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A Prominent Antigenic Surface Polypeptide Involved in the Biogenesis and Function of the Vaccinia Virus Envelope[1]

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Polypeptides of the vaccinia virus envelope exposed on the surface were identified by means of sulfo-*N*-hydroxysuccinimidobiotin as a surface tag. Among surface expressed polypeptides is the 35-kDa antigen, previously designated Ag35. Both monoclonal (mAb) and monospecific affinity pure antibodies directed against Ag35 neutralized vaccinia infectiousness, indicating that this prominent surface antigen has a function during early virus–host cell interactions. The binding of several monoclonal antibodies to various regions of Ag35 was tested by reacting CNBr fragments, derived from the polypeptide, employing Western blotting. All mAbs tested reacted with the same region of Ag35. Estimation of the molecular weights (MW), based on migration of the CNBr peptides in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, revealed that those partial digestion products which contained a proline-rich 99 amino acid limit digest fragment were present at a position approximately 12.5 kDa larger than that predicted from the DNA sequence. By contrast, partial and limit digest products lacking the proline-rich fragment migrated to the MW position expected from the length of the DNA sequence. This observation demonstrates that departure from a predicted 22.3 kDa to an anomalous MW of Ag35 is conferred by the proline-rich peptide. The surface location of Ag35 was confirmed by immune electron microscopy. In a competition test the binding specificity of mAb and affinity-purified antibodies at the surface of virions could be demonstrated. Evidence for an association of Ag35 with the virus envelope at various stages during biogenesis of vaccinia was obtained by immune electron microscopy of whole mounts and thin sections. Presence of Ag35 as an early component of immature and mature virions, probably residing in the bilayer membrane structure was detected. A distinction can, therefore, be made between Ag35 and several other vaccinia envelope polypeptides which are synthesized as late functions and added during late stages of envelope assembly.

INTRODUCTION

The orthopoxviruses as exemplified by vaccinia virus have been subjected to intensive study because they possess great structural and functional complexity, follow a unique scheme of replication, and are versatile as recombinant vectors for transfer of foreign genes into animal cells (Dales and Pogo, 1981; Fenner *et al.*, 1989; Panicali *et al.*, 1983; Smith *et al.*, 1983). Orthopoxvirus replication is confined to the cytoplasm of host cells mandating the encoding by the virus of a large spectrum of enzymatic activities to initiate uncoating and transcriptional events (Reviewed in Dales and Pogo, 1981; Fenner *et al.*, 1989). The morphogenesis of vaccinia can be subdivided into a temporally regulated series of stages. One particular area of interest in this laboratory has been the biogenesis of the vaccinia viral envelope which occurs through a unique *de novo* mechanism within the viroplasmic matrix also termed "factory." We define as *the envelope* the membraneous lipoprotein tegument which encloses the virion (Dales and Pogo, 1981). Unlike the situation with other membrane-enclosed eukaryotic viruses, formation of the vaccinia envelope is not connected with preexisting cellular membranes (Dales and Mosbach, 1968). The first step in envelope assembly evident by electron microscopy is the appearance of curved segments consisting of a unit membrane configuration backed by an external layer of spicules, which provide rigidity and confer spherical shape to the immature form of the virion (Dales and Mosbach, 1968). The early stages of envelope formation can occur normally in the absence of viral DNA synthesis, which may be blocked by inhibitors such as hydroxyurea (HU) (Pogo and Dales, 1971). During normal morphogenesis, when DNA synthesis is allowed to occur, nucleoprotein complexes are packaged within the envelopes and then become differentiated into characteristic biconcave cores and associated lateral bodies. At a late stage during maturation the virus surface is modified by exchange of the spicule layer for a coating of surface tubular elements (STE) (Stern and Dales, 1976a; Essani *et al.*, 1982).

Although our understanding to date about the formation of the vaccinia envelope is rudimentary, it is known that the mixtures of phospholipids and glycolipids which become incorporated are different in compo-

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sition than those present in uninfected cells but correspond closely to the changed composition which is significantly altered after infection (Stern and Dales, 1974; Anderson and Dales, 1978). Concerning envelope polypeptides, the spicules consist of a 65-kDa polypeptide which accumulates in large quantities during early stages of infection (Sarov and Joklik, 1973; Essani *et al.*, 1982). Little information is available, however, about the other proteins of the formative envelope.

Much attention is currently focused on the potential use of vaccinia virus recombinants as effective vaccines against a wide variety of pathogens (reviewed by Mackett and Smith, 1986; Moss and Flexner, 1987; Piccini and Paoletti, 1988). The foreign polypeptides expressed by such recombinants are compartmentalized within the cell according to signal sequences encoded by the gene inserted but do not become part of the virion. Analysis of the vaccinia envelope polypeptides may identify structural motifs within these polypeptides having a role in envelope assembly. Furthermore, thorough understanding of the antigenic components of the viral envelope will have a bearing on future developments in creating safe and efficacious vaccines capable of modulating the host's immune response, so as to circumvent a major limiting factor in revaccination of individuals with such vaccinia recombinants.

One prominent envelope component of mature vaccinia which becomes antigenically significant during infection in animals is a 35-kDa polypeptide designated Ag35. This polypeptide can be readily solubilized with the nonionic detergent NP-40 (Wilton *et al.*, 1986). Ag35 was identified as an early virus function because it is synthesized when DNA replication was stopped by HU (Gordon *et al.*, 1988). The gene encoding Ag35 has been mapped within the 8.7-kbp *Hin*dIII fragment H (Gordon *et al.*, 1988) and localized to the open reading frame (ORF) designated H6R according to the convention of Rosel *et al.* (1986). The relevant ORF encodes a polypeptide with a predicted molecular weight (MW) of only 22.3 kDa. The product of the gene when expressed in *Escherichia coli* comigrates with the authentic polypeptide at 35 kDa implying that the observed anomalous electrophoretic migration in a conventional gel system could result from some property of the polypeptide itself and is not due to any post-translational event.

MATERIALS AND METHODS

Cells and viruses

HeLa and Mouse L2 cells were maintained in nutrient media (NM) consisting of Eagle's modified MEM supplemented with 10% fetal bovine serum. Vaccinia virus strain IHD-W and the temperature-sensitive mutant 1085 (Dales *et al.*, 1978) were propagated and titrated by plaque assay using L cells at 37 and 32°, respectively. Virus of high purity was obtained from infected L cells by centrifugation through potassium tartrate gradients, as previously described (Stern and Dales, 1974).

Protein gels and electroblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970). For immunoblotting, transfer of polypeptides to nitrocellulose filters was performed as described by Towbin *et al.* (1979). Antigen was revealed by means of antibodies which were marked using ^{125}I-conjugated protein A (ICN) as described by Batteiger *et al.* (1982) and modified by Wilton *et al.* (1986).

Labeling of vaccinia surface polypeptides

Polypeptides on the surface of vaccinia particles were identified by reacting them with the sulfo-*N*-hydroxysuccinimide ester of biotin (sulfo-NHS-biotin; Pierce), using a modification of the procedure previously described for *Dictyostelium discoideum* plasma membranes (Ingalls *et al.*, 1986; Goodloe-Holland and Luna, 1987). Purified virions were suspended in 10 m*M* NaHEPES, pH 8.2, at a concentration of 250 μg of protein/ml and reacted for 30 min on ice with the sulfo-NHS-biotin at a concentration of 12.5 mg/ml. To terminate the reaction, lysine was added at a final concentration of 0.1 *M*. The biotinylated virus polypeptides were separated by SDS–PAGE and transferred to a nitrocellulose filter as above. The material was exposed to 5% FBS in 10 m*M* Tris–HCl, pH 7.3, 115 m*M* NaCl, 0.05% Tween-20 (TBS-Tw) for 1 hr as a blocking step, washed three times with TBS-Tw, then reacted with a 1/3000 dilution of alkaline phosphatase–streptavidin complex (Amersham) for 1 hr. After 3 washes with TBS-Tw, the biotinylated proteins were visualized using an alkaline phosphatase color development system, as described by the manufacturer (Biorad).

