribed using mCAP mRNA Capping kit (Stratagene, La Jolla, CA) as described by the manufacturer in the presence of 1 μl RNase inhibitor (Boehringer Mannheim Biochemical, Germany). DNA was eliminated by addition of 10 units of DNase I (Stratagene) for 5 min at 37°, and the RNA was then resuspended in RNase-free TE and *in vitro* translated with the *in vitro* Express Translation kit (Boehringer Mannheim Biochemical). Approximately 2 μg of RNA was incubated (45 min at 31°) with 60 μl of rabbit reticulocyte lysate in the presence of 240 μCi of [^{35}S]methionine (NEN-Dupont, Wilmington, DE) and 3 μl of RNase inhibitor. Immunoprecipitation was performed as described elsewhere (*22*). (A) Full-length p72 products immunoprecipitated with mAb 135D4 (lane 1) and mAb OH_3NT (anti-African horsesickness virus, a gift from W. W. Laegreid, PIADC, USDA) (lane 2). Note the three different *in vitro* translation products from *Sal*I linearized pGEM Ad 62-18 (I, II, III). (B) Truncated p72 products obtained by linearization of the pGEM Ad 62-18 plasmid with *Cla*I, *Bst*EII, *Dra*I, and *Pvu*II were immunoprecipitated with mAb 135D4 (lane 1), mAb OH_3NT (lane 2), an ASFV convalescent pig serum (lane 3), or a nonimmune pig serum (lane 4). The diagram above depicts the p72 products tested by immunoprecipitation. Numbers in parentheses indicate the p72 amino acid residues represented in each protein product. (+) and (−) indicate presence or absence of reactivity of each product with mAb 135D4.

(Fig. 1B). This result identifies the region between amino acids 393 and 442 as necessary for mAb 135D4 reactivity.

To further identify critical amino acids associated with antibody reactivity within the 393–442 region of p72, additional p72-truncated products were produced using PCR with pGEM Ad 62-18 as template. In all cases, the forward primer was upstream and contained the T7 promoter sequences present in pGEM Ad 68-18, while the reverse primer began at the codon for the desired carboxy-terminal amino acid. PCR products were then *in vitro* transcribed and translated. Though all truncated p72 translation products of 393 (V0), 399 (VA), 404 (VB), 409 (V1), 427 (V2), and 445 (V3) amino acids were specifically immunoprecipitated by an ASFV convalescent swine serum, only VB, V1, V2, and V3 were recognized by mAb 135D4 (Fig. 2). The fact that VB is reactive and VA is not indicates that residues 400 to 404 are necessary for mAb 135D4 reactivity.

