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Vaccinia virus L1 binds to cell surfaces and blocks virus entry independently of glycosaminoglycans

Chwan Hong Foo[a,*], **Huan Lou**[a], **J. Charles Whitbeck**[b], **Manuel Ponce-de-León**[a], **Doina Atanasiu**[a], **Roselyn J. Eisenberg**[b], and **Gary H. Cohen**[a]

a*Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, 19104, USA*

b*Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, 19104, USA*

Abstract

L1 and A28 are vaccinia virus (VACV) envelope proteins which are essential for cellular entry. However, their specific roles during entry are unknown. We tested whether one or both of these proteins might serve as receptor binding proteins (RBP). We found that a soluble, truncated form of L1, but not A28, bound to cell surfaces independently of glycosaminoglycans (GAGs). Hence, VACV A28 is not likely to be a RBP and functions after attachment during entry. Importantly, soluble L1 inhibited both binding and entry of VACV in GAG-deficient cells, suggesting that soluble L1 blocks entry at the binding step by competing with the virions for non-GAG receptors on cells. In contrast, soluble A27, a VACV protein which attaches to GAGs but is non-essential for virus entry, inhibited binding and entry of VACV in a GAG-dependent manner. To our knowledge, this is the first report of a VACV envelope protein that blocks virus binding and entry independently of GAGs.

Keywords

poxvirus; vaccinia; entry; attachment; L1; A28; receptor-binding

INTRODUCTION

Vaccinia virus (VACV) is a complex, enveloped virus with an ~ 189 kbp double stranded DNA genome. It is the prototypic member of the *Poxviridae* family and was used successfully as a vaccine in the worldwide effort to eradicate smallpox from the global human population. Multiple infectious forms of VACV (Condit, Moussatche, and Traktman, 2006), each with a different number of envelope layers, are produced during infection, including the mature virion (MV/IMV) and the enveloped virion (EV/EEV). The MV consists of a single membrane envelope and is released from the infected host cell during lysis. A portion of MV particles are also enwrapped by endosomal and/or trans-Golgi-derived membranes and are subsequently exocytosed to become EV particles. The EV has an additional outer membrane layer and thus exhibits different proteins on its surface compared to the MV.

*Corresponding author: Address: Department of Microbiology, University of Pennsylvania, 240 S. 40th St, Levy Rm 233, Philadelphia, PA 19104, Phone: (215) 898-6553, FAX: (215) 898-8385, Email: E-mail: chfoo@mail.med.upenn.edu.

The majority of studies investigating the entry of VACV into host cells have focused on the MV since they are more abundant and easier to purify than EV particles (Ichihashi, 1996). Moreover, proteins on the MV surface are important for entry of both forms of VACV (Law et al., 2006; Senkevich et al., 2005). The envelope of the MV consists of approximately twenty five integral or peripheral membrane proteins (Chung et al., 2006), at least sixteen of which have been implicated to function in virus entry and/or fusion (A27, A17, H3, D8, L1, A28, H2, A21, L5, G3, G9, A16, J5, F9, I2 and A26) (Brown, Senkevich, and Moss, 2006; Chiu et al., 2007; Ichihashi, Takahashi, and Oie, 1994; Izmailyan et al., 2006; Kochan et al., 2007; Lai, Gong, and Esteban, 1990; Lai, Gong, and Esteban, 1991; Lin et al., 2000; Nichols et al., 2008; Ojeda, Domi, and Moss, 2006; Ojeda, Senkevich, and Moss, 2006; Senkevich and Moss, 2005; Senkevich, Ward, and Moss, 2004; Townsley, Senkevich, and Moss, 2005a; Townsley, Senkevich, and Moss, 2005b). Of these, eight proteins (A28, H2, A21, L5, G3, G9, A16, J5) form a conserved, multiprotein entry-fusion complex (EFC) that is essential for the penetration of viral cores and VACV-induced cell-cell fusion (Izmailyan et al., 2006; Ojeda, Domi, and Moss, 2006; Ojeda, Senkevich, and Moss, 2006; Senkevich and Moss, 2005; Senkevich et al., 2005; Senkevich, Ward, and Moss, 2004; Townsley, Senkevich, and Moss, 2005a; Townsley, Senkevich, and Moss, 2005b). Monoclonal antibodies to L1, another conserved MV envelope protein, diminish VACV-induced cell-cell fusion at low pH (Ichihashi, Takahashi, and Oie, 1994), and exhibit potent virus-neutralizing activity in plaque reduction assays (Aldaz-Carroll et al., 2005; Ichihashi, 1996; Wolffe, Vijaya, and Moss, 1995). For this reason, L1 is a key component of several subunit vaccine candidates currently in development for protection against lethal orthopoxvirus diseases (Fogg et al., 2004; Heraud et al., 2006; Hooper et al., 2004; Xiao et al., 2007). Despite some recent progress, the specific functions of many of these proteins during entry remain poorly defined. It is currently unclear if they function during or prior to membrane fusion, such as attaching to cell surface molecules. In addition, the host cell receptor(s) responsible for triggering internalization and/or membrane fusion of VACV have not been identified.