Affinity purification of antiserum

Antibody specific to Ag35 was separated from the polyclonal antiserum, raised in rabbits against viral envelope components released by NP-40 (Gordon *et al.*, 1988), using affinity purification (Ball and Kovala, 1988). Briefly, an NP-40 extract from purified virus was prepared as described (Wilton *et al.*, 1986) and separated by preparative SDS–PAGE using a full-width comb. Following electrophoresis the proteins were transferred to nitrocellulose and the band correspond-

ing to Ag35 was localized by staining with amido black and excised from the nitrocellulose sheet. The excised strip was cut into small pieces and transferred to the barrel of a 5-ml syringe. All subsequent steps were carried out by drawing 1 ml of the appropriate buffer into the syringe through an 18-g needle and mixing gently. The nitrocellulose was blocked by treating with phosphate-buffered saline + 0.05% Tween 20 (PBS-Tw) containing 3% casein for 4 hr at 24°. Nitrocellulose pieces were washed extensively with PBS-Tw (six to eight washes) then reacted with the antiserum overnight at 4°. Once again the pieces were washed extensively with PBS-Tw. The bound antibodies were eluted for 1 min with 0.1 *M* glycine, pH 2.4, 0.15 *M* NaCl, the eluate was immediately neutralized with 1 *M* Tris-HCl, pH 8.2, then dialyzed against PBS. The concentration of immunoglobulin protein in the final sample was determined by the method of Lowry *et al.* (1951).

Selection of hybridomas producing monoclonal antibodies

Proteins present in the envelope of vaccinia were solubilized with NP-40 and 2-mercaptoethanol, as described previously (Stern and Dales, 1976) and used for immunizing 8-week-old Balb/c mice. For initial immunizations the antigen was obtained from 300 μg viral protein and was administered intraperitoneally in complete Freund's adjuvant. Two booster immunizations were administered at 2-week intervals. Suspensions of splenocytes prepared 3 days after the last injection were fused with cells of the SP2/0 myeloma (10:1) in the presence of 40% polyethylene glycol (PEG) 1000 in Iscoves modified D-MEM (IMDM) containing 5% DMSO.

Hybridomas were selected by the procedure of Koehler and Milstein (1975). Selection was carried out in HAT medium, culture fluids in which hybridoma colonies developed were screened for antibodies to vaccinia virus polypeptides by means of ELISA, immunofluorescence, and Western blotting.

Established, antibody-producing, twice cloned hybridomas were grown intraperitoneally in syngeneic mice and ascites fluid was obtained. The Ig fraction was purified from the ascites fluid by HPLC using a DEAE 5PW (Beckman) column employing in sequence as binding buffer 20 m*M* Tris HCl (pH 8.5) and 20 m*M* Tris–HCl (pH 7.0) + 0.5 *M* Na acetate as the eluting buffer.

Neutralization assays

Neutralization of virus infectiousness was assayed by a plaque reduction test. Samples of the antiserum or monoclonal antibody (mAb) being tested were diluted in PBS, then mixed with a standard number of plaque forming units (pfu) of vaccinia virus in a final volume of 36 μl. Following incubation for 30 min at 37° to bind the antibody, the mixture was serially diluted with NM and applied to indicator monolayers of L cells for plaque assay.

Cyanogen bromide (CNBr) cleavage of polypeptide Ag35

The viral protein released by the method of Stern and Dales (1976) with NP-40 and 2-mercaptoethanol (2-ME) from 2 mg of purified vaccinia virus protein were separated on a 11% SDS–PAGE and identified by limited staining with Coomassie blue. The Ag35 band was excised, destained, and cut into pieces 1 cm in length. Samples were reacted on a rotary mixer at room temperature for 30 min in sequential steps with 10 ml of the following solutions: H_2O, 0.1 *N* HCl, 0.1 *N* HCl containing 0, 10, or 30 mg CNBr, two washes with H_2O, 0.25 *M* Tris–HCl, pH 6.8. They were then equilibrated with dissociation buffer for use with SDS–PAGE.

The reacted gel pieces and MW markers were electrophoresed in duplicate through 15% SDS–PAGE. One gel was stained with Coomassie blue and the other used for Western blot analysis.

Immunogold labeling of virions

Antigens were identified on virions and formative virus intermediates by immunogold labeling. All reactions were carried out by floating formvar and carbon coated grids on droplets of the appropriate reagent diluted in PBS containing 0.5% bovine serum albumin (PBS–BSA). After attachment of virus material to the films, the grids were placed for 30 min in PBS–BSA to block nonspecific binding of antibodies. The primary antibody was diluted as desired and allowed to react for 30 min and then was removed by three washes of 2 min each with PBS–BSA. The secondary antibodies, were goat anti-rabbit (Au–anti-rabbit) or goat anti-mouse (Au–anti-mouse) conjugated with 10 nm colloidal gold particles (Sigma). They were diluted 1:20 and applied for a further 30 min. The grids were washed as above with PBS–BSA and then 3 times with 10 m*M* HEPES, pH 7.3. In some cases the material was negatively stained using 1% phosphotungstic acid (PTA).

Immunogold reactions on cell lysates

Cytoplasmic lysates from vaccinia virus infected cells were prepared for immunogold EM by a modification of the procedure used by Stern and Dales (1974). Infected monolayers were scraped, the cells pelleted at 700 *g* and resuspended in MEM diluted 1:8 with water to induce swelling. The cells were lysed by re-

peatedly (10 times) forcing them through a No. 25 needle of a syringe. Remaining intact cells and large cell debris were removed by centrifugation at 200 *g* for 20 min. The supernatant suspension was adjusted to 0.15 *M* NaCl to stabilize osmotically sensitive structures. Dilutions of the supernatant fraction were applied to EM grids, the material was then fixed for 2 min with 0.1% glutaraldehyde in PBS, washed in PBS, and reacted with antibodies in the manner described above.

Intracellular immunogold labeling

Intracellular virus components containing Ag35 were localized by immunolabeling as described by Dales *et al.* (1983). Monolayers of HeLa cells grown in organ culture dishes (Falcon 3037) pretreated with poly-L-lysine, to enhance cell attachment, were infected with vaccinia virus at an m.o.i. of 10 PFU/cell. Cells undergoing infection were washed in 5 m*M* Na-PIPES, pH 7, 2 m*M* $MgCl_2$, 2 m*M* EGTA, 0.115 *M* NaCl (PiBS), fixed for 10 min in freshly prepared 1% paraformaldehyde in PiBS, permeated by exposure to three changes of 0.01% saponin in PiBS (PiBS-S) for 3 min. The PiBS-S was removed by washing twice with PiBS. The primary antibodies, either mAb $7C_{11}$ or the affinity purified rabbit anti-Ag35 Ig, each diluted 1 in 10 in PiBS, were applied at room temperature for 1 hr. After removal of unadsorbed antibodies by washing three times with PiBS of the appropriate secondary antibody-gold conjugates, diluted 1 in 10, were added for 1 hr. After five washes with PiBS, the cell material was fixed additionally with 2% glutaraldehyde and 1% osmium tetroxide and then was dehydrated through graded ethanol series and embedded in epoxy resin mixtures. Thin sections were prepared and stained by conventional methods.

RESULTS

External polypeptides of vaccinia virus

Polypeptides exposed on the surface of vaccinia virus were identified by biotinylation of intact virions, using the sulfo derivative of *N*-hydroxysuccinimidobiotin (sulfo-NHS-biotin). This agent effects the transfer of biotin to primary amines, most notably the ϵ group of lysine residues, within polypeptides. The charged sulfate group on this compound prevents penetration of the compound into hydrophobic portion of membranes, thereby limiting reactivity to externally exposed regions of envelope polypeptides. The biotinylated virus was subjected to SDS–PAGE followed by electrophoretic transfer to nitrocellulose. The biotinylated polypeptides were visualized by reaction with a streptavidin–alkaline phosphatase (AP) complex and the use of an AP color development system. This procedure identified six prominent polypeptides with molecular weights of 58, 39, 35, 31, 24, 23, and 22 kDa (Fig 1A, lane 2) as derived by comparison with biotinylated markers. One band present just above the 58-kDa polypeptide and evident in all lanes is probably an artifact of the gel system and not an authentic viral polypeptide because it traversed the entire gel. When biotinylated virus polypeptides were separated on a 15% polyacrylamide gel, an additional surface polypeptide of 14 kDa was evident (data not shown); this component corresponds to the 14-kDa external polypeptide described by Rodriguez *et al.* (1987).