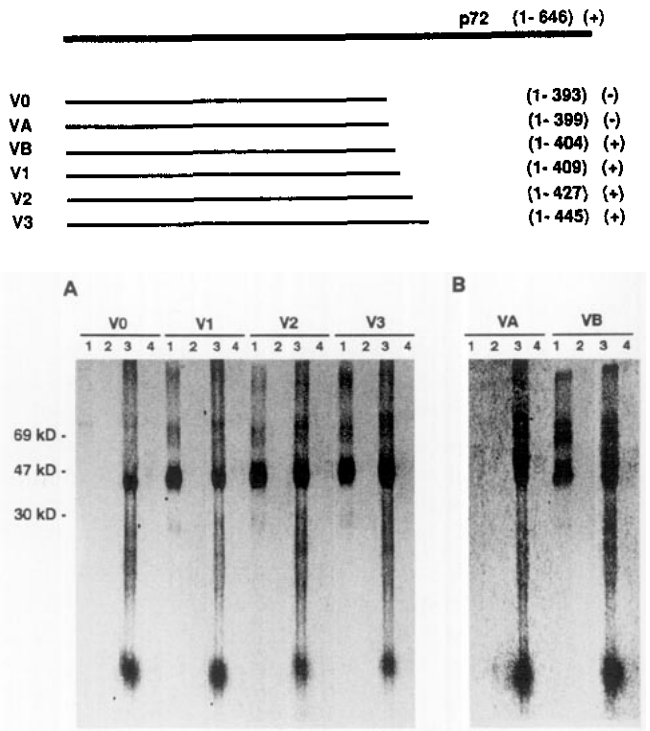


FIG. 2. Reactivity of mAb 135D4 with PCR-derived truncated p72 protein products. p72-truncated DNAs were produced by PCR using the pGEM Ad 62-18 plasmid as template. In all cases the forward primer was upstream of the T7 promoter sequences present in pGEM Ad 62-18. Reverse primers began at the last codon for the desired carboxy-terminal amino acid residue. PCR products used, showing the forward and the reverse primers, respectively, and the amino acid position of the truncated carboxy terminus were designated as follows: (V_0) 5′ GTTGTAAAACGACGGCCAGTGAATTG 3′, 5′ AAAGCGTATATTGCGTCTACTGGGGC 3′, residue 393; (V_1) 5′ CATTCAGGCTGCGCAACTGT 3′, 5′ ATTCGTGAGCGAGATTTCATTAATG 3′, residue 409; (V_2) 5′ GTTGTAAAACGACGGCCAGTG 3′, 5′ AAAAAGGTTGTGTATTTCAGGGGT 3′, residue 427; (V_3) 5′ CTATTACGCCAGCTGGCGAAAG 3′, 5′ GTGGGTCACCTGCGTTTTATGG 3′, residue 445; (V_A) 5′ GTTGTAAAACGACGGCCAGTGAATT 3′, 5′ TGGGATAAACCATGGTTTAAAGCGTATA 3′, residue 399; (V_B) 5′ GTTGTAAAACGACGGCCAGTGAATT 3′, 5′ TTCATTAATGACTCCTGGGATAAACCAT 3′, residue 404. PCR products were *in vitro* transcribed/translated and immunoprecipitated as described in the legend to Fig. 1. Each of the p72-truncated protein products was immunoprecipitated with mAb 135D4 (lane 1), mAb OH_3NT (lane 2), an ASFV convalescent pig serum (lane 3), or a nonimmune pig serum (lane 4). The diagram above depicts the p72 products tested by immunoprecipitation. Numbers in parentheses indicate the p72 amino acid residues represented in each protein product. (+) and (−) indicate presence or absence of reactivity of each product with mAb 135D4.

