Structural and Functional Characterization of a Cell Surface Binding Protein of Vaccinia Virus*

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The nature of the interaction between the enveloped DNA-containing poxviruses and the surfaces of host cells as a first step in virus infection is not known. In this investigation we have identified and defined structural and functional properties of a 32-kDa protein of vaccinia virus. This protein is part of the virus envelope and binds to the cell surface of various cultured cells. The gene encoding the 32-kDa viral protein was mapped and sequenced. It was found to code a 35,426-Da protein with a large N-terminal domain with sequence homology to carboxic anhydrase and a C-terminal domain with sequences similar to those of the attachment-glycoprotein VP7 of rotavirus and to transmembrane proteins. A potential cell surface binding domain was within the last 50 amino acid residues of the C terminus. The 32-kDa protein is basic, predicted pl 8.67, is synthesized at late times post-infection, may form dimers held by disulfide bonds at the single cysteine 262, and is apparently non-glycosylated. The 32-kDa protein is a vaccinia virus antigen, with predicted antigenic sites located near amino acids 108–110 (carboxic anhydrase domain) and 298–299 (transmembrane domain). Several lines of evidence suggest that the 32-kDa protein is needed for efficient virus replication in cultured cells but that in addition to this protein other viral proteins are involved in the process of virus entry into cells.

Vaccinia virus has potential as a recombinant vaccine against many diseases of human and veterinary importance (1, 2). However, very little is known about the mode of virus penetration into cells. The genome of vaccinia virus encodes information for some 230 polypeptides of which approximately 100 are incorporated into the mature virion (3, 4). Based on the structure of the virus, the virion proteins can be further divided into two groups, one associated with the outer envelope and the other with the core (5, 6). The biological activities and function of most of these protein are unknown. In particular, it is not known which polypeptides are involved in the early interactions with the host cell which lead to entry of the virus. It is believed that such proteins are localized in the outer envelope. The results of morphological studies suggest that vaccinia may obtain entry by endocytic mechanisms and by fusion of the viral envelope with the host plasma membrane (7–9). The results of biochemical studies suggest that the low pH endocytic pathway described for simpler enveloped viruses is not important for vaccinia virus entry, but fusion of the virus with the plasma membrane plays a major role in virus penetration (10). Based on studies with specific neutralizing antibodies it has been proposed that an abundant 54-kDa surface tubule protein may mediate entry of the virus (11), while a role for a 14-kDa envelope protein on virus penetration at the level of fusion with the cell membrane has been suggested (12, 13). On the other hand, based on protease studies and on specificity of neutralizing antibodies, it has been suggested that vaccinia envelope proteins of 59 and 34 kDa and an aggregate of 17–25-kDa proteins may have an essential role in the virus penetration. Two other envelope proteins of 32 and 29 kDa and a cleavage fragment of 54 kDa may be adsorption proteins (14, 15).

Despite the fact that there is no clear agreement on the specific proteins involved in viral entry, the above studies indicate that they are localized in the outer envelope and that viral binding and subsequent entry can be dissociated as separate events. Because the initial event in the life cycle of a virus is attachment to specific receptors on a host cell and because this interaction will play a major role in virus pathogenesis (16), the present study was undertaken to establish which of the envelope proteins of vaccinia virus has specificity for binding to mammalian cells. We have identified a 32-kDa envelope protein of vaccinia virus that binds to the surface of cells of various origins. Biochemical and genetic properties of the 32 kDa vaccinia virus cell surface binding protein are described.

EXPERIMENTAL PROCEDURES

Virus and Cells—The wild-type strain WR of vaccinia virus was propagated in HeLa S3 cells and purified by sucrose gradient centrifugation (17). The particle to plaque forming unit (pfu) ratio was about 30:1. The virus was titrated in BSC-40 cells. Binding experiments were carried out with monolayer cultures of monkey cells (BSC-40), mouse cells (L-929), human cells (HeLa), and canine MDCK cells.

Induction of Envelopes of Vaccinia Virus—Vaccinia virus envelopes were prepared from 5 μg of purified vaccinia virus suspended in 1 ml by sonicating in 50 mM Tris hydrochloride (pH 8.5), 10 mM MgCl₂, 10 mM dithiothreitol, 0.5% Nonidet P-40. In some experiments 1 The abbreviations used are: pfu, plaque forming unit(s); PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropylthio β-D-galactoside; kb, kilobase(s).