Four MV proteins are known to mediate attachment of MV to cell surface molecules. A27 and H3 bind to the cell surface glycosaminoglycan (GAG) heparan sulfate (HSPG) (Chung et al., 1998; Lin et al., 2000), D8 binds to chondroitin sulfate proteoglycan (CSPG) (Hsiao, Chung, and Chang, 1999), while A26 attaches to the extracellular matrix protein laminin (Chiu et al., 2007). However, none of these proteins are solely essential for cell-binding or entry because other MV proteins can complement adsorption (Chiu et al., 2007; Hsiao, Chung, and Chang, 1999). On some cell types, high concentrations of soluble GAGs do not inhibit VACV infectivity (Carter et al., 2005). Moreover, mutant MV particles lacking A26 still enter cells which do not express GAGs, even in the presence of soluble laminin (Chiu et al., 2007), strongly suggesting the existence of another unidentified MV protein which binds to non-GAG, non-laminin receptor(s) on the plasma membrane.

In this study, we hypothesized that L1 and/or the EFC component A28 mediates attachment of VACV to cells by binding to non-GAG receptor(s) on the plasma membrane. Several criteria are often used to assess whether a viral surface protein is a receptor-binding protein (RBP): (i) antibodies to the viral protein inhibit virus entry, (ii) soluble forms of the protein block virus entry at the adsorption step and (iii) the soluble protein binds to cell surfaces. Hence, we produced soluble, truncated forms of VACV L1 and A28 proteins using a baculovirus system and tested for their abilities to interact with cell surface molecules and to inhibit virus entry. Since a soluble, recombinant form of A27 had been reported to bind to cell surfaces and block VACV entry in a manner that is dependent on its GAG-binding domain (Hsiao, Chung, and Chang, 1998; Lai, Gong, and Esteban, 1990), we incorporated A27 in our study for comparison. We found that soluble L1, but not A28, binds to cell surfaces independently of GAGs. Therefore, A28 is not likely to be a RBP. Interestingly, unlike soluble A27, soluble L1 blocks

binding and entry of VACV in GAG-deficient cells. This suggests that VACV L1 may function in entry as a RBP by engaging host receptor(s) which are unique from GAGs.

RESULTS

Purification of soluble forms of VACV MV envelope proteins

To compare the roles of L1, A27 and A28 in the entry of VACV, we employed a biochemical approach, testing soluble forms of each of the MV proteins for their abilities to interact with cell surface components and to perturb virus entry. The cloning and purification of soluble forms of L1 and A27 using the baculovirus expression system were described previously (Aldaz-Carroll et al., 2005; Xiao et al., 2007). To generate a soluble form of A28, we cloned the ectodomain of A28L (residues 23-146) into baculovirus. We appended the sequences for the melittin signal peptide and a six histidine tag to the N-terminus of A28 (Fig. 1A). The protein was expressed and secreted from infected insect cells and purified from the supernatant by nickel-chelate affinity chromatography. Purified A28 was analyzed using SDS-PAGE, followed by silver staining or Western blotting (Fig. 1A). Under reducing and denaturing conditions, soluble A28 migrated as four major bands of 14-19 kDa. The three slowest migrating bands represent N-glycosylated forms, since they were absent when the protein sample was pretreated with PNGase F (data not shown).

To assess if recombinant A28 was structurally similar to its genuine counterpart from VACV, we used a rabbit hyper-immune serum generated against VACV to probe for discontinuous epitopes in soluble A28. The anti-VACV serum recognized soluble A28 when assessed by Western blotting (Fig. 1B), indicating that at least a subset of the antibodies in the rabbit serum detected A28-specific epitopes which are common in both forms of A28. Importantly, these epitopes are conformation-dependent, as the rabbit serum only recognized soluble A28 when it was analyzed under native, but not under denaturing conditions. In contrast, a different VACV protein, soluble A33 (Xiao et al., 2007), was still revealed under denaturing conditions (Fig. 1B). Hence, the recombinant A28 shares some conformational epitopes with the authentic VACV A28.

Antibodies to L1, A27 and A28 inhibit virus entry

Viral structural proteins which are involved in entry into host cells, such as RBPs or membrane fusion proteins for enveloped viruses, are common targets of neutralizing antibodies. Therefore, we immunized rabbits with purified A27 or A28 to obtain antisera for virus neutralization studies. Anti-L1 rabbit polyclonal antibodies (PAbs) were already available, as described previously (Aldaz-Carroll et al., 2005). Although several investigators have reported the neutralization capability of antibodies to A27 (Czerny et al., 1994; Rodriguez, Janeczko, and Esteban, 1985), these studies used the traditional plaque reduction neutralization test (PRNT), which measures virion infectivity but not early events such as virus entry. To determine if PAbs directed against L1, A27 or A28 inhibit VACV infectivity at the stage of entry, we employed a β-galactosidase entry assay. This assay utilizes a recombinant VACV with the *lacZ* reporter gene regulated by a VACV dual early/late promoter. During early events of VACV infection, viral encoded genes regulated by early promoters are expressed shortly after release of the cores into the cytoplasm, hence entry of the recombinant virus can be determined by measuring β-galactosidase activity. Antibodies to L1, A27 and A28 neutralized entry of VACV into BSC-1 cells in a dose-dependent fashion (Fig. 2). Antibodies to A28 were especially effective at neutralization, with an IC_{50} value of 400 ng/ml IgG. In contrast, the IC_{50} values for the antibodies to L1 and A27 were 3.1 and 6.2 μg/ml IgG respectively. These results suggest that L1, A27 and A28 all play a role at an early step of VACV infection, and are in agreement with previous studies which characterized the functions of these proteins in virus entry using a variety of approaches (Chung et al., 1998; Hsiao, Chung, and Chang,

1998; Nelson et al., 2008; Rodriguez, Paez, and Esteban, 1987; Senkevich, Ward, and Moss, 2004; Wolffe, Vijaya, and Moss, 1995).