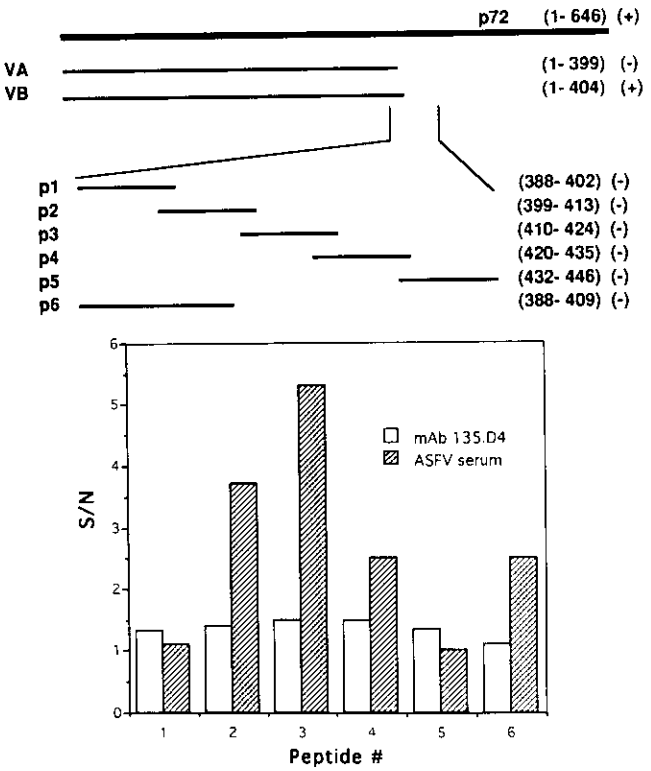


FIG. 3. Reactivity of synthetic p72 peptides with mAb 135D4. Synthetic peptides were designed using the amino acid sequence of the p72 gene of ASFV isolate Malawi Lil20/1 (Lu *et al.*, unpublished data) and obtained from Chiron Mimotopes U.S. (Raleigh, NC). Peptide denomination, sequence, and location on the p72 amino acid sequence are as follows: (p1) RRNIRFKPWFIPGVI, 388–402; (p2) PGVINEISLTNNELY, 399–413; (p3) NELYINNLFVTPEIH, 410–424; (p4) PEIHNLFVKRVRFSL, 420–435; (p5) RFSLIRVHKTQVTHT, 432–446; (p6) RRNIRFKPWFIPGVI-NEISLTNCys, 388–409. ELISA plates (Immulon IV, Dinatech, Denmark) were coated overnight at room temperature with peptides (2 μg/ml) and then blocked with 1% OVA in PBS. Undiluted mAb 135D4 or mAb OH_3NT and ASFV convalescent or nonimmune pig serum diluted $1/10^4$ were incubated for 1 hr at room temperature. Alkaline phosphatase anti-mouse or pig Ig (KPL, Gaithersburg, MD) was used as the secondary antibody. Plates were read at 405 nm in an ELISA plate reader, and results are presented as a signal/background ratio: OD obtained with the mAb 135D4 or ASFV convalescent pig serum/OD obtained with their respective controls. The diagram above depicts the relative location of the peptides tested by ELISA on the p72 amino acid sequence. Numbers in parentheses indicate the p72 amino acid residues represented in each peptide. (+) and (−) indicate reactivity of each product with mAb 135D4.

To analyze whether the residues 400–404 truly represent the complete epitope for mAb 135D4, five partially overlapping 15mer peptides covering the p72 residues 388 to 446 and a 23mer peptide covering the critical area between residues 388 and 409 were synthesized (Chiron Mimotopes U.S., Raleigh, NC) and tested in direct ELISA (Fig. 3). Though peptides 2, 3, 4, and 6 were recognized by an ASFV convalescent pig serum, none of the peptides showed any reactivity with mAb 135D4. Further attempts to detect any reactivity between the peptides and mAb 135D4 using inhibition/competition experiments between the binding of viral p72 and mAb 135D4 in ELISA, immunoprecipitation, and immunocytochemistry yielded negative results (data not shown). These results indicate that although residues 400–404 are indeed critical for the recognition of p72 by mAb 135D4, they do not completely constitute by themselves the mAb 135D4 binding site, suggesting that this epitope may have conformational characteristics.

Failure of peptides 2 and 6 (amino acids 399–413 and 388–409, respectively) to react with mAb 135D4 may be due to lack of immediately adjacent protein regions that are important for introducing some critical structure necessary for binding or stabilizing the antigen–antibody interaction. To examine this possibility, larger segments of the p72 protein, including one containing this region (HH; amino acids 360–508), were cloned in frame into pET plasmids (Novagen, Madison, WI), *in vitro* transcribed and translated, and examined for reactivity with mAb 135D4 using immunoprecipitation (Fig. 4). Although all p72 protein regions were specifically immunoprecipitated by an ASFV convalescent pig serum, none were recognized by mAb 135D4. Thus, the absence of immediately adjacent protein regions is not associated with failure of peptides 2 and 6 to react with mAb 135D4. This