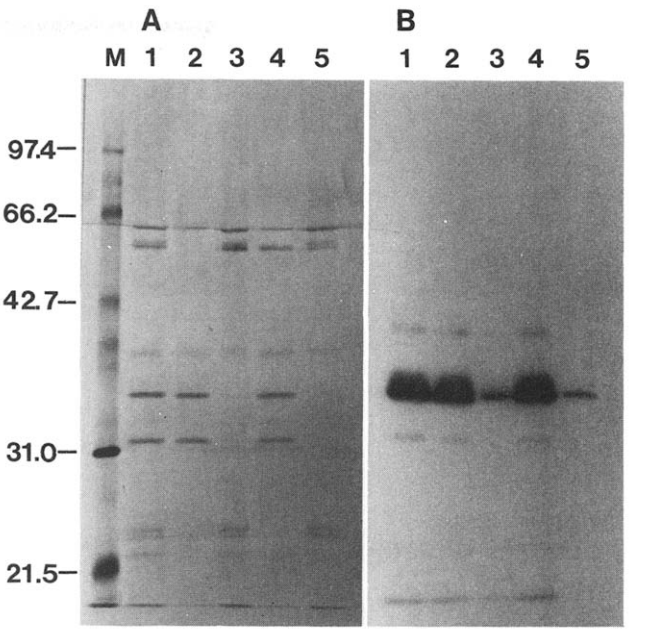

FIG. 1. Distribution of vaccinia polypeptides following surface biotinylation. Purified IHD-W vaccinia, at a protein concentration of 250 μg/ml, was reacted for 30 min on ice with sulfo-NHS biotin at a concentration of 12.5 mg/ml in 10 m*M* HEPES, pH 8.2. Following biotinylation, the virus was extracted with 1% NP-40 or 1% NP-40 plus 125 m*M* 2-ME. Samples of whole virions, the soluble extracts and the insoluble material were subjected to separation by SDS–PAGE, then transferred to nitrocellulose membranes which were reacted for identification of biotinylated polypeptides (A), or processed for immunoblotting using rabbit antiserum directed against the NP-40-soluble envelope components (B). (Lane M) biotinylated MW standards (from Biorad); (lane 1) purified whole virus; (lane 2) envelope fraction released with NP-40; (lane 3) NP-40-insoluble material; (lane 4) envelopes solubilized with NP-40 and 2-ME; (lane 5) core material insoluble in NP-40 and 2-ME.

Identification by Western blotting of vaccinia virus antigens associated with the envelope has been described previously (Wilton *et al.*, 1986); two groups of antigens were identified, those released by the neutral detergent NP-40 alone and those solubilized along

with the entire envelope by NP-40 plus 2-ME as described in Pogo and Dales (1968). The biotinylated virus surface polypeptides were separated in the same manner. Extraction with 1% NP-40 released the bulk of the 35- and 31-kDa polypeptides into the detergent phase (Fig. 1A, lane 2) leaving behind the 58- and 22-kDa biotinylated polypeptides with the NP-40 insoluble pellet (Fig. 1A, lane 3). The 39-kDa antigen was about equally abundant in the NP-40 soluble and insoluble fractions. After extraction of virions with NP-40 and 125 m*M* 2-ME, the envelopes disappeared leaving behind cores with attached lateral bodies (See Fig. 6D). In this preparation the 58-, 35-, 31-, and 22-kDa polypeptides, appeared in the soluble fraction (Fig. 1A, lane 4). The 31-kDa polypeptide released by NP-40 alone should not be confused with a similarly migrating 32-kDa polypeptide described previously (Wilton *et al.*, 1986). The latter was not synthesized by the IHD-W strain of vaccinia used in the current study but was present when the IHD-J strain was reacted with NHS–biotin (data not shown), indicating that the 32-kDa polypeptide is also a surface exposed component. One should note the faint signals of 60-, 24-, and 23-kDa biotinylated polypeptides which remained with the viral cores (Fig. 1A, lane 5). Slight biotinylation of these highly abundant core polypeptides (Dales and Pogo, 1981) could have been due to presence of a minor fraction of damaged virions in the purified suspensions.

The location of the prominent antigen of the envelope, Ag35 was compared on duplicate samples by means of Western blotting with that of other surface-labeled polypeptides. The antigens were identified by polyvalent rabbit antibodies raised against components of the viral envelope released by NP-40 (Gordon *et al.*, 1988). Distribution of Ag35 between detergent soluble and insoluble fractions, detected by immunoblotting (Fig. 1B) was identical to that revealed by the biotinylated polypeptides. More efficient solubilization of Ag35 achieved here, as compared to that reported by us previously (Wilton *et al.*, 1986) was probably due to carrying out the extraction at a pH 8.3 rather than 7.3 and use as starting material of a lower concentration (250 μg/ml not 1 mg/ml) of virus protein. Among minor components observed by Western blotting employing these polyvalent antibodies was an antigen migrating faster than Ag35 (Fig. 1B), which comigrated with a biotinylated polypeptide (Fig. 1A) and a second antigen, above Ag35 which did not have a biotinylated counterpart.

Characterization of Ag35 by monospecific antibodies

From data on the location of the biotinylated 35-kDa polypeptide and its ready solubilization by NP-40 it became evident that this surface component corresponds to Ag35, detected previously by mouse hyperimmune sera (Wilton *et al.*, 1986) and rabbit serum against all vaccinia antigens solubilized by NP-40 (Gordon *et al.*, 1988). To define the antigen further affinity purified rabbit antibodies were prepared, as described under Materials and Methods. Specificity of these antibodies is evident in immunoblotting analysis, illustrated in Fig. 2, where only a single prominent band at 35-kDa is evident. To obtain an approximate estimate on the amount of monospecific antibodies after affinity purification, in relation to the starting material, serial dilutions of the original rabbit antiserum were reacted with standard amounts of vaccinia antigen and compared with the affinity-purified antibodies (Fig. 2). It should be noted that a very weak additional band at a higher MW position is also evident in the lane corresponding to reaction with purified and nonpurified antibodies. The minor antigenic component may result from retardation in the electrophoretic field due to an unreduced dimer or a contaminant associated with Ag35 because following excision from the gel and reelectrophoresis, this antigen migrated to the 35-kDa position (data not shown). As described below, similar observations were made with MAb against Ag35.

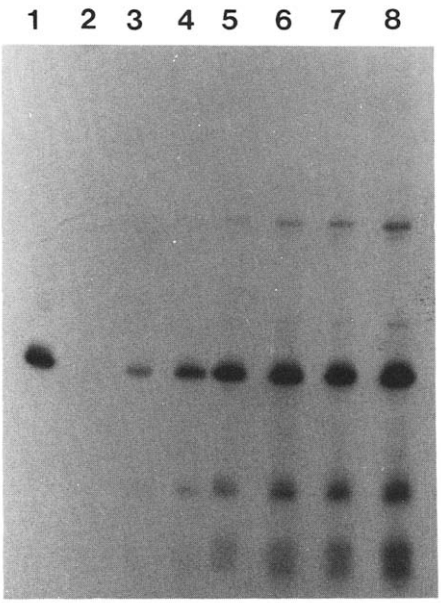

FIG. 2. Specificity of affinity-purified rabbit antibodies against Ag35 tested by Western blotting. Each strip contained polypeptides from 5 μg of purified virus and the reactions were carried out in final volumes of 2 ml. 20 μl of affinity-purified antibodies (lane 1) was compared with (in lane 2) 0.1 μl; (3) 0.5 μl; (4) 1 μl; (5) 5 μl; (6) 10 μl; (7) 50 μl; and (8) 100 μl of the rabbit antiserum against NP-40-soluble envelope components.

To ascertain whether Ag35 plays a role in infectivity during early cell–virus interactions, the affinity-purified

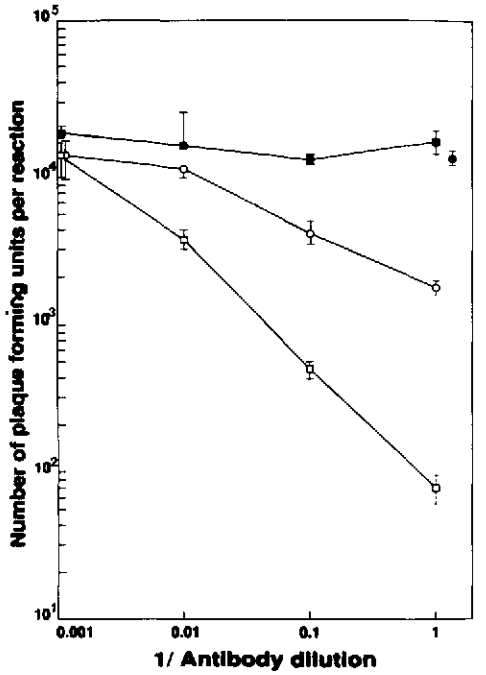

FIG. 3. Reduction of vaccinia infectiousness by anti-envelope polyspecific and anti-Ag35 monospecific rabbit antibodies. Either PBS alone or varying dilutions of antisera in PBS were incubated in a 40 μl reaction volume at 37° for 1 hr with a standard number of PFUs of vaccinia virus. Then the virus-antibody complexes were diluted and assayed on L cell monolayers. Open squares, antiserum to NP-40 extract; closed squares, preimmune rabbit serum; open circles, affinity-purified monospecific antibodies; closed circle, control in PBS.