Soluble L1 and A27, but not A28, block virus entry

When added exogenously during viral infection, soluble, recombinant forms of viral RBP typically inhibit entry of mammalian viruses by competing with the virion proteins for binding to the host receptors (Chu et al., 2005; Jassal et al., 2001; Johnson, Burke, and Gregory, 1990). We probed the ability of soluble L1, A27 and A28 to block virus entry into several mammalian cell types: BSC-1, HeLa, B78H1 and Vero cells. Our data indicate that soluble A28 did not significantly affect VACV entry into all cell types tested (Fig. 3A black bar, 3B, 3C and data not shown). In contrast, 20 μM of soluble L1 and A27 blocked entry of VACV into BSC-1 cells by 73% and 60% respectively (Fig. 3A, black bars). Inhibition of *lacZ* expression by soluble L1 and A27 was detected as early as 2 h p.i., suggesting that at least early gene expression of VACV was affected (data not shown). The results for A27 recapitulate those previously reported for recombinant A27 that was expressed in bacteria (Hsiao, Chung, and Chang, 1998). The blocking effect by the bacterial A27 was attributed to its interference with viral adsorption onto cell surface HSPG (Hsiao, Chung, and Chang, 1998). We also repeated these experiments in HeLa human epithelial cells (Fig. 3B), B78H1 murine melanoma cells (Fig. 3C), and Vero African green kidney monkey cells with similar results (data not shown). In all cases, the soluble proteins inhibited entry of VACV in a dose-dependent manner. At a concentration of 10 μM, soluble L1 inhibited VACV entry by 60% to 80% depending on the cell type tested. In contrast, the blocking effect of soluble A27 was noticeably weaker in B78H1 cells (39% at 10 μM) than in BSC-1, HeLa or Vero cells (58%, 75%, and 62% respectively at 10 μM). This disparity could be due to differences in expression levels of heparan sulfate moieties among the different cell types, or because VACV can utilize alternative, non-HSPG molecules on the plasma membrane of B78H1 cells for attachment.

To assess the specificity of the blocking phenotype of soluble L1, we generated soluble forms of two homologs of L1 and tested them in the entry assay. F9 is a MV protein that is essential for virus entry and VACV-induced cell-cell fusion (Brown, Senkevich, and Moss, 2006). F9 is structurally homologous to L1, in that the two proteins have similar membrane topology, share invariant cysteines, and have 25% sequence identity (Fig. 1C) (Brown, Senkevich, and Moss, 2006; Su et al., 2005). This sequence identity is most pronounced in the ectodomains of the proteins, which are of similar lengths. M1 is the variola virus ortholog of L1, sharing all but one amino acid residue with L1 in its predicted ectodomain (Fig. 1D). The purified F9 and M1 proteins were analyzed using SDS-PAGE, followed by silver staining or Western blotting (Fig. 1C and D). Under reducing and denaturing conditions, both proteins resolved as several closely migrating bands of 19-25 kDa, possibly representing differentially glycosylated forms of the proteins. At 10 μM, soluble M1 blocked entry of VACV by approximately 72%, whereas soluble F9 did not have any significant effect, even though it was structurally homologous to M1 and L1 (Fig. 3D). The results for M1 were not surprising since the single amino acid difference between the two proteins is conservative (Lys-177 in L1 compared to Arg-177 in M1).

To address the possibility that soluble L1 or A27 inhibits β-galactosidase expression by affecting cellular functions such as protein translation, we asked if soluble L1 or A27 could reduce enzymatic activity when added to cells at, or shortly after the initiation of penetration. Virions were preadsorbed to BSC-1 cells at 4 °C and then soluble proteins were added either as soon as (Fig. 3A, grey bars) or 30 minutes after (Fig. 3A, white bars) the incubation temperature was shifted to 37 °C. Soluble L1, A27 and M1 significantly inhibited β-galactosidase expression only if added to cells prior to virus adsorption (Fig. 3A, black bars). This indicates that blocking occurred at an early stage of entry, perhaps during virus adsorption.

Moreover, the experiment shows that the blocking results were not due to non-specific, broad cytotoxic effects caused by the proteins.

In addition to the β-galactosidase expression assay, we employed a different, established approach to measure the entry of VACV. In this penetration assay, intracellular VACV cores are visualized and quantified using confocal immunofluorescence microscopy (Vanderplasschen, Hollinshead, and Smith, 1998). Consistent with our findings obtained using the β-galactosidase assay, the addition of soluble L1 or A27 prior to virus adsorption significantly reduced the number of intracellular cores by 62% and 54% respectively (Fig. 4). Similarly, the presence of soluble A28 did not alter the number of VACV cores (101% of control) that were detected after virus entry into HeLa cells.