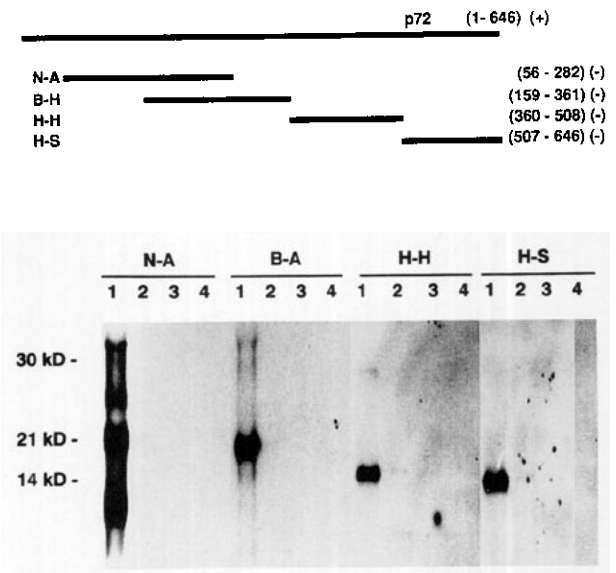
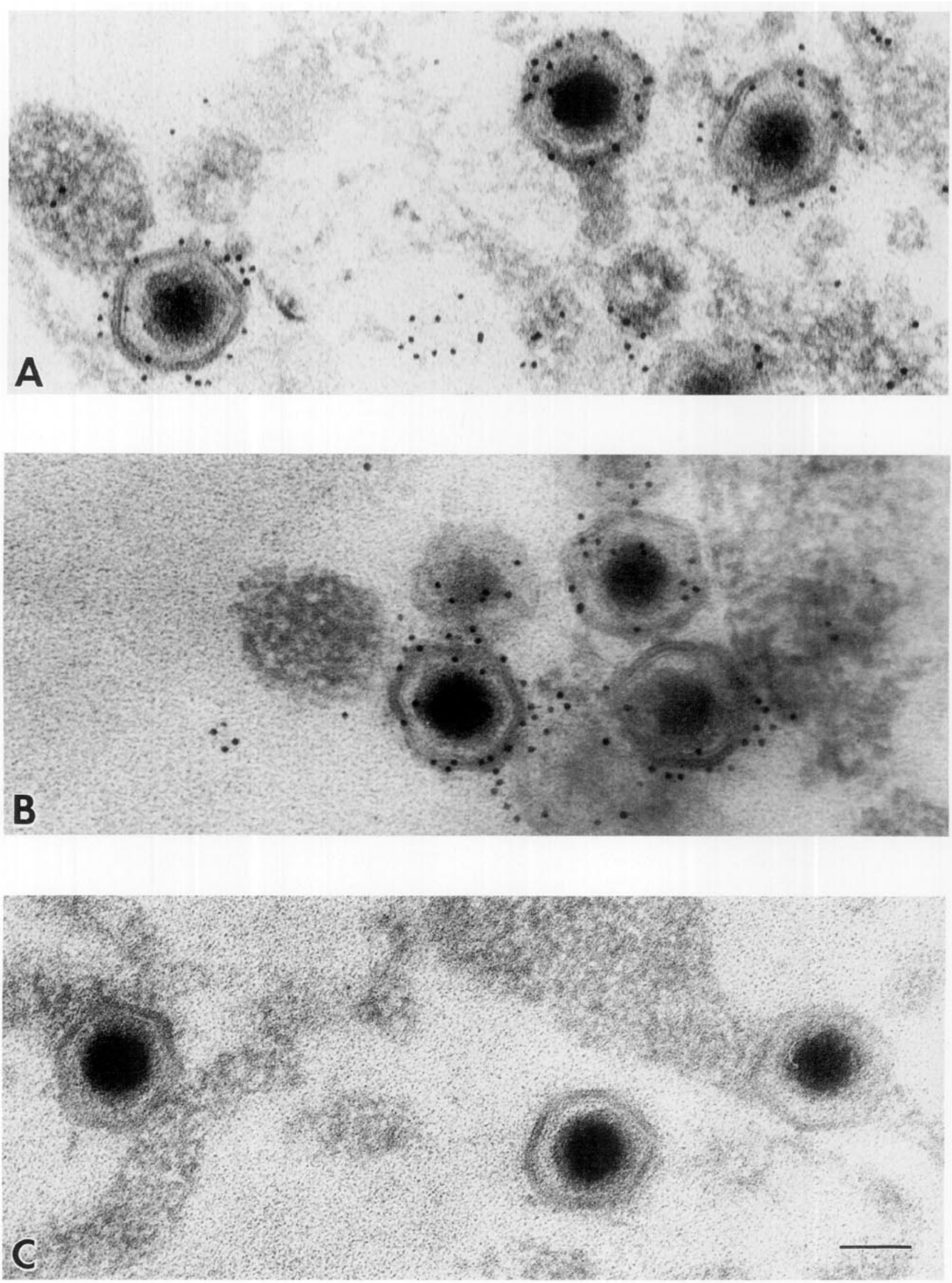