monospecific antibodies were tested in a neutralization assay. For comparison we also used the serum directed against the NP-40 extracted virion components. As evident from plaque reduction data seen in Fig. 3, at an optimum dilution the polyvalent serum decreased vaccinia virus infectivity approximately 400-fold, and the affinity-purified serum by about 10-fold. Preimmune control rabbit serum had no significant effect on infectivity. As described above in Fig. 2, the level of reactivity specific for Ag35 was also evaluated by Western blotting. Assessment of the comparative antibody reactivity is based on affinity for SDS-denatured Ag35 antigen. From these data we estimated that the reactivity of 20 μl of the affinity-purified antibodies corresponded approximately to that obtained with 5 μl of the polyvalent antiserum, suggesting that reactivity of purified anti-Ag35 antibodies could be several-fold higher than actually observed by the neutralization assay. This still made them less efficient than the polyvalent serum. Loss of potency may occur because the affinity purification procedure relied on immobilization of antigen on Western blots. Therefore, the antibodies released after binding must have been primarily against linear epitopes, rather than some additional, conformational epitopes, possessing perhaps greater significance for neutralization of infectiousness. A second and more important consideration is the presence in the serum, directed against the entire NP-40 extract, of antibodies to a spectrum of antigens interacting with the host cell surface, including the important 14-kDa polypeptide described by Rodriguez *et al.* (1985).

Specificity of monoclonal antibodies against Ag35

To further define antigenicity of the 35-kDa polypeptide we selected a panel of mAbs, following immunization of mice with vaccinia virus components extracted with NP-40 and 2-ME, as described under Materials and Methods.

Among 158 colonies which grew into cultures 99 proved to be positive against vaccinia antigens by ELISA and/or immunofluorescence testing. Among these, 32 gave a signal by Western blotting, of which 18/32 had affinity for Ag35 to a variable degree. mAbs which gave the strongest signals with Ag35 were chosen for further studies.

Although in the NP-40/2-ME extract of virus three prominent protein bands are usually present (see Fig. 1B), most of the antivaccinia mAbs selected were against Ag35. This finding can be attributed to either an abundance of Ag35 and/or to its effectiveness as an antigen. Table 1 summarizes the characterization of some selected and purified monoclonal antibodies reactive against Ag35. It should be noted that only one mAb ($1F_7$) of all those tested was found to be of the IgM,k isotype. The degree of immunofluorescence

TABLE 1

PROPERTIES OF MONOCLONAL ANTIBODIES DIRECTED AGAINST Ag35

mAb	Isotype[a]	Intensity of immunofluorescence[b]	% PFUs neutralized[c] antibody dilutions		
			1	1/10	1/100
$7C_{11}$	IgG_1, k	++++	70	62	53
$4D_6$	IgG_1, k	++	0	0	0
$1F_7$	IgM, k	++++	NA[d]	NA	NA
$6C_2$	IgG_1, k	++++	NA	NA	NA

[a] Isotyping was carried out using INNO LIA mouse mAb isotyping kit (Innogenetics), as described by the manufacturer.

[b] Immunofluoresence was assayed on acetone permeabilized L cells using rhodamine-conjugated goat anti-mouse immunoglobulin as the second antibody.

[c] Tissue culture media from growing hybridomas were assayed by plaque reduction on L cell monolayers, as described under Materials and Methods.

[d] NA, not assayed.

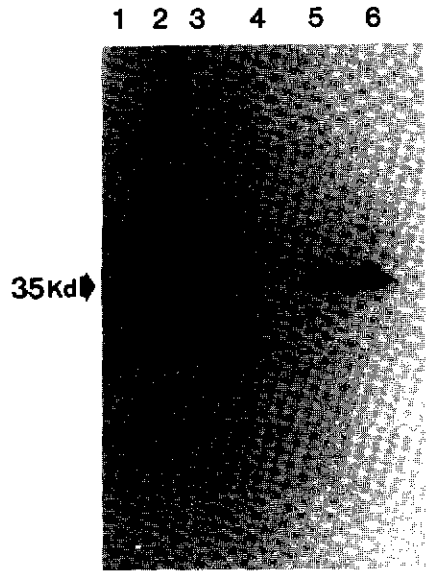

FIG. 4. Specificity of monoclonal antibodies for Ag35 demonstrated by immunoblotting. The reactions were conducted as described under Materials and Methods. (Lanes 1 and 4) extract from uninfected L2 cells; (lanes 2 and 5) extracts from vaccinia infected cells; (lanes 3 and 6) pure whole virus; lanes 1–3 reacted with mAb $7C_{11}$; lanes 4–6 reacted with mAb $4D_6$.

was variable and only one of mAbs tested showed any appreciable capacity for neutralization of infectivity.

The specificity of the mAbs giving the strongest reactions by immunofluorescence and Western blotting was tested further following purification of ascites fluid by HPLC. As evident from Fig. 4, $4D_6$ and $7C_{11}$ when applied to material from infected cells (lanes 2, 5) and pure virus (lanes 3, 6) gave a strong signal specifically with Ag35, whereas uninfected cell lysates were negative (lanes 1, 4).

In view of the frequency with which mAbs against Ag35 were occurring in our selection we wished to ascertain whether some particular domain(s) on this antigen was immunogenic preferentially. The 35-kDa polypeptide could be broken selectively into a few fragments by CNBr because in the amino acid sequence only three internal methionines are available for cleavage. The fragments derived from CNBr digestion of purified Ag35, as described in Materials and Methods, were resolved by PAGE separation. A graphic presentation showing the location of N-terminal and internal methionines, as predicted from sequence of Ag35 (Gordon *et al.*, 1988) and the possible peptides which can be derived therefrom, is provided in Fig. 5B. This should be compared with the actual peptides resolved by PAGE and stained by Coomassie blue (Fig. 5A) and binding mAbs as revealed by immunoblotting (Fig. 5C). Due to the anomalous migration of Ag35 which, based on the sequence data, should be 22.3 kDa rather than 35 kDa (Gordon *et al.*, 1988) it is not possible to predict the exact MWs for individual peptides obtained by CNBr cleavage. However, it is possible to relate the diminution in MW as a consequence of the removal of one or more fragments. It is evident from data in Table 1 that for CNBr fragments CB6, CB7, and CB8 the MW obtained from the gel (Fig. 5A), matches closely that predicted from the sequence. For the intact Ag35 and CB 1-5 fragments the apparent MWs in Fig. 5A and Table 2 are about 12–13 kDa greater than predicted from the sequence. Therefore, the anomalous migration in PAGE must be due to a sequence motif residing in the limit digest peptide CB5, as this represents the only fragment common to all peptides showing the anomalous migration. A prominent 20-kDa band in Fig. 5A could be a cleavage or degradation product of one of the larger fragments.

Affinity of mAb $7C_{11}$ for CNBr peptides is revealed in Fig. 5C from which it is evident that this antibody identifies an epitope on fragment CB5. Three other mAbs, $4D_6$, $1F_7$, and $6F_9$ among those tested showed the same binding pattern as $7C_{11}$ (data not shown), indicating that CB5 contains a highly immunogenic sequence of Ag35.

Similar Western blotting utilizing the monospecific rabbit antibodies described above and hyperimmune antisera from mice immunized with whole vaccinia virus (Wilton *et al.*, 1986), gave identical or very similar affinity-labeling patterns against the CB fragments (data not shown). This result confirms the importance of the antigenic domain(s) in CB5. Analysis by computer program for probable antigenic determinant(s) revealed, in Fig. 5D that there is a high correlation between predicted regions and the amino acid sequence in fragment CB5.