Soluble L1 and A27, but not A28, bind to surfaces of cells

It is possible that recombinant L1 inhibits virus entry by binding to proteoglycans or to a specific cell surface receptor, thereby preventing the authentic virion L1 from performing its task of receptor-binding in entry. In addition, because soluble A28 did not block virus entry (Fig. 3 and 4), we hypothesized that it would not bind to cell surfaces, or at least not in a manner that is relevant for entry. To test these hypotheses, we used a protein-cell binding assay (CELISA) to determine if soluble L1 or A28 binds to cell surfaces (Connolly et al., 2002). Herpes simplex virus (HSV) gD was used as a positive control because it is a well-characterized viral RBP (Johnson, Burke, and Gregory, 1990;Nicola et al., 1996). We found that soluble L1, A27 and HSV gD bound to cell surfaces in a dose-dependent fashion (Fig. 5A). As predicted, we detected no appreciable cell-binding by A28. The affinity of soluble A27 to cell surfaces appeared to be higher than those of soluble L1 or HSV gD. Of note, the affinity of soluble L1 seemed to be in the sub-micromolar range. The cellular binding of A27 reached saturation at a concentration of about 1 μM, while L1 and gD did not achieve saturable binding at 10 μM. In general, the results for A27 agree with those from previous studies (Chung et al., 1998;Hsiao, Chung, and Chang, 1998). As a second approach, we employed indirect immunofluorescence microscopy to visualize binding of the proteins to cells (Fig. 5B and Supplementary Fig. 1). In agreement with the CELISA data, soluble A28 did not bind to cells, even at 10 μM. Here we conclude that A28 is not likely to be a high-affinity attachment factor or a RBP, consistent with findings of other reports (Senkevich, Ward, and Moss, 2004;Turner et al., 2007). In contrast, soluble L1, A27 and control gD bound to cell surfaces, with preferential association of soluble A27 at cell-cell junctions.

Soluble L1 and A27 bind to cell surfaces independently of glycosaminoglycans

Studies have shown that cellular HSPG is the binding partner of A27 (Chung et al., 1998; Ho et al., 2005; Hsiao, Chung, and Chang, 1998; Lin et al., 2002). VACV L1 may attach to proteoglycans akin to A27. Alternatively, it may bind to important and distinct non-GAG receptor(s) that activate subsequent steps of entry. Indeed, soluble L1 appear to have a cell-binding pattern which is unlike that of soluble A27, with A27 localizing preferentially to cell-cell junctions when visualized by microscopy (Fig. 5B). To determine if soluble L1 binds to proteoglycans or non-proteoglycan receptors, we first compared the binding of soluble L1 to gro2C and sog9 cells, which are L cell mutants deficient in HSPG, or HSPG and CSPG synthesis respectively (Banfield et al., 1995; Gruenheid et al., 1993). Soluble HSV gC, which adheres to HSPG (Tal-Singer et al., 1995), served as a control while soluble A27 was also tested for comparison. Soluble L1 bound less well to gro2C cells than parental L cells (1.6 fold decrease in binding at 10 μM) (Fig. 6A). In addition, soluble L1 bound less well to sog9 cells than gro2C cells (1.8 fold decrease at 10 μM). Hence, our data suggest that both HSPG and CSPG contribute to cell surface attachment of L1. However, there was significant residual binding of L1 and A27 to sog9 cells, in contrast to what was found for HSV gC (Fig. 6C). In fact, soluble L1 and A27 still bound to the surface of these GAG-deficient cells in a dose-

dependent manner (Fig. 6A and B), suggesting that L1 and A27 also bind to non-GAG receptor (s) on cells. Similar results were reported for bacterial A27 in another study using FACS analysis (Hsiao, Chung, and Chang, 1999).

To pursue this hypothesis further, we next asked if pre-incubation with soluble GAGs could eliminate cell surface binding of soluble L1 or A27. If they bind to non-GAG receptors, these proteins should still attach to cells even in the presence of high concentrations of GAGs. Again, we used HSV gC as a control. Cell surface attachment by L1 or A27 was partially blocked by incubation with increasing quantities of soluble heparin (Fig. 6D, filled diamonds and filled circles, respectively). However, complete abrogation of cell surface binding was never achieved. 66% of the binding of soluble L1 and 39% of the binding of A27 could not be competed by heparin added at a high concentration (6.25 μg/ml). Similar results were obtained in experiments using heparin at concentrations as high as 25 μg/ml (data not shown). On the other hand, binding of HSV gC to cells was virtually abolished by its incubation with soluble heparin (Fig. 6D, filled triangles), in agreement with published data (Tal-Singer et al., 1995). Pre-incubation of L1 or A27 with soluble chondroitin sulfate had no effect on binding of the proteins to cells (Fig. 6D, open diamonds and open circles respectively). When L1 was pre-incubated with a combination of heparin and chondroitin sulfate, its binding was not reduced further than it was with heparin alone (data not shown). In sum, although L1 and A27 exhibit GAG-binding properties, they also bind to cell surface molecules which are not proteoglycans.

Soluble L1, but not A27, blocks virus entry independently of glycosaminoglycans at the adsorption step

Our comparisons of L1 and A27 thus far suggest that both proteins may function similarly in entry. Antibodies to L1 or A27 inhibit virus entry, soluble forms of both proteins bind to cell surfaces independently of GAGs and both proteins also block entry of VACV. Since it has been reported that the ability of A27 to block entry depends on its GAG-binding domain (Ho et al., 2005; Hsiao, Chung, and Chang, 1998), it should not block VACV entry into cells devoid of proteoglycans. On the other hand, if soluble L1 blocks entry by sequestering non-GAG entry receptors, it should still inhibit virus entry into GAG-deficient cells. More importantly, this inhibition by soluble L1 in GAG-deficient cells should occur specifically at the step of virus adsorption. In a virus-cell binding assay, soluble L1 should be able to hinder the binding of virions to cell surfaces which are devoid of GAGs. To test these possibilities, we compared the abilities of soluble L1, A27 and A28 to block cell surface binding and entry of VACV in parental L and mutant sog9 cells.