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1569
Antibodies—A genomic library used for antibody screening by Dr. R. W. Moyer. Phage (2 X 10^6) with 5% Blotto for 2 h, and then incubated for 2 h with rabbit anti-32-kDa antibodies. Thereafter, 20 μl of culture solution were added to 20 μl of goat anti-rabbit IgG conjugated with peroxidase. After washing with PBS, antigen-antibody complexes were visualized with 0.02% 4-chloro-1-naphthol and 0.006% hydrogen peroxide in PBS.

Binding of Envelope Proteins to Cells—Confluent growing cells in cultured dishes (24 wells) were placed over ice for 1 h, then cells were washed with cold PBS and 2 X 10^6 μl of G-50 excluded labeled envelopes were added in 0.2 ml of PBS/well. At time intervals, cells were washed five times with cold PBS, lysed directly with 100 μl of SDS sample buffer (62.5 mM Tris HCl, pH 6.8, 4% SDS, 10% mercaptoethanol, 10% glycerol, and 0.2% bromphenol blue), heated at 100 °C for 3 min, and analyzed on 12% SDS-PAGE.

Immunoblotting—Proteins were fractionated by one-dimensional 12% SDS-PAGE (18) and by two-dimensional SDS-PAGE (19). The proteins were transferred to nitrocellulose paper (20), which was soaked for 2 h at room temperature with 5% Blotto (nonfat dry milk) in PBS and then incubated overnight with rabbit anti-32-kDa antibodies. Thereafter, the paper was washed with PBS four times for 15 min each and then incubated in Blotto for 2 h at room temperature with goat anti-rabbit IgG conjugated with peroxidase. After washing with PBS, antigen-antibody complexes were visualized with 0.02% 4-chloro-1-naphthol and 0.006% hydrogen peroxide in PBS.

Identification of a 32-kDa Vaccinia Virus Cell Surface Binding Protein—To examine which of the vaccinia envelope proteins could serve as cell surface attachment proteins, envelopes were separated from purified virus cores by centrifugation at 37 °C, then incubated with rabbit anti-32-kDa antibodies. Thereafter, the paper was washed with PBS four times for 15 min each and then incubated in Blotto for 2 h at room temperature with goat anti-rabbit IgG conjugated with peroxidase. After washing with PBS, antigen-antibody complexes were visualized with 0.02% 4-chloro-1-naphthol and 0.006% hydrogen peroxide in PBS.

Antibody Screening of a λgt11 Expression Library and Selection of Anti-32-kDa Antibodies—A genomic library used for antibody screening was prepared from randomly generated rabbit poxivirus DNA fragments in the expression vector λgt11 (21) and was kindly provided by Dr. R. W. Morgan (2 x 10^7) for screening were plated in 10 μl isopropylthio-β-D-galactoside (IPTG) and incubated at 37 °C for 4 h. Therefore, filters were removed, washed at room temperature in PBS, blocked in PBS with 0.5% BSA and 15% fetal calf serum for 1 h, washed with PBS, and incubated overnight with hyperimmunized rabbit anti-vaccinia serum (12) with a solution of 0.5% BSA, 15% fetal calf serum for 1 h. Antibody reacting plaques were detected by immunoperoxidase staining and by reactivity with 32-kDa protein A. Positive plaques were cloned and then sequenced. The amount of total radioactivity bound to cells is shown in the left panel, while the proteins bound to cells are shown in the right panel. The amount of total radioactivity bound to cells is shown in the left panel, while the proteins bound to cells are shown in the right panel. If the labeled plaque proteins could serve as cell surface attachment proteins, envelopes were separated from purified virus cores by centrifugation at 37 °C.