Localization of Ag35 on vaccinia virions by immune electron microscopy

The presence of the Ag35 in the envelope as determined by its release with NP-40, as well as the presence of a prominent surface reactive biotinylated polypeptide at 35 kDa suggests that at least a part of Ag35 is exposed. To confirm this assumption and ascertain whether Ag35 is distributed randomly or at specific loci on the virus surface we reacted highly purified intact virions with antibodies and marked these antibodies with immunoglobulin–colloidal gold conjugates. Extensive labeling over the entire surface of virions was obtained with mAb $7C_{11}$ (Fig. 6A). The apparent increase in concentration of gold particles near the perimeter of virions which was often evident, was most likely due to the nature of the EM image which be-

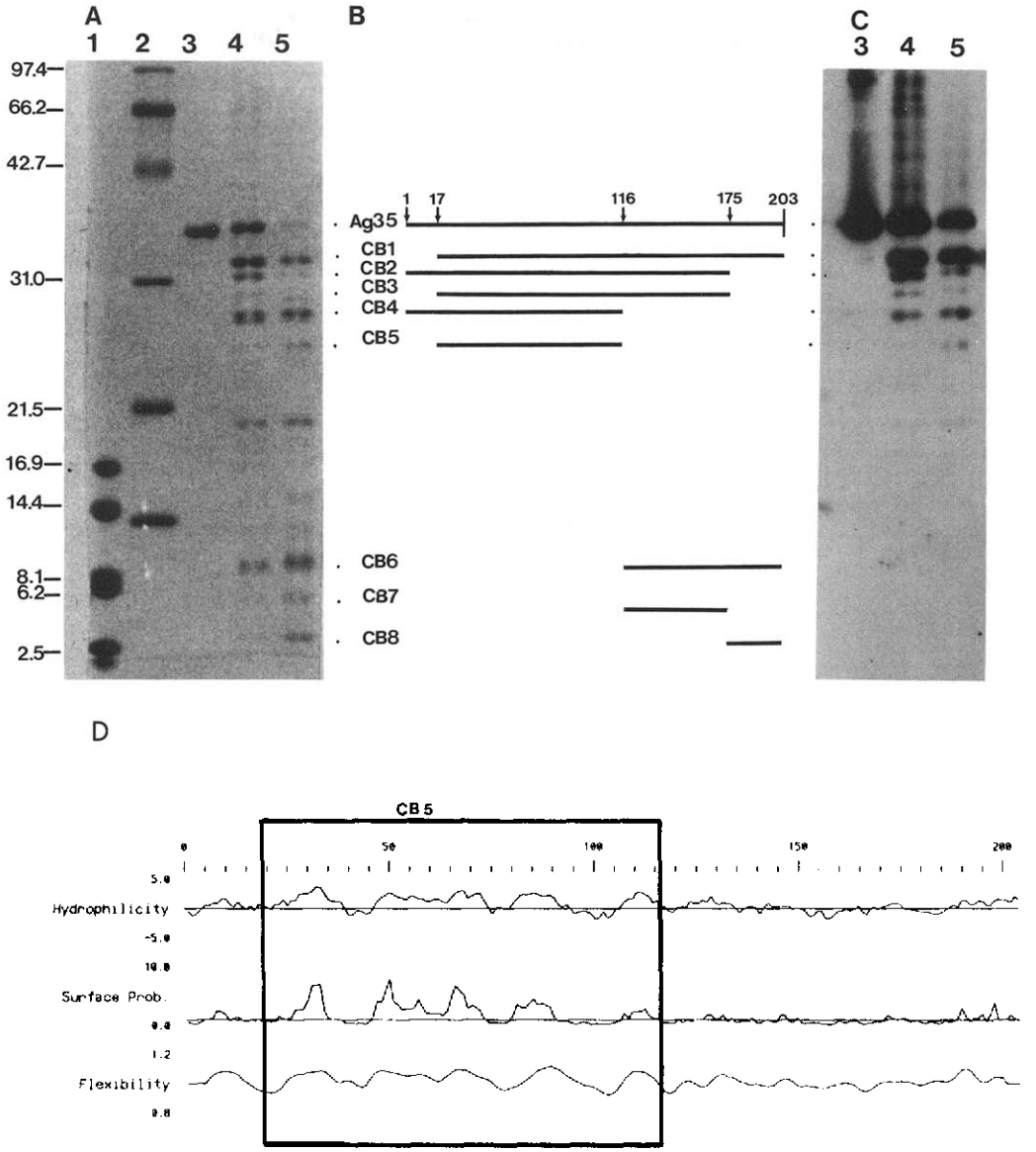

FIG. 5. Epitope mapping of Ag35 by CNBr hydrolysis. Envelope material extracted with NP-40 and 2-ME was separated by preparative SDS–PAGE and lightly stained with Coomasie blue. The band corresponding to Ag35 was excised from the gel, destained and either mock-reacted (lane 3) or reacted with 10 mg/ml (lane 4) or 30 mg/ml (lane 5) of CNBr, as described under Materials and Methods. Following CNBr treatment, the peptides were separated by SDS–PAGE on 15% gels. Duplicate samples were either stained with Coomassie blue (A) or subjected to Western blotting, employing mAb $7C_{11}$ (C). (B) diagrams CNBr cleavage sites and the sizes of all fragments which could possibly be derived by partial or complete digestion. MW markers in lane 2 included phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) and in lane 1 CNBr digest of myoglobin (16.9, 14.4, 8.2, 6.2, 2.5 kDa). (D) The sequence of Ag35 was analyzed for the presence of predicted regions of hydrophilicity, surface orientation, and flexibility (Devereux *et al.*, 1984) and the plots of these analyses are illustrated. Note that the boxed region, spanning CB5 fragment, residues 18 to 116, indicates the mapped antigenic determinant and shows a high degree of correlation with predicted regions of all three parameters.

comes a two-dimensional visualization of a three-dimensional structure. As a control of the specificity, we applied an irrelevant mAb, 46 B.2, against the coronavirus nucleocapsid protein (kindly provided by Dr. M. Buchmeier, Scripps Clinic and Research Fdn., La Jolla, CA), which failed to bind to vaccinia virions (Fig. 6B).

Parallel experiments conducted with affinity-purified monospecific antibodies against Ag35, also showed specific binding to the surface of virions in whole mounts (Fig. 6C) and thin sections (Fig. 6E), whereas, rabbit antiserum to tubulin of chicken (a gift of Dr. E. Ball, Department of Biochemistry, University of West-

TABLE 2

COMPARISON OF MOLECULAR WEIGHTS OF PEPTIDES GENERATED BY CNBr CLEAVAGE OF Ag35 ESTIMATED FROM SDS–PAGE AND PREDICTED FROM THE DNA SEQUENCE

CNBr fragment	Observed kDa[a]	Predicted kDa[b]	Discrepancy in kDa[c]	Binding of mAb in Western blot	
				$7C_{11}$	$4D_6$
Ag35	35.0	22.3	12.5	+	+
CB1	32.5	20.4	12.0	+	+
CB2	31.0	19.3	11.5	+	+
CB3	30.0	17.3	12.5	+	+
CB4	26.0	12.9	13.0	+	+
CB5	24.5	10.9	13.5	+	+
CB6	9.5	9.4	0	−	−
CB7	6.5	6.3	0	−	−
CB8	3.5	3.0	0.5	−	−
CB9	n.o.[d]	2.0	—	−	−

[a] Observed MW was determined by comparison with the migration of molecular weight markers coelectrophoresed with the CNBr digest of Ag35.

[b] Predicted MW of the fragment derived from the sequence of Ag35 (Gordon *et al.*, 1988).

[c] Differences between MW of fragments estimated from SDS–PAGE and those predicted from the DNA sequence.

[d] n.o. means that the indicated band was not observed perhaps because the peptide had migrated beyond the limit of the gel.

ern Ontario) used as a control did not label the surface of vaccinia (data not shown).

Following treatment of purified vaccinia virus with NP-40 and 2-ME the viral envelopes are removed leaving behind intact the viral cores and associated lateral bodies (Pogo and Dales, 1968). After exposing stripped virus to mAb $7C_{11}$ the number of gold particles evident was only a small fraction (less than 5%) of those present with intact virions (compare Figs. 6A and 6D). The gold particles in Fig. 6D could have been due to specific binding of antibodies to Ag35 on residual fragments of envelope, as well as to nonspecific labeling.

The specific association of Ag35 with envelopes of mature virions was confirmed by means of immunotagging of infected cells, using permeation to antibodies with saponin. Both the intracytoplasmic virions and those at the surface became coated with antibodies to Ag35, identified by the presence of gold particles (Fig. 6E). However, virions released from cells in wrapping membranes derived from the Golgi of the host cell (Dales and Pogo, 1981) were not labeled (Fig. 6E). Absence of labeling from such shrouded virions indicated a total absence of Ag35 from the surface of the wrapping membrane and also demonstrates the inability of the antibodies to penetrate the enclosing membrane.