FIG. 4. Reactivity of different regions of the p72 protein with mAb 135D4. DNA restriction fragments encoding various regions of the p72 protein were excised from pGEM Ad62-18 and cloned in frame into pET21 (Novagen, Madison, WI). Cloned p72 gene fragments were as follows: (NA) *Nde*I–*Ava*I fragment, residues 56–282; (BH) *Bam*HI–*Hin*dIII fragment, residues 159–361; (HH) *Hin*dIII–*Hin*dIII fragment, residues 360–508; (HS) *Hin*dIII–*Sty*I fragment, residues 507–646. Cloned DNA fragments were *in vitro* transcribed/translated as described in the legend to Fig. 1 and immunoprecipitated with an ASFV convalescent pig serum (lane 1), a nonimmune pig serum (lane 2), mAb 135D4 (lane 3), and mAb OH_3NT (lane 4). The diagram above depicts the p72 regions examined. Numbers in parentheses indicate p72 amino acid residues represented in each product. (+) and (−) indicate reactivity of each product with mAb 135D4.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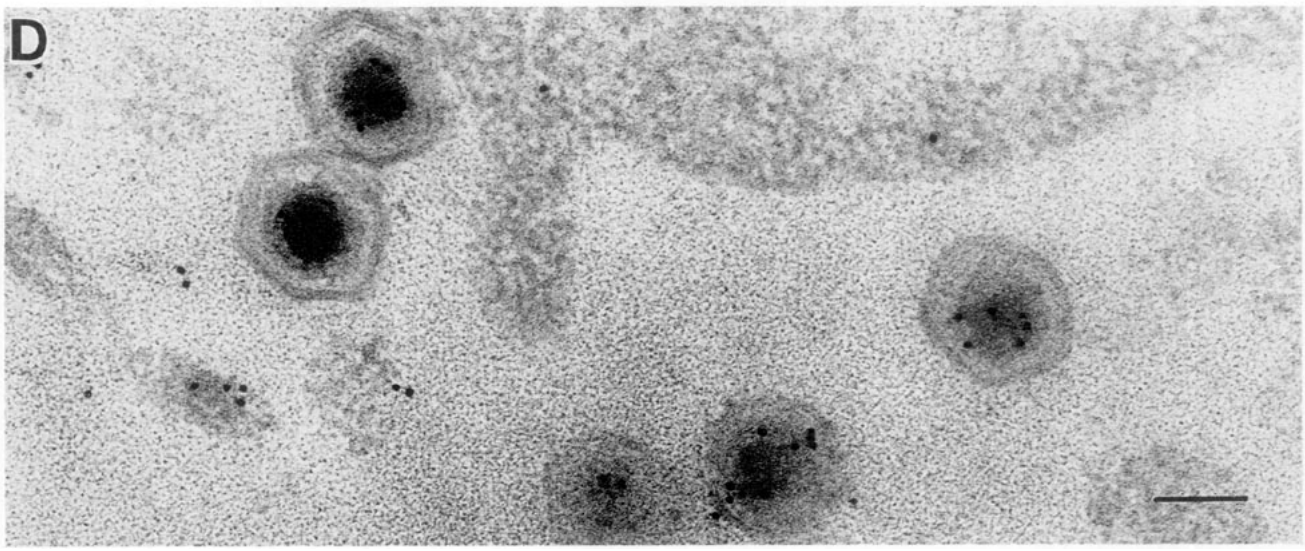

FIG. 5. Location of p72 in the ASFV virion. Anti-p72 rabbit antiserum was prepared as follows. ASFV p72 was immunoprecipitated from extracts of Vero cells infected with ASFV isolate E75-V5 using mAb 135D4 as previously described (*22*). Immunoprecipitated 72-kDa protein was then excised from SDS–PAGE gels and used for rabbit immunization. Resulting sera specifically immunoprecipitated a 72-kDa protein from ASFV-infected cells (data not shown). Immunogold labeling of ultrathin sections of pig macrophage cultures infected with ASFV isolate Malawi Lil-20/1 with anti-p72 antiserum (A and B). Primary cultures of swine macrophages were infected with ASFV Malawi Lil-20/1 (m.o.i. = 10). Infected cells were scrapped at 20 hr post infection and fixed overnight at 4° with 2% formaldehyde (freshly made from paraformaldehyde) and 0.1% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4. Cells were then washed twice with 0.1 *M* sodium cacodylate buffer with 10% sucrose, dehydrated to 90% ethanol, and embedded in Lowicryl K4M resin at room temperature. Thin sections were collected on Formvar-coated, nickel grids, blocked with BSA, and incubated with a 1/100 dilution of the anti-p72 rabbit serum. Rabbit antibodies were detected using anti-rabbit antiserum conjugated to 10 nm gold as directed by the manufacturer (Amersham). (C) Control grids incubated with anti-rabbit Ig conjugated to gold alone and (D) a rabbit antibody directed to an ASFV nucleoid-associated protein LMW5AR. Spatial distribution of p72 and LMW5AR antibody labeling was quantified in two compartments, near the surface of the virion and over the nucleoid. p72 antibody-labeled virions ($N = 26$) averaged 12.5 ± 4.7 gold grains associated with the surface of the virion and only 4 ± 0.2 grains located near the nucleoid. In contrast, LMW5AR antibody-labeled virions ($N = 100$) averaged 4.7 ± 2.8 grains over the nucleoid and only 0.44 ± 0.8 grains over the virus surface. When the secondary antibody was used alone, no gold label was seen over either compartment. Bar, 100 nm.