Binding of 32-kDa Protein of Vaccinia Virus to Cells of Different Origins—Vaccinia virus has a wide host range, infecting cells of many different origins. As shown in Fig. 3, vaccinia 32-kDa envelope protein was the only envelope protein specifically bound to mouse L-929 cells and human HeLa cells as well as to monkey BSC-40 cells. The differences in extent of binding was not due to differences in number of cells. Significant, when similar binding experiments were carried out with confluent cultures of the polarized epithelial Madin-Darby canine kidney (MDCK) cells, mutant S111, very little binding of 32-kDa protein was detected (data not shown). Table I summarizes for the different cell lines the extent of binding of virions, virus yields, and binding of 32-kDa protein. These results reveal that 32-kDa protein binds to the various cell lines differently, with poor binding to a polarized epithelial cell line.

Identification of an Antibody Against the Vaccinia Virus 32 kDa Protein—We previously showed that screening a λgt11 expression vector pT7-7 was kindly provided by Dr. S. Tabor (Harvard Medical School, Boston, MA). An Alu fragment (3.34 kb) containing the entire 32-kDa gene was excised from pUC19 plasmid that contains a 3.6-kb EcoR1 insert of vaccinia HindIII DNA fragment. The Alu fragment extends 5.3 nucleotides upstream from the start codon (ATG) and 370 nucleotides downstream from the translation codon (TAG). The plasmid pT7-7 was linearized with EcoRI and blunt-ended with Klenow fragment of E. coli DNA polymerase I. The linearized plasmid was ligated with the Alu fragment of vaccinia DNA. A plasmid pT7-32K with the correct 32-kDa reading frame was isolated. This plasmid was used to transform E. coli BL21 (DE3) that contains one copy of T7 RNA polymerase (25), and exponentially growing cells were induced with 400 μM of IPTG. Various times after induction 5.0-ml aliquots of growing cells were removed, cells pelleted, and resuspended in 250 μl of 50 mM Tris HCl, pH 8.0, 5 mM EDTA, 70 μg/ml NaCl, 1 mM dithiothreitol. The resulting cultures were brought to 0.1 M NaCl and 1% SDS, freeze-thawed three times, sonicated, and the supernatant was collected after centrifugation. The proteins in the supernatant were analyzed by immunoblot.
Fig. 1. A 32-kDa envelope protein of vaccinia binds to the cell surface of monkey (BSC-40) cells. Solubilized and Sephadex G-50-excluded 125I-labeled vaccinia virus envelopes (2 x 10^6 cpm) were added to cells growing in monolayer (24 wells) and after intervals the radioactivity associated with the cells was measured as described under “Experimental Procedures.” A, shows total radioactivity associated with the cells (○) and radioactivity bound to the plastic well (■). B, shows a lighter exposure of the autoradiogram of the labeled proteins bound to cells as analyzed on a 12% SDS-PAGE. Lane WR, represents the profile of total labeled envelope proteins prior to their addition to cells. The times of binding are given in minutes. C, shows the same results as B, but a darker exposure of the lanes corresponding to the bound 32-kDa protein and a lighter exposure of lane WR. The optical density of the 32-kDa band as measured by laser densitometry was: 0.38 (5 min), 0.45 (15 min), 0.58 (30 min) 0.61 (60 min), and 0.71 (90 min).

expression library of genomic rabbit poxvirus DNA with hyperimmune rabbit anti-vaccinia virus serum is an efficient method to isolate monospecific antibodies against immunogenic proteins of vaccinia virus (26). We also showed that animals immunized with vaccinia virus develop antibodies against a 32-kDa vaccinia virus protein (26). After screening the λgt11 rabbitpox DNA library with rabbit anti-vaccinia virus serum, we identified two recombinant phages that, after E. coli infection, induced fusion proteins capable of selecting antibodies with reactivity against a 32-kDa protein. The reactivity of one of these antibodies (selected by phage E14) is shown in Fig. 4A. To verify that antibodies were directed against the 32-kDa protein that binds to cells, we carried out two-dimensional SDS-PAGE analysis. 125I-Labeled vaccinia envelope proteins were bound to BSC-40 cells as described above, and the bound material was analyzed by non-equilibrium two-dimensional SDS-PAGE followed by autoradiography of a Western blot. A single labeled protein of 32 kDa was
We calculated that the adsorbed to cells was estimated with \[3H\]thymidine-labeled virus; it was about 5% of the input virus. We determined that the extent of binding of virions to cells, while the virus titer obtained at 24 h post-infection represents virus yields. The virus titers are given in plaque-forming units (pfu)/ml and are from duplicate cultures of about 5 x 10^6 cells each. Binding of 32-kDa protein to different cells was carried out as described for Fig. 3. The extent of binding of 32-kDa protein was determined after radiolabeled virus envelopes were bound to cells for 120 min, and the amount of radioactivity incorporated into the 32-kDa band, excised from a polyacrylamide gel, was determined by scintillation counting. The 32-kDa binding is given in counts/min/2 x 10^6 cells. The canine MDCK-5D11 is a mutant cell line that establishes tight junctions in culture, and was kindly provided by Dr. G. Ojakian (Health Science Center, Brooklyn, NY).