As was observed in BSC-1, HeLa and B78H1 cells (Fig. 3 and 4), soluble A28 had no effect on the entry of VACV into L or sog9 cells (Fig. 7A). Consistent with this observation, A28 also did not alter the binding of VACV to these cells (Fig. 7D). As predicted, soluble A27 blocked VACV entry in sog9 cells to a much lesser extent than in L cells (2.3 fold difference in efficacy at 10 μM) (Fig. 7B). The difference in efficacy observed between the two cell types was more drastic when the inhibition of virus-cell binding was assessed (6.7 fold difference in efficacy at 20 μM) (Fig. 7E). In fact, there was minimal loss of VACV binding in sog9 cells when soluble A27 is added.

Interestingly, soluble L1 blocked entry to a similar extent in both cell types (Fig. 7C). 10 μM of soluble L1 blocked entry of VACV into L and sog9 cells by 48% and 51% respectively. Importantly, this inhibition of entry by soluble L1 is due to its ability to block the binding of VACV to surfaces of cells (Fig. 7F), including cells devoid of proteoglycans. The reduction in virus-cell binding also appeared to be more efficacious in the presence of proteoglycans. 20 μM of soluble L1 abrogated virus binding to L cells by 81% while the same amount of L1 reduced virus-cell association by 63% in sog9 cells.

Our data demonstrate that the ability of A27 to inhibit VACV entry is primarily due to its binding to proteoglycans (Ho et al., 2005; Hsiao, Chung, and Chang, 1998). On the contrary, the ability of L1 to block cellular binding and entry of VACV is independent of its GAG-binding property, supporting the hypothesis that unlike the HSPG-binding VACV A27, VACV L1 functions in entry by binding to non-GAG molecule(s) on cell surfaces.

Anti-L1 MAbs block virus entry independently of glycosaminoglycans

A panel of monoclonal antibodies (MAbs) to L1 have previously been characterized (Aldaz-Carroll et al., 2005; Ichihashi, 1996; Wolffe, Vijaya, and Moss, 1995). Based on competitive-binding assays of peptides of L1, the MAbs have been classified into two neutralizing groups and one non-neutralizing group (Aldaz-Carroll et al., 2005). VMC-2, VMC-3 and VMC-5 belong to the neutralizing Group 1 while VMC-4 and VMC-35, which bind to discontinuous epitopes, are in non-neutralizing Group 2. 7D11, which also binds to non-linear epitopes (Su et al., 2007), belongs to another group of neutralizing MAbs (Group 3). We wanted to test whether the neutralizing activity of these antibodies is contingent upon the presence of cell surface GAGs. If these neutralizing MAbs obstruct binding of VACV L1 to its cell surface non-GAG receptor(s), but not to proteoglycans, their neutralization profiles should be uniform in all L cell types, regardless of differences in proteoglycan expression. As illustrated in Figure 8, the neutralizing activity of both groups of neutralizing MAbs virtually remained unchanged in all L cell types assessed (compare Fig. 8A and B), indicating that anti-L1 MAbs block virus entry via a mechanism that is independent of cell surface proteoglycans.

Furthermore, we considered the possibilities that the neutralizing MAbs hinder entry of VACV either by forming non-functional L1 dimers on viral surfaces, or by aggregating virions together. We therefore generated Fab fragments of the antibodies and examined their ability to inhibit virus entry. The neutralization activities of Fab antibodies from Group 1 (VMC-2 and VMC-3) were retained in cells which do not express proteoglycans (Fig. 8C and data not shown). The IC_{50} values for VMC-2 Fab and IgG were approximately 4 and 0.07 μg/ml respectively. The diminished neutralization strength of the Fab antibodies is not surprising, since avidity is lost once an antibody is converted from a divalent to a monovalent form. In agreement with plaque reduction studies using 7D11-Fab (Su et al., 2007), we conclude that MAbs do not neutralize virus by causing inter and intra-virion crosslinking of L1. Rather, we suggest virus entry is impeded by MAbs blocking binding of L1 to its non-GAG molecule(s). These results, together with our other observations, strongly suggest that unlike VACV A27, VACV L1 may function in virus entry by binding to cellular receptor(s) which are unique from proteoglycans.

DISCUSSION

Role of L1 in the entry of VACV

Central to our current understanding of the entry of animal viruses is the concept that it is a highly regulated, sequential process that is triggered by signal(s) originating from the host cell (Weissenhorn, Hinz, and Gaudin, 2007). One common trigger for entry of enveloped viruses is the binding of a specific viral RBP to its host entry receptor. Upon binding, the RBP undergoes conformational changes to effect subsequent steps of the entry process, resulting in the fusion of the viral and host membranes (Dimitrov, 2004; Reske et al., 2007; Weissenhorn, Hinz, and Gaudin, 2007). Previous studies have shown that several MV envelope proteins, including A27, H3, D8 and A26, attach to cell surface GAGs or the extracellular matrix protein laminin, and that soluble forms of A27, H3 and D8 block entry of VACV (Chiu et al., 2007; Chung et al., 1998; Hsiao, Chung, and Chang, 1999; Lin et al., 2000). These studies have highlighted the importance of proteoglycans and laminin for the attachment phase of the VACV entry process, but it is unclear whether these interactions between the VACV envelope proteins

and their cognate receptors also serve as signals to activate membrane fusion. In any case, these MV proteins are non-essential for entry of VACV, and entry in GAG-deficient cells cannot be completely blocked by soluble laminin (Chiu et al., 2007), implying the presence of an alternative receptor-ligand interaction that triggers VACV entry.