Since both the monospecific rabbit and mAb directed against Ag35 were avidly bound at the surface, it was predicted that prior binding of one specific antibody should interfere with binding of the secondarily applied antibody, if the relevant epitopes are identical or closely contiguous. Virions were reacted in a quantitative analysis with the monospecific rabbit antibodies first, then with mAb $7C_{11}$. The relative amount of mAb antibodies at the surface was estimated by counting gold particles on randomly selected virions. Primary application of the rabbit antibodies decreased by a factor of 2 the number of gold particles identifying mAb $7C_{11}$ (Table 3). However, to achieve this degree of competition it required the use of high concentrations of the rabbit antibodies, implying that mAb was present in excess. This was confirmed by competition experiments employing for primary binding a constant amount of the rabbit antibodies followed by a range of dilutions of mAb $7C_{11}$. This procedure resulted in effective competition against $7C_{11}$ when 250 ng/μl of rabbit antibodies were used to compete with 12.5 ng/μl or 25 ng/μl of mAb, as shown by the data in Table 4. Competition between the same constant amount of rabbit antibodies and 50 ng/μl of mAb reduced binding by approximately 50%, a value similar to that obtained when rabbit primary antibodies were 500 ng/μl and mAb at 300 ng/μl (see Table 3). These data indicate that mAb $7C_{11}$ outcompeted the affinity-purified rabbit antibodies for closely positioned sites on the virion surface.

In the absence of either antibody, the background binding of Au–anti-mouse to vaccinia virus was negligible. The background was only slightly higher in the presence of the competing rabbit antibody. This in-

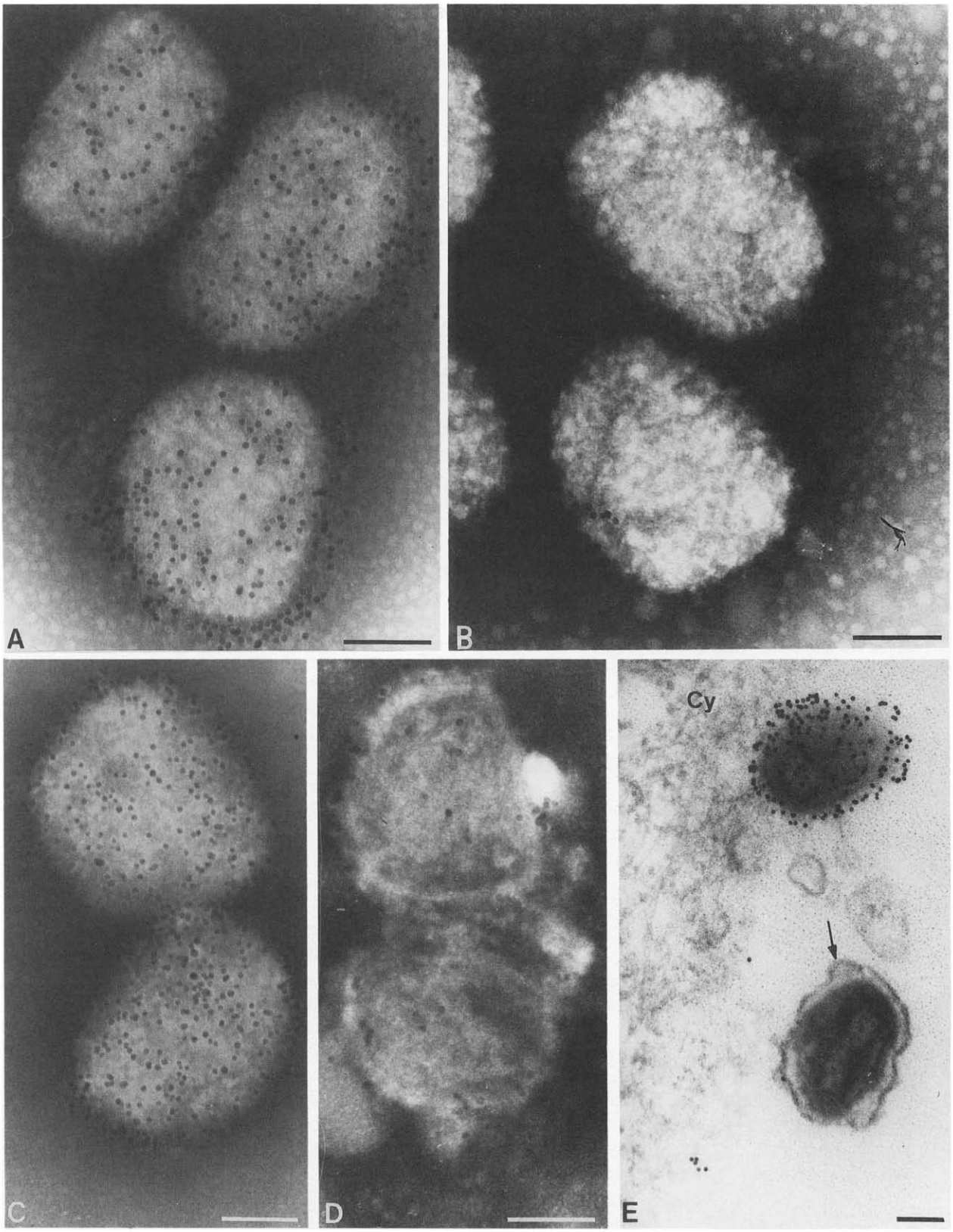

FIG. 6. Surface localization by immunogold electron microscopy of Ag35 on purified vaccinia virions. (A), Three virions reacted with mAb $7C_{11}$ followed by goat anti-mouse immunoglobulin–gold conjugate (Au–anti-mouse). (B) Two virions reacted with mAb against coronavirus nucleocapsid protein and Au–anti-mouse. (C) Virions reacted with affinity purified anti-Ag35 antibodies followed by Au-anti rabbit. (D) Viral cores

creased background could have been due to some minor cross-reactivity between the goat anti-mouse Ig and rabbit antibodies.

Efficient competition for Ag35 binding by antibodies from different sources confirms the stringency of localization of Ag35 to the surface of the virion by the immunogold technique.

Association of Ag35 with vaccinia envelopes during virus development

We took advantage of a procedure to introduce antibodies into infected cells, by permeation of the cell membranes, to determine whether Ag35 is present on envelopes during the formative stages as well as in mature particles. Although permeation of individual cells in cultures by means of saponin was highly variable, examples were found in which tagging of mAb $7C_{11}$ or the monospecific rabbit antibodies by gold–antibody conjugates was adequate to demonstrate in thin sections the specificity of binding to immature forms of vaccinia. In one example of virus-infected cells sampled 12 hr after inoculation, numerous immature virions labeled by gold particles were evident in the cytoplasm (Figs. 7A and 7B). Intermediates in envelope assembly possessing the characteristic rigid, spicule-backed hemispherical conformation were also specifically labeled, as evident in the examples shown in Figs. 7B and 7C. Reactivity with Ag35 was frequently evident at the free ends of segments of envelope, where the spicule-backed membrane structures are terminated, suggesting that specific antibodies became attached to the bilayer structure of envelopes (Fig. 7C). It is quite possible that an external layer of spicules, described in detail previously (Essani, *et al.*, 1982) was a hindrance to the ready access of antibodies to the subjacent bilayer. Very few gold particles were present within the nonmembraneous viroplasmic matrix material. The specificity of antigen–antibody interaction was shown by absence of gold particles when mAb directed against the variable surface glycoprotein of *Trypanosoma bruceii* (A gift of Dr. M. W. Clarke, Department of Microbiology, University of Western Ontario) was used as a control (data not shown).

Since Ag35 is known to be an early polypeptide, which can be synthesized despite an inhibition of DNA replication by HU (Gordon *et al.*, 1988), a drug causing arrest of virus assembly at the enveloped but immature particle stage (Pogo and Dales, 1971), we used this drug to ascertain whether Ag35 occurs in immature virions. Immature virions were released from cells which had either been treated with HU or were untreated during infection. The intact particles were subjected to labeling with gold conjugates in the manner used with isolated mature vaccinia. It was found that anti-Ag35 antibodies became attached to the immature virus particles regardless whether they originated from controls or HU-treated cells (Fig. 7E). This result further demonstrated that Ag35 is, indeed, a component of the envelope.

Association of Ag35 with the membrane bilayer of the envelope was also investigated by means of the temperature-sensitive mutant ts1085 of vaccinia. At the restrictive temperature envelopes of this mutant are assembled as flexible bilayers lacking the attached external spicule layer. Such membrane sheets surround the viroplasmic assembly sites within the cytoplasm (Dales *et al.*, 1978). In order to determine whether Ag35 could be closely associated with these free membranes, *in situ*, tracing by immunogold was carried out on permeabilized cells which had been infected with ts1085 at 39° for 24 hr. The results, exemplified by the illustration in Fig. 7D, demonstrated that the rabbit anti-Ag35 monospecific antibodies became bound predominantly to membraneous structures surrounding foci of viroplasm. This observation supports the conclusion that Ag35 is an intimate component of the membrane bilayer of the envelope.