**TABLE I**

| Cell lines | Binding of virions | Virus yields | 32-kDa binding protein |
|------------|-------------------|--------------|------------------------|
|            | pfu/ml            | cpm          |                        |
| L-929      | 1 x 10^6          | 5 x 10^9     | 1120                   |
| BSC-40     | 2 x 10^6          | 3.2 x 10^6   | 3627                   |
| HeLa-G     | 4.5 x 10^6        | 1.8 x 10^6   | 6285                   |
| MDCK-5D11  | 4 x 10^6          | 4.2 x 10^6   | 243                    |

Vaccinia Virus 32-kDa Binding Protein Is Synthesized at Late Time Post-Infection—Vaccinia virus gene expression is coordinately regulated (6). So, it was of interest to define the time course of synthesis of the 32-kDa binding protein during virus infection. This was examined by immunoblot analysis. Cells were infected in the absence or presence of a specific inhibitor of virus DNA synthesis (cytosine arabinoside) or an inhibitor of virus assembly (rifampin). At various times post-infection cell extracts were prepared and analyzed by SDS-PAGE and immunoreactivity with antibodies against the 32-kDa protein (Fig. 5). The 32-kDa protein was not detected at 4 h post-infection but was apparent at 8 h, indicating that it belongs to the late class of viral genes. The protein was not synthesized in the presence of cytosine arabinoside; it accumulated at late times post-infection even in the presence of rifampin. The 32-kDa binding protein was not processed; it remained as a single 32-kDa polypeptide throughout infection.

**A Large Fragment of the Vaccinia Virus 32-kDa Binding Protein Is Removed from Virus Particles by Trypsin**—It has been shown that digestion of wild type vaccinia virus with low doses of trypsin enhances infectivity 2-3-fold (15, 27, 28). We examined the sensitivity of the 32-kDa vaccinia virus envelope protein to trypsin digestion (Fig. 6). In the virus pellet fraction (lanes T1 and T10), trypsin digestion reduced the amount of the 32-kDa protein and increased the amount of a 30-kDa protein (lanes T1 and T10 compared with wild-type). The band appearing at 39 kDa results from reactivity with a contaminating antibody against a 39-kDa protein, an immunodominant core protein (28). Trypsin appears to degrade the 32-kDa protein to a 30-kDa fragment that remained virus-associated (lanes T1 and T10) or a 29-kDa fragment recovered in the supernatant solution (lanes S1 and S10).

There was concomitant release of the 29-kDa protein and increase in virus titers (see legend to Fig. 6). We conclude that the 32-kDa protein in vaccinia virions has a large external fragment of about 29 kDa that is released after trypsin digestion and that this enzyme treatment increases virus infectivity.
Vaccinia Virus Cell Surface Binding Protein