In this study, we found that soluble L1 bound to cell surfaces and blocked virus entry at the adsorption step (summarized in Table 1). Based on our binding curves, the affinity of L1 with cell surfaces was in the micromolar range and the binding did not saturate. The binding affinity between a viral RBP and its cognate receptor typically varies depending on the abundance as well as the type of receptor molecules on the particular cell type assessed. In some instances, such as HIV, the affinity between CD4 and gp120 is relatively high (nM range) and binding of CD4 to cells is saturable (Lasky et al., 1987). For HSV gD, the affinity is low (μM range) and receptor abundance differs according to the cell type (Fig. 5) (Spear, 2004;Willis et al., 1998). It is likely the abundance of the cellular binding partner(s) of L1 is relatively low, at least in the cell types we studied.

Our data suggest that a fraction of the cell surface association of L1 is due to proteoglycans, since cellular binding by L1 is partially inhibited by soluble heparin and is reduced in GAG-deficient cells. Indeed, soluble L1 adhered to heparin directly (Supplementary Fig. 2), although its affinity to heparin seemed more modest than that of A27. Nevertheless, a significant portion of the cell surface binding by L1 cannot be accounted for by GAGs, indicating the presence of alternative interactions between L1 and cell surface targets which are non-GAG in nature. The bifunctional binding behavior of L1 is not surprising since there are precedence from other viral RBPs (Bender et al., 2005; Summerford, Bartlett, and Samulski, 1999; Vives et al., 2005). Importantly, soluble L1 still blocked both entry and virion binding in GAG-deficient cells. This finding distinguishes L1 from A27, which exhibited GAG-dependent characteristics in these assays. To our knowledge, this is the first report of a VACV envelope protein that blocks cellular binding and entry independently of GAGs. Since the inhibition of entry by soluble L1 occurs specifically at the step of virus adsorption, we propose that VACV L1 functions in entry as a RBP by engaging a cognate non-proteoglycan cellular receptor.

Although it was previously reported that L1 is required for assembly and morphogenesis (Ravanello and Hruby, 1994), a new study recently characterized a conditional lethal L1 null mutant virus which demonstrated a defect in entry and VACV-induced cell-cell fusion, but not in assembly (Bisht, Weisberg, and Moss, 2008). Our hypothesis regarding L1 would be more consistent with the phenotype of the L1 null mutant virus described in the later study by Bisht, Weisberg, and Moss (2008).

The crystallographic structure of the L1 ectodomain offers some important insights into its function(s) (Su et al., 2005). The protein has five α-helices packed against two double-stranded β-sheets (Fig. 8D). It lacks structural features that are characteristic of known viral membrane fusion proteins (Heinz and Allison, 2001; Skehel and Wiley, 2000). Even though intermolecular interactions between L1 and components of the proposed entry-fusion complex have been reported (Bisht, Weisberg, and Moss, 2008), L1 is not required for the stability or assembly of the complex and hence is not considered to be an integral component of the complex (Bisht, Weisberg, and Moss, 2008; Brown, Senkevich, and Moss, 2006; Senkevich et al., 2005). Furthermore, a recent study demonstrated that the A17-A27 protein complex is sufficient to induce cell-cell fusion (Kochan et al., 2007). Thus, it is unlikely that L1 plays a direct role in membrane fusion as a viral fusion protein during entry.

Interestingly, neutralizing epitopes of L1 are confined to loops 1, 2 and 3 of its structure. These discontinuous loops may serve as domains for interaction with the non-GAG receptor (Fig. 8D, red residues) (Aldaz-Carroll et al., 2005;Ichihashi and Oie, 1996;Su et al., 2007). We also

showed that the virus neutralizing activity of anti-L1 MAbs does not rely on the presence of proteoglycans. The nature of the L1 receptor and the domains on L1 which bind the receptor are subjects for future investigations.

Role of A28 in the entry of VACV

It was proposed that components of the EFC, such as A28, are unlikely to mediate attachment to cells since viruses lacking these proteins bind to cell surfaces normally even though they cannot enter cells (Izmailyan et al., 2006; Ojeda, Domi, and Moss, 2006; Ojeda, Senkevich, and Moss, 2006; Senkevich and Moss, 2005; Senkevich et al., 2005; Senkevich, Ward, and Moss, 2004; Townsley, Senkevich, and Moss, 2005a; Townsley, Senkevich, and Moss, 2005b). However, functional redundancy in the cell surface binding machinery of VACV may compensate for any potential loss in binding by these mutant virions. Such a mechanism was proposed for an A27-null mutant virus that bound to cells as well as its parental virus strain (Hsiao, Chung, and Chang, 1999). Hence, experiments that assess the cell-surface binding of purified, soluble whole EFCs or its individual components are warranted to address this issue.

In this study, we found that the isolated A28 protein did not bind to cell surfaces nor did it block VACV binding and entry (summarized in Table 1). It is unlikely that the N-linked oligosaccharides found on the recombinant A28 masked any inherent cell binding motifs and interfered with our analyses, since recombinant A28 which was deglycosylated with PNGase F also did not exhibit any phenotype in our cell-binding or inhibition of entry assays (Supplementary Fig. 3). Therefore, we conclude that A28 is unlikely to be a RBP.