DISCUSSION

New data derived from the present study further characterize the prominent vaccinia virus antigen Ag35 (Gordon *et al.*, 1988), with respect to its involvement in the structure and formation of this virus. Localization of Ag35 by means of biotinylation of surface polypeptides and by immunoelectron microscopy revealed the unambiguous location to be on the external surface of the envelope.

Surface labeling in this study confirms and extends the work of Sarov and Joklik (1972), who used radioiodination to identify surface exposed vaccinia polypeptides. The use of a NHS–biotin type of probe for surface polypeptides has one major advantage over the use of radioiodination of the target amino acid tyrosine; the NHS–biotin reacts with primary amino groups most notably the ϵ group of lysine, a much more prevalent and widely distributed amino acid in proteins. Sarov and Joklik (1972) observed a strong signal correspond-

produced by treatment with NP-40 and 2-ME reacted with mAb $7C_{11}$ and Au–anti-mouse. (E) Thin section of an infected HeLa cell illustrating two virions near the surface. The cell monolayer was reacted with mAb $7C_{11}$ followed by Au–anti-mouse. Note the high concentration of gold particles on the surface of one vaccinia particle and absence of labeling from the other virion enclosed by a wrapping membrane (arrow). Cy, cytoplasm. *Magnification* (A, B, D) ×151,000, (C) ×131,000, (E) ×78,000. Bar represent 0.1 μm.

TABLE 3

COMPETITION BETWEEN AFFINITY-PURIFIED RABBIT ANTIBODIES AND mAb $7C_{11}$ FOR BINDING TO Ag35 ON VIRIONS

Concentration of competing antibody (ng/μl)	Gold particles per virion[a]
0	226 ± 28 (15)[b]
50	174 ± 21 (13)
125	156 ± 34 (11)
500	99 ± 22 (10)

[a] Vaccinia virions immobilized on EM grids were reacted with various concentrations of the affinity purified anti-Ag35 antibodies followed by the 300 ng/μl of mAb and Au–anti-mouse. All reactions were incubated at 24° for 30 min.

[b] The number of virions examined in the sample are given in parentheses.

ing to low-MW polypeptides designated VP10 migrating at 14–16 kDa, as well as weaker signals which likely correspond to the 58- and 35-kDa biotinylated polypeptides reported in the present study. In our investigation we identified four additional surface exposed polypeptides migrating at 39, 31, 23, and 22 kDa. The 58-kDa surface polypeptide most probably corresponds to the component of the surface tubular elements or STE of Stern and Dales (1976a). Unlike Ag35 which can be solubilized by NP-40 alone, STE are only released when the entire envelope is dismembered by extraction with NP-40 and 2-ME.

Localization of Ag35 to the structure termed viral *envelope* (Dales and Pogo, 1981) was confirmed by immunogold labeling of intact virions using both affinity purified antibodies, derived from a polyclonal antiserum directed against envelope components released by NP-40 and mAbs reacting with Ag35. The specificity of immunogold labeling of the vaccinia envelope itself was confirmed in thin sections revealing the absence of antigen reactivity from the wrapped, extracellular form of the virion. This result demonstrates that antibodies to Ag35 are unable to penetrate the bilayer of the wrapping membrane. Furthermore, the absence of reactivity from virions released by exocytosis after becoming wrapped in a membrane derived from the golgi (Dales, 1973) shows that Ag35 is not a component of the membrane of cellular origin.

Neutralization of vaccinia infectivity by either affinity-purified rabbit monospecific antibodies or a mAb directed against Ag35, suggests that this polypeptide participates intimately in the cell–virus interaction. Not surprisingly, the polyvalent affinity-purified antibodies could diminish infectiousness more efficiently, to approximately 1/10 the original value, than the mAbs since the affinity-purified serum would be likely to react with more than one epitope on the polypeptide. Neutralization observed with the polyclonal anti-envelope serum was much more effective than with the monospecific antibodies indicating that in suppression of infectiousness several polypeptide antigens are probably participants. The relatively low efficiency in plaque reduction tests of monospecific rabbit antibodies against Ag35 is in keeping with neutralization data obtained using antibodies against other vaccinia envelope polypeptides (Rodriguez *et al.*, 1985) and may be the consequence of a very large surface area and antigenic complexity of this virus. In addition, inefficient neutralization by mAb and monospecific antibodies may indicate that several polypeptides play a role at the initial stage of the infection.

As a consequence of the specific immunogold tagging of Ag35 we could examine the involvement of this polypeptide with the developing viral envelope. Ag35 has been identified as an early protein (Gordon *et al.*, 1988), and therefore one synthesized even in the absence of viral DNA synthesis which can be blocked with hydroxyurea. Thus Ag35 is in a different class of vaccinia envelope polypeptides than the 14-kDa fusogenic protein described by Rodriguez and Esteban (1987) and Rodriguez *et al.* (1987), the cell surface-binding 32-kDa polypeptide of Niles and Seto (1988) and Maa *et al.* (1990), and the 58-kDa STE component described by Stern and Dales (1974b, 1976b) all of which are late proteins. This temporal distinction in regulation of Ag35 expression is wholly compatible with the mechanism of assembly of the vaccinia envelope

TABLE 4

COMPETITION FOR BINDING TO Ag35 ON VIRION SURFACE BETWEEN VARIOUS CONCENTRATIONS OF mAb $7C_{11}$ AND MONOSPECIFIC RABBIT ANTIBODIES

Prior addition of rabbit antibody[a]	Concentration of mAb (ng/μl)[b]	Number of gold particles per virion[c]
−	50	230 ± 32
+	50	104 ± 26
−	25	197 ± 37
+	25	62 ± 27
−	12.5	246 ± 28
+	12	24 ± 17
−	0	3 ± 3
+	0	12 ± 5

[a] Affinity-purified rabbit anti-Ag35 antibodies (250 ng/μl) in PBS + 0.5% BSA were reacted for 30 min with the virions immobilized on grids for 30 min prior to the addition of the mAb. The uncompeted controls were handled in the same way except that rabbit antibodies were omitted.

[b] MAb $7C_{11}$ was diluted in PBS + 0.5% BSA and reacted with virus 30 min after incubation with the competing rabbit antibody.