FIG. 4. Vaccinia virus 32-kDa binding protein is a basic polypeptide. Antibodies reactive against a 32-kDa vaccinia virus protein were generated as described under “Experimental Procedures.” A, Western blot of one-dimensional SDS-PAGE. Proteins of purified vaccinia virus (10 μg) were electrophoresed on a 10% SDS-polyacrylamide gel, blotted, and reacted with rabbit anti-vaccinia virus serum (lane 1), recombinant phage E14-selected anti-32-kDa antibodies (lane 2) and monoclonal antibody C3 (lane 3) that recognizes the vaccinia 14-kDa fusion protein (19). Antibody reactivity was detected by immunoperoxidase staining. B, Western blots of two-dimensional non-equilibrium pH polyacrylamide gel electrophoresis (NEPHGE). The left panel is an autoradiogram of 125I-labeled 32-kDa protein bound to cells. Binding of labeled envelopes to BSC-40 cells was for 60 min; cells were then collected, lysed, and mixed with 20 μg of purified vaccinia virus. A lane shown to the left contains total labeled envelope proteins electrophoresed only on one dimension. The right panel is the immunoblot of the same gel shown to the left. The blot was reacted with antibodies against the 32-kDa protein and antibody reactivity was detected by immunoperoxidase staining. The arrows point migration of the 32-kDa protein. The first dimension was run on non-equilibrium pH polyacrylamide gel electrophoresis (NEPHGE). The left panel is an autoradiogram of 125I-labeled 32-kDa protein bound to cells. Binding of labeled envelopes to BSC-40 cells was for 60 min; cells were then collected, lysed, and mixed with 20 μg of purified vaccinia virus. A lane shown to the left contains total labeled envelope proteins electrophoresed only on one dimension. The right panel is the immunoblot of the same gel shown to the left. The blot was reacted with antibodies against the 32-kDa protein and antibody reactivity was detected by immunoperoxidase staining. The arrows point migration of the 32-kDa protein. The first dimension was run on non-equilibrium pH (7 to 9) gel and the second dimension was run under standard SDS-PAGE conditions.

to cultured cells. Similar results have been observed with a polyclonal antiserum raised against a 32-kDa protein (29).

Physical Mapping, Sequence, and Homologies of the Vaccinia Gene Encoding the 32-kDa Binding Protein—To determine the map position of the gene for the 32-kDa protein, Southern blot hybridization analyses were carried out with vaccinia virus DNA that was digested with various restriction enzymes. The blots were hybridized with nick-translated specific DNA probes. The hybridization results are shown in Fig. 7. The probes were total vaccinia DNA (Fig. 7A) and a 1.5-kb EcoRI insert from a recombinant phage (Fig. 7, B and C). The sequence encoding the 32-kDa protein mapped to the middle region of vaccinia HindIIID DNA fragment. Since the entire HindIIID DNA fragment has been sequenced (30), finer mapping was carried out. By sequencing the EcoRI inserts from two recombinant phages and the overlapping sequences from cloned vaccinia DNA fragments, we identified the 32-kDa encoding gene as open reading frame 8 (30). To illustrate a number of properties of the 32-kDa encoding gene, the nucleotide sequence, deduced amino acid sequence, and hydrophathy plot are shown in Fig. 8. The protein has 304 amino acids in length giving a molecular mass of 35,426 daltons, it has a large fragment with 40–50% high degree of protein sequence homology with carbonic anhydrase, several putative glycosylation sites (underlined in Fig. 8) and a potential transmembrane domain at the C terminus (amino acids 276–294). Near the transmembrane domain is a region with sequences similar to the VP7 attachment glycoprotein of rotaviruses (Fig. 9). The predicted isoelectric point is 8.63, very similar to that established by two-dimensional SDS-PAGE (Fig. 5). Because the 32-kDa encoding gene was isolated from an expression library using rabbit anti-vaccinia virus serum, the protein must contain immunogenic sites. Computer analysis of the predicted most antigenic sites on the 32-kDa protein revealed a strongly antigenic site between amino acids 108 and 110 and another antigenic site at position 298–299.

Vaccinia Virus 32-kDa Protein Is Non-glycosylated—As deduced from the amino acid sequence (Fig. 8), the 32-kDa binding protein has four potential glycosylation sites. Since this is a membrane protein, and many membrane proteins are glycosylated, we examined glycosylation. First, we examined the sensitivity of 32-kDa protein to in vivo inhibitors of N- and O-linked glycosylation (tunicamycin, monensin). As measured by immunoblot analysis with anti-32-kDa antibodies, these glycosylation inhibitors did not alter the size of the 32-kDa protein when extracts of virus infected cells were analyzed on SDS-PAGE (data not shown). Next, we examined the sensitivity of 32-kDa from purified virions and envelopes to N- and O-linked glycosidases. Neither endo-H, endo-F, nor O-glycanase had any effect on size of the 32-kDa protein when virion lysates were examined by immunoblots of one-dimensional SDS-PAGE. Similarly, there was no reactivity of the 32-kDa protein with lectins (data not shown). An expression library using rabbit anti-vaccinia virus serum, the protein must contain immunogenic sites. Computer analysis of the predicted most antigenic sites on the 32-kDa protein revealed a strongly antigenic site between amino acids 108 and 110 and another antigenic site at position 298–299.