We found that PAbs to A28 inhibit entry of VACV, suggesting that this protein functions at a post-receptor binding step such as membrane fusion. Nelson, Wagenaar and Moss (2008) recently provided evidence for a direct interaction between A28 and another component of the EFC, H2 (Nelson, Wagenaar, and Moss, 2008). Their findings showed that this interaction is dependent on a conserved sequence in H2 which shares some similarity with fusion peptides from other viral membrane fusion proteins. The authors postulated that A28 stabilizes the putative fusion peptide in H2 during the prefusion state of the EFC by concealing it from the aqueous environment. It may be possible that PAbs to A28 neutralize virions by impeding the dissociation between A28 and H2 when the EFC converts from its prefusion to fusogenic intermediate state.

Interestingly, while antibodies to L1 and A27 blocked virus entry as much as 95% at the highest dose examined (100 μg/ml) (data not shown), antibodies to A28 inhibited entry by no more than 60%, even at high concentrations. Similar results were obtained for a different anti-A28 PAb which we harvested from another A28-immunized rabbit (data not shown). It is possible there is an alternative entry mechanism or pathway that is resistant to anti-A28 antibodies. Overall, our results corroborate key findings from a recent study which also tested neutralization effects of different PAbs to A28 (Nelson et al., 2008).

Role of A27 in the entry of VACV

A27 is a well studied MV envelope protein and was one of the first identified cellular attachment proteins of VACV (Lai, Gong, and Esteban, 1990), even though it is non-essential for virus entry (Hsiao, Chung, and Chang, 1999). A recombinant form of A27 which was expressed in bacteria blocked binding and entry of VACV (Hsiao, Chung, and Chang, 1998). The inhibition of entry by A27 was dependent upon its N-terminal GAG-binding region, since a mutant A27 protein lacking this domain did not block entry (Hsiao, Chung, and Chang, 1998). Here, we found that recombinant A27 derived from insect cells also blocked cellular binding and entry of VACV. The inhibitory effects are primarily due to the A27-GAG interaction because they were alleviated in GAG-deficient cells. Nevertheless, we observed

some residual inhibition of entry by soluble A27 in these cells (Fig. 7B), which could be due to high concentrations of soluble A27 disrupting the non-covalent interactions between the virion A17 and A27 complex.

Our cell surface binding data for A27 also agree with those for a bacterial form of A27 (Hsiao, Chung, and Chang, 1998; Hsiao, Chung, and Chang, 1999). Binding of soluble baculovirus A27 to cells is saturable, suggesting specific interactions with unique proteoglycans, such as heparan sulfates with specific configurations. In addition, the affinity of A27 to cell surfaces appeared to be relatively high, with estimated K_d values varying within the nanomolar range depending on the cell type analyzed.

In conclusion, our studies show that L1 fulfills three criteria for a viral RBP: (i) antibodies to L1 inhibit virus entry, (ii) soluble forms of L1 block virus entry at the adsorption step and (iii) the L1 protein binds to cell surfaces. We postulate that VACV L1 functions in entry by engaging specific cell surface receptor(s) which are unique from proteoglycans. Further investigations with L1 may facilitate the discovery of novel poxvirus entry receptor(s) and improve our understanding of how poxviruses trigger membrane fusion.

MATERIALS AND METHODS

Cells and viruses

BSC-1, HeLa, Vero, B78H1, murine fibroblast parental L, mutant gro2C and sog9 cells (Banfield et al., 1995; Gruenheid et al., 1993) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). VACV vSIJC-20 (Western Reserve strain, **NC_006998**) (S. N. Isaacs, unpublished data), a recombinant virus with the *lacZ* gene under the control of the P7.5 early-late VACV promoter was used in this study. For preparation of gradient-purified virus, BSC-1 cells were infected at a MOI of 0.05 PFU/cell and incubated at 37°C until cytopathic effect was complete (3-4 days). The infected cell cultures were homogenized on ice and its supernatant was clarified of cellular debris by centrifugation at 2000 × g for 30 min at 4°C. The supernatant was then centrifuged at 25,000 × g for 2 h at 4° C and the recovered pellet was resuspended in cold 10 mM Tris (pH 9.0), sonicated and loaded atop a stepwise gradient of 60%, 36% and 15% sucrose prepared in 10 mM Tris (pH 9.0). The step gradient was centrifuged at 40,000 × g for 4 h and banded virus was harvested via side puncture of the tube. Collected virus was further concentrated by pelleting through a cushion of 36% sucrose for 2 h at 5000 × g. The resulting pellet was finally resuspended in 1 ml of 10 mM Tris (pH 9.0), sonicated, stored at − 80 °C and titers were determined by plaque assay with BSC-1 cells (Aldaz-Carroll et al., 2005). L cells and its mutant derivatives were generous gifts from F. Tufaro and B. W. Banfield. VACV vSIJC-20 was kindly provided by S. N. Isaacs.