finding directly supports the suggestion that the mAb 135D4 epitope is conformational in nature.

To localize p72 within the virion, immunoelectronmicroscopy of ASFV-infected macrophages was performed using a polyclonal rabbit antiserum prepared against mAb 135D4 affinity-purified p72. Significant and specific labeling with the p72 antiserum was observed in association with the surface of unenveloped virus particles (Fig. 5).

These results indicate that the ASFV-neutralizing mAb 135D4 recognizes an epitope present on the virion structural protein p72. The neutralizing epitope has conformational characteristics that likely involve two regions of the protein. One region located between amino acids 400 and 404 is necessary but not sufficient for mediating antibody binding. A second region located between amino acid residues 221 and 360 is also likely involved. Its involvement is suggested by the fact that while the truncated amino terminus p72 product spanning residues 221–646 was recognized by mAb 135D4 (Fig. 1A III) the protein fragment comprising residues 360–508, which included the critical amino acids 400–404, was not (Fig. 4).

Consistent with the prior observation that mAb 135D4 immunoprecipitated a 70- to 72-kDa protein from all ASFV isolates tested and neutralized several heterologous virulent virus isolates (*1*), the structural protein p72 is known to be highly conserved among ASFV isolates; a panel of p72 mAbs exhibited similar reactivities with virtually all ASFV isolates tested (*23*), and p72 genes from three different isolates showed more than 98% conservation at the amino acid level (*18, 21,* Lu *et al.,* unpublished data).

Unlike virulent or low passaged ASFV isolates, highly passaged tissue culture-adapted ASFV variants are either poorly neutralized or not neutralized at all by mAb 135D4, even though the antibody precipitates a 72-kDa protein from all infected cell extracts. Conformational changes in the p72 protein itself, or in other structural proteins that affect p72 structure or conformation in the virion, were suggested as being responsible for converting the epitope from a neutralizing to a nonneutralizing one (*1*). The conformational nature of the mAb 135D4-neutralizing epitope described here makes this suggestion even more plausible. Absence of proper p72 conformation may also explain why highly purified p72 obtained by denaturing SDS–PAGE gels failed to induce neutralizing antibody in immunized pigs (*19*).

p72 has previously been localized in both the interme-

diate layers of the viral capsid (*24*) and on the surface of unenveloped virions (*20*) using immunoelectronmicroscopy. Immunogold labeling with the polyclonal p72 antiserum described here supports the observation of Whyard *et al.* (*20*) and places the protein at the surface of unenveloped virions, a location consistent with its involvement in virus neutralization (Fig. 5). This is of possible significance because unenveloped ASFV virions are fully infectious, and they do not appear to differ functionally from enveloped virions, which acquire a loose and irregular envelope while budding from infected cell membranes (*25–28*).

The role of neutralizing antibodies in the protective immune response to ASF is not known; however, it is reasonable to suggest that they may be of significance. Anti-viral antibodies alone protect pigs from lethal ASFV infection (*8*) and neutralizing antibodies are produced in pigs following infection (*1, 13*). Identification here of the first ASFV protein to contain a virus-neutralizing epitope may be the first step toward development of a protective immunogen. However, the conformational characteristics of this p72-neutralizing epitope will have to be taken into account when designing and evaluating experimental p72 antigens as protective immunogens.

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