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FIG. 5. Synthesis of vaccinia virus 32-kDa protein in the course of virus infection. BSC-40 cells were infected with 5 pfu/cell of vaccinia virus in the absence or presence of cytosine arabinoside (40 μg/ml) or rifampin (100 μg/ml). At various times post infection, cells were collected and lysates were analyzed by 10% SDS-PAGE. The gel was blotted and reacted with 32-kDa antibodies. Antibody reactivity was detected with 125I-protein A, after autoradiography. The times post-infection, in hours, are indicated on the top. Ar, cytosine-arabinoside, and Ra, rifampin. The band appearing on top of 32-kDa represents an immunodominant vaccinia 39-kDa core protein (26), as a result of low levels of anti-39-kDa antibodies present during preparation of the antibody.
Further prove lack of glycosylation in the 32-kDa binding protein, we expressed this protein in E. coli, where glycosylation does not occur. The entire gene encoding the 32-kDa protein was cloned in the prokaryotic expression vector pT7-7 (24). The constructed pT7-32K plasmid was transformed into BL21 (DE3) competent cells which contained one copy of phage T7 RNA polymerase under the control of an inducible repressor Lac UV5 (25). After IPTG induction, the expression product was revealed by immunoblot with anti-32-kDa antibodies. With time after IPTG induction a 32-kDa protein is expressed in increasing amounts (Fig. 10). The size of the E. coli expressed 32-kDa protein is the same as that of the 32-kDa protein present in purified vaccinia virions (Fig. 10), regardless of the percent of acrylamide used (not shown). The above findings suggest that the 32-kDa vaccinia virus binding protein is non-glycosylated.

Vaccinia Virus 32-kDa Protein Forms Disulfide-linked Dimers—It is known that a number of the structural proteins of vaccinia virus can form aggregates held by disulfide bonds (31). Since we had identified a 14-kDa vaccinia envelope protein that forms disulfide linked trimers through linkage of 2 contiguous cysteine residues (13, 32), it was of interest to determine whether the 32-kDa protein that has a single cysteine residue at position 262 could also form oligomers. Purified virions were disrupted in the absence or presence of the reducing agent β-mercaptoethanol, and the Western blot of one-dimensional SDS-PAGE was reacted with antibodies against the 32-kDa protein (Fig. 11A). In the presence of the reducing agent, there was a single 32-kDa band (lane 1), but in the absence of the reducing agent there were two bands of about 64 and 32 kDa (lane 2). This suggests disulfide linkage of 32 kDa as a dimer. This was further documented by a diagonal immunoblot analysis using purified 35S-labeled virus.

The first dimension was run under nonreducing conditions and the second dimension was run under reducing conditions. The Western blot was reacted with antibodies against the 32-kDa protein and the same blot was also exposed to x-ray film. From the autoradiogram (Fig. 11B), it is evident that a number of virion proteins form disulfide linked oligomers (those proteins that migrate to the left of the diagonal). By superimposing the autoradiogram (panel B) with the corresponding immunoblot (panel C), we established the position of the monomer and dimer forms of the 32-kDa binding protein as those indicated by the arrows. We conclude that the 32-kDa protein of vaccinia virus is present in the virion as a disulfide-linked dimer.