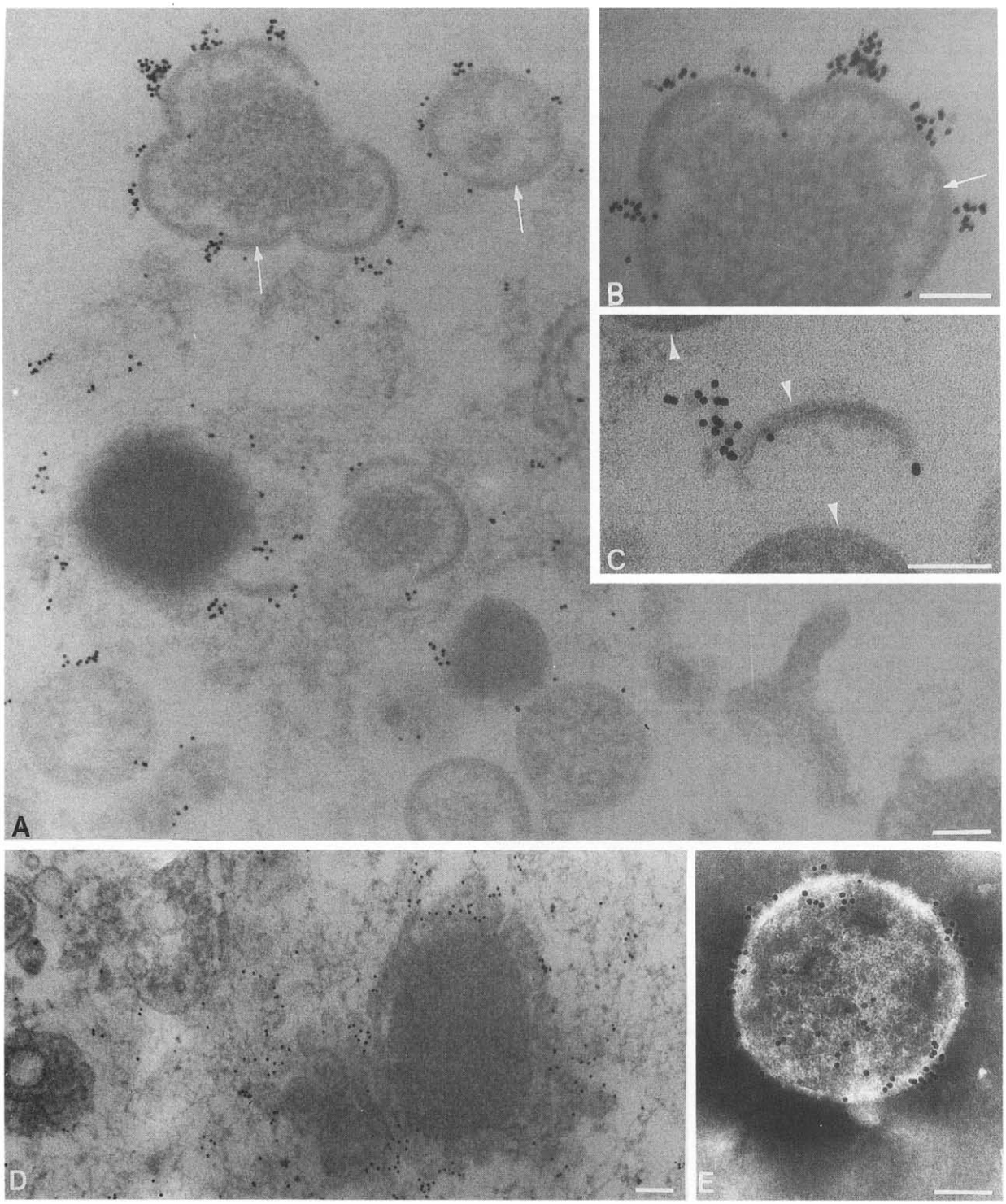

FIG. 7. Intracytoplasmic localization by immune electron microscopy of Ag35 on developing vaccinia particles. Infected HeLa cells were fixed, permeabilized, and reacted with affinity-purified monospecific anti-Ag35 antibodies, followed by Au–anti-rabbit, as described under Materials and Methods. (A) A portion of the cytoplasm also shown in (B) and (C) at a higher magnification illustrates intermediates in virus formation in a cell infected for 12 hr. Note the spherical shapes of the envelopes possessing an external layer of spicules (arrowheads). Note also in (C) that Ag35 is present in a short segment of envelope at the free ends. (D) A section through cytoplasm of a HeLa cell infected for 12 hr at 39° with vaccinia ts1085 and reacted as in (A–C). The gold particles are most abundant at or near the membranes surrounding viroplasm areas. (E) A whole mount of a negatively stained immature virion isolated from cells infected for 18 hr in the presence of 5 m*M* HU. The preparation was reacted on grids with mAb $7C_{11}$ and Au–anti-mouse. Magnifications (A) ×96,000; (B) ×122,000; (C) ×139,000; (D) ×56,000; (E) ×96,000. Bar represents 0.1 μm.

which proceeds through a complex series of steps, commencing prior to the onset of viral DNA synthesis with the formation of segments of membrane bilayers with a backing of spicules. Ag35 was identified in the immature, spherical vaccinia, which can be formed in the presence of HU (Pogo and Dales, 1971), illustrating that Ag35 participates during the earliest phases of envelope assembly.

In the normal assembly process, the nucleoprotein material accumulated in viroplasmic foci undergoes complex differentiation into mature virions within the envelope. An additional late step in the assembly of the vaccinia virion is the replacement of the spicule layer by STE, characteristic of the mature envelope (Dales and Pogo, 1981). Apparently, the late surface polypeptides are incorporated at the time of virus maturation while Ag35 is part of the envelope throughout virus development.

During morphogenesis, in several vaccinia ts mutants, the attachment of the external spicule layer onto the envelope fails to occur (Dales *et al.*, 1978). As a consequence, accumulations of flexible pleomorphic membranes surround viroplasmic foci. Demonstration of an association of Ag35 with such membrane domains and in immature virions reveals that in normal development Ag35 is, indeed, associated with the envelope bilayer and not with the spicules.

The mechanism(s) for integrating vaccinia polypeptides into the envelope bilayer appears to be distinct from those which occur with conventional enveloped viruses. To date none of the recognized vaccinia envelope polypeptides for which DNA sequence data are available, among them the 14-kDa fusion protein (Rodriguez and Esteban, 1987), the 32-kDa component (Niles and Seto, 1988; Maa *et al.*, 1990), and Ag35 (Gordon *et al.*, 1988) possess an amino-terminal hydrophobic signal sequence involved in insertion into or through a bilayer membrane. Although vaccinia virions contain two or more glycoproteins, none are present in the envelope (Holowczak, 1970; Garon and Moss, 1971). The absence of envelope glycoproteins in vaccinia, in contrast with other enveloped viruses, is the consequence of synthesis of envelope proteins in the cytosol rather than on the endoplasmic reticulum. Hence the integration of nascent polypeptides must occur subsequent to their synthesis and not as a cotranslational event.

Details of the integration of Ag35 into the envelope are yet to be clarified. Although the C-terminal 1/3 portion of the polypeptide is hydrophobic (Gordon *et al.*, 1988) it is devoid of a conventional transmembrane segment composed solely of hydrophobic amino acids. Analyses by either the GOR (Garnier *et al.*, 1978; Garnier and Robson, 1989) or Chou–Fassman (Chou and Fassman, 1974, 78) protocols indicate that, in terms of secondary structure, the hydrophobic portion possesses an α-helical configuration. From a model of the helical region (Fig. 8) it becomes clear that there is amphipathicity whereby selectively hydrophobic amino acids occur on one side of the helix, providing sites for interaction with the lipids of the bilayer and polar charged amino acids occur on the opposite side of the helix. Involvement of amphipathic helices in lipid bilayers structures, analogous to the model presented here, has been reviewed by von Heijne (1988), who considers two types of interactions with membranes: (1) as either homo- or heterodimers in which two or more helices simultaneously span the membrane having the hydrophilic portions of the helices oriented inward away from the hydrophobic portion of the lipid or (2) as monomers "floating on the membrane surface having the hydrophobic portion inserted within but not through the membrane". The latter model fits the association of myelin basic protein with myelin membranes (Mendz *et al.*, 1990).

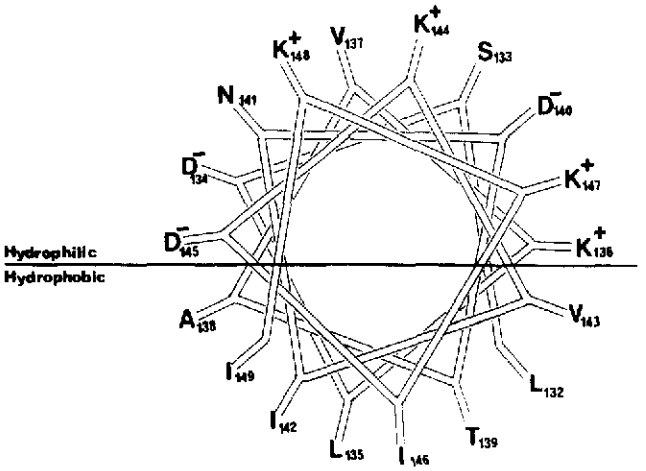

FIG. 8. Helical wheel representation of amphipathic helix in Ag35.

One interesting characteristic of Ag35 is its anomalous migration by SDS–PAGE. Although the polypeptide migrates to a position corresponding to 35 kDa, the open reading frame (ORF) encoding Ag35 is sufficient to encode a polypeptide of only 22.3 kDa. This discrepancy was anticipated by Rosel *et al.* (1986) from the length of the ORF for H6R. The inconsistency cannot be due to post-translational modification of the polypeptide since the ORF when linked to a prokaryotic promoter and expressed in *E. coli* yielded a polypeptide that retained the anomalous migration. When examining the behavior of CNBr cleavage products of Ag35, in SDS–PAGE it became clear that fragments containing the peptide CB5, corresponding to a unit peptide spanning the region between methionines at residues 18

A E I R A H L K N S A
E N K D K N E D I F P
E D V I I P S T K P K
T K R A T T P R K P A
A T K R S T K K E E V
E E E V V I E E Y H Q
T T E K N S P S P G V
S D I V E S V A A V E
L D D S D G D D E P M

FIG. 9. Amino acid sequence of peptide CB5, residues 18–116, as predicted from DNA sequence of Ag35. Prolines are underlined.

and 116 in the predicted amino acid sequence (see Fig. 9), were found at positions 12–13 kDa larger than anticipated from the DNA sequence, while peptides which do not contain this fragment were positioned correctly, according to their predicted MW. Within CB5 is a stretch of 22 amino acids, residues 39–60, in which 5 prolines occur, consistent with the explanation we have suggested (Gordon *et al.*, 1988), as the cause of the anomalous MW of this polypeptide in SDS–PAGE.

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