**DISCUSSION**

In this investigation we have identified and defined structural and functional properties of a vaccinia virus envelope protein that it has an apparent molecular mass of about 32 kDa on SDS-PAGE and a mass of 35,426 calculated from the
Fig. 8. Nucleotide sequence, deduced amino acid sequence, and hydropathy plot of vaccinia 32-kDa gene. The nucleotides are numbered above the sequence in the 5' to 3' direction; nucleotide 1 is the A of the ATG codon for the initiator methionine. Hydropathy analysis is based on the hydrophobic studies of Kyte and Doolittle (40). Hydrophobic regions are above the x axis and hydrophilic regions are below it. The C. A. DOMAIN is the region of amino acid sequence homology with carbonic anhydrases of human, Rhesus macaque, horse, bovine, sheep, and rabbit. The VP-7 domain is the region of homology with VP7 glycoprotein of rotavirus. The TX domain is the region of amino acid sequence homology with transmembrane proteins. The possible glycosylation sites are underlined. Sequence analysis was carried out using the International Biotechnologies program.

![Hydropathy plot of vaccinia 32-kDa gene](image)

Fig. 9. Homologous sequences between the 32-kDa protein of vaccinia virus and VP7 glycoprotein of rotavirus. VV-32K, 32-kDa protein of vaccinia virus; VGXRS2, rotavirus type 2; VGXRS7, rotavirus strain Hu/Australia/5/77; VGXRCB, rotavirus strain UK; VGXRD, rotavirus strain NCDV; VGXIS, simian 11 rotavirus; VGXR7H, rotavirus. Identical residues in all sequences are boxed.

![Alignment of sequences](image)

The binding of 32-kDa protein to cells was vaccinia virus greatest binding, followed by monkey and mouse cells. Binding experiments with a mutant of the polarized epithelial cell line MDCK revealed very little binding of 32-kDa protein to cells which correlated with poor binding of virions to cells (Table I). This negative result can be explained if the 32-kDa protein is involved in the polarity of viral entry as suggested by inability of the virus to replicate in these cells (Table I). Elsewhere we will show that entry of vaccinia virus is restricted to basolateral surfaces of polarized epithelial cells. The binding of 32-kDa protein to cells was vaccinia virus

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1. D. Rodriguez, J. R. Rodriguez, G. Ojakian, and M. Esteban, manuscript in preparation.
2. D. Rodriguez, J. R. Rodriguez, G. Ojakian, and M. Esteban, manuscript in preparation.
specific, since purified vaccinia virus (but not by vesicular stomatitis virus or BSA) competed. With saturating concentrations of vaccinia virus particles, competition was nearly complete, indicating a finite number of binding sites on the cell surface (Fig. 2).

The 32-kDa protein has a large fragment at the N terminus with high degree of amino acid sequence homology with carbonic anhydrases. This sequence homology was identified previously after sequencing the 16-kb HindIIId fragment of vaccinia DNA and finding a gene, D8, with an overall similarity of greater than 50% with carbonic anhydrases (30). This is the gene that we have identified here as coding for the 32-kDa protein. Competition experiments with carbonic anhydrases A and B (from Sigma) showed less than 10% competition when up to 100 μg were competed with 5 × 10^8 cpm of the 32-kDa protein for binding to cells. Carbonic anhydrases had no effect on virus infectivity (data not shown). These experiments suggested that the carbonic anhydrase domain was not essential for binding. An interesting region at the C terminus has the potential transmembrane sequence (amino acids 277-299) (30), but it also has a region with sequence homology to the attachment glycoprotein (amino acids 163-183) VP7 of rotaviruses (Fig. 9). It has been shown that VP7 is an attachment protein with virus neutralizing properties (34, 35). Based on several observations we suggest that the C terminus of the 32-kDa protein serves the cell surface binding domain. First, it is structurally similar to another virus attachment protein (Fig. 9). Second, a large external (29 kDa) is removed from the 32-kDa after treatment of purified vaccinia virus particles with trypsin and the virus gains infectivity (29; cf Fig. 6). Third, effective neutralization of trypsin-treated vaccinia virus has been observed with a monoclonal antibody that recognizes a 32-kDa envelope protein of vaccinia virus IHD-J strain (15). This led to the suggestion that the small portion of the 32-kDa protein that remained after trypsin digestion had an essential role in infection of the virus (15). Comparison of our data with that of vaccinia IHD-J 32-kDa (15) suggests that the vaccinia 32-kDa protein is the same for both virus strains, IHD-J and WR.

Although the above findings suggested that the vaccinia 32-kDa attachment domain is located within 50 amino acid residues at the C terminus and that this domain is required for efficient virus infection to cultured cells, significant virus replication can still be obtained with viruses that lack this domain. In fact, variants of vaccinia virus (IHD strain) have been found that apparently lack the 32-kDa protein (14), and variants of vaccinia virus (WR strain) have been generated that lack the C-terminal domain of the 32-kDa protein (29). All of these variants were infectious, although their titers had been reduced severalfold (14). The apparently nonessential role of 32-kDa binding protein for virus multiplication in cells in culture is also supported by our competition experiments with E. coli-produced 32-kDa protein. With saturating concentrations of cold 32-kDa protein added to cells we could only obtain about 25% reduction in vaccinia virus plaque formation. The ability of vaccinia virus to be infectious in cells in culture in the absence of 32-kDa cell surface binding domain indicates that this virus uses more than one mechanism to bind to cells. This is not surprising due to the complexity of the vaccinia virus particle and to about 30 polypeptides present in the vaccinia virus envelope (35). Definitive proof for the role of the 32-kDa protein on vaccinia virus tropism will be obtained through generation of deletion mutants on the corresponding gene and assays of virus infectivity both in cultured cells and in animals. The later experiments will be facilitated by generating vaccinia recombinants expressing luciferase, as we have shown this marker to be a highly sensitive indicator to measure virus tropism in vivo (41).

The vaccinia 32-kDa protein is synthesized at late times during virus infection (Fig. 5). Although 32-kDa is a membrane protein, its synthesis might occur in membrane free ribosomes and not involve the endoplasmic reticulum. This could result in an unglycosylated protein, despite of the fact that the 32-kDa protein contains four possible glycosylation sites (Fig. 8). Apparent lack of glycosylation in the 32-kDa protein has been suggested with in vitro inhibitors of glycosylation, in vitro treatment with glycosidases (data not shown) and after expression of the 32-kDa encoding gene in E. coli cells (Fig. 10). It is of interest to note that other vaccinia membrane proteins are also non-glycosylated, like a 35-kDa (36) and a 14-kDa fusion protein. This may turn out to be a general property of vaccinia membrane proteins that, by their unique characteristics of interacting with host lipids during the virus assembly process that occurs in the cytoplasm (5), these proteins are unable to be glycosylated.

The vaccinia virus 32-kDa protein may form disulfide-linked dimers (Fig. 11). From the deduced amino acid sequence, dimerization must occur at the single cysteine residue at position 262. It is predicted that this site is close to the trypsin-sensitive cleavage site (possibly Arg at position 259) that releases the external 29-kDa fragment. Removal of the external fragment by trypsin should generate a virus particle that has two identical halves composed of two small peptides (45 amino acids in length). The overall folding of these two peptides may favor a more stable interaction of the virus with a cell membrane component(s) through the α-helix (position 270-280).

The vaccinia virus 32-kDa protein is an immunogenic polypeptide, since antibodies against 32-kDa protein are easily obtained from rabbit anti-vaccinia virus serum after selection with extracts of E. coli cells that had been infected with a λ recombinant phage expressing a fragment of the 32-kDa protein (Fig. 4). Moreover, antibodies reacting with a 32-kDa vaccinia virus protein readily can be detected by immunoblot analysis in serum from mice immunized with vaccinia virus (26). The predicted antigenic sites of the 32-kDa protein are found near positions 108-110 (carboxy anhydride site) and 298-299 (transmembrane site). Because the 32-kDa protein is a membrane protein, characterization of the antigenic domains will be important to understand immune responses to vaccinia virus.

Virus attachment to cells is a major determinant in virus tropism. Thus, it will be important to unravel the structural features during interaction of the vaccinia virus 32-kDa protein with a putative cellular receptor. In this regard it is of major interest that among the animal virus receptors that have been clearly identified, three have properties of the immunoglobulin superfamily (picornavirus and retrovirus HIV), while a sialic acid is involved in influenza virus-receptor interaction (37). Although it has been suggested that the epidermal growth factor receptor can act as vaccinia virus receptor (38), it seems unlikely that epidermal growth factor receptor interacts with the 32-kDa protein since binding of labeled 32-kDa protein to cells could not be competed by epidermal growth factor (data not shown).

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J S Maa, J F Rodriguez and M Esteban

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