Antibodies and reagents

Rabbit polyclonal antibodies (PAbs) R193/R194 and R203/R204 were produced by immunizing rabbits with purified recombinant A27 and A28 proteins respectively, using procedures described previously for raising PAbs against herpes simplex virus (HSV) glycoproteins (Krummenacher et al., 1998). Preimmune antibody (control) was harvested from a rabbit prior to immunization. Productions of rabbit PAbs R180 and R47, raised against recombinant L1 and HSV-1 gC respectively, were reported in previous studies (Aldaz-Carroll et al., 2005; Eisenberg et al., 1987). Rabbit serum R8 was raised against HSV-2 gD (Isola et al., 1989) while R236 was raised against peptides derived from the VACV A4 core protein (J. C. Whitbeck et al., submitted for publication). Monoclonal antibodies (MAbs) VMC-2, VMC-3, VMC-4, VMC-5 and VMC-35 were selected from a panel of monoclonal antibodies against purified recombinant L1 (Aldaz-Carroll et al., 2005). MAb 7D11, raised against VACV, was kindly provided by A. L. Schmaljohn (A. L. Schmaljohn, J. W. Hooper and S. A.

Harrison at the 1989 Annual Meeting of the American Society for Virology) (Wolffe, Vijaya, and Moss, 1995). Immunoglobulin G (IgG) was purified from rabbit sera and murine ascites fluid with HiTrap protein G 1-ml columns (Amersham Pharmacia Biotech) and dialyzed against phosphate buffered saline (PBS). Fab fragments were generated from purified IgG using the Immunopure® Fab Preparation Kit (Pierce). The anti-tetrahistidine MAb was purchased from Qiagen Inc. Horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit secondary antibodies were from Kirkegaard and Perry Laboratories. Anti-VACV rabbit hyper-immune serum was kindly provided by S. N. Isaacs (Isaacs, Kotwal, and Moss, 1992).

Production and purification of recombinant proteins

(i) Novel proteins - VACV A28, F9 and variola virus M1—The A28L and F9L open reading frames (ORF) were PCR amplified from the respective cloned HindIII A and F fragments of the VACV (Western Reserve strain, **NC_006998**) genome. The putative transmembrane domains of the full-length A28L (residues 4-26) and F9L ORFs (residues 174-196) were excluded and only their ectodomains (residues 23-146 and 1-175 respectively) were expressed. For A28, the forward primer used for PCR amplification was: 5'-gcg**ggatcc**acaccaccaccaccaccacggttactcaatatatgaaaat-3'(adds six histidine codons (underlined) to the 5' end of A28L ORF as well as a BamHI site (bold)) and the reverse primer was 5'-gcg**gaattc**aaagtacagattttagaaa-3' (adds EcoRI (bold)). Similarly for F9, the forward primer for PCR amplification was: 5-gc**tgatca**catggcggaaactaaagagttt-3' (adds BclI site (bold)) and the reverse PCR primer was 5'-gcgc**gaattc**agtgatgatggtgatgatgatcgtaatacggcaatcggttt-3' (adds 6 histidine codons (underlined) to the 3' end of F9L ORF as well as an EcoRI site (bold)). The final PCR products were either digested with BamHI and EcoRI or BclI and EcoRI respectively, and subsequently ligated into the baculovirus transfer plasmid, pVT-Bac (Tessier et al., 1991) which was digested with the same set of restriction enzymes. Therefore, the resulting recombinant plasmids contained ORFs consisting of the melittin signal sequence followed, in frame, either by the six histidine codons and the ectodomain of A28 (codons 23-146), or the F9 ectodomain (codons 1-175) and then six histidine codons. A different cloning strategy was used for generating the pVT-Bac plasmid which encodes recombinant variola virus M1. The ectodomains of variola virus M1 (Variola major Bangladesh-1975 strain, L22579) and VACV L1 (Western Reserve strain, **NC_006998**) differ in a single amino acid residue (R177 in M1 versus K177 in L1). Hence, the K177 codon in the truncated L1R ORF in a previously created recombinant pVT-Bac plasmid (Aldaz-Carroll et al., 2005) was mutated to an arginine codon using the QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems) as described previously (Connolly et al., 2002). Thus, similar to recombinant L1(185t), the putative transmembrane domain of the full-length M1R ORF (residues 186-204) was excluded and only the ectodomain (residues 1-185) was expressed. The forward primer for QuikChange mutagenesis was: 5-ctcaaatagcacct**aga**caagttgctgg-3' (substitutes into arginine codon (bold)) and the reverse primer was 5'-ccagcaacttg**tct**aggtgctatttgag-3' (substitutes into arginine codon (bold)). The resulting plasmid contained ORF consisting of the melittin signal sequence followed, in frame, by the M1 ectodomain (codons 1-185 with arginine codon at position 177) and then six histidine codons.

The resulting pVT-Bac plasmids encoding recombinant A28, F9 and M1 were co-transfected with baculovirus DNA (BaculoGold, BD-Pharmingen) into Sf9 insect cells. After one week, recombinant baculoviruses were harvested. The viruses were then plaque purified and high titer stocks were prepared (Rux et al., 1998). The recombinant proteins were secreted from infected insect cells and purified from supernatants using nickel-chelate affinity chromatography, as described previously (Rux et al., 1998). For Western blot and silver stain analysis, purified recombinant proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under native (0.1% SDS, no reducing agent,

no boiling) or reduced (boiled 10 min in 2.5% SDS, 350 mM β-mercaptoethanol) conditions in precast Tris-glycine gels (Invitrogen). Following SDS-PAGE, separated proteins were either visualized by staining with silver nitrate (Pharmacia) or were transferred to a nitrocellulose membrane and then probed with antibodies and visualized by ECL (Amersham).

(ii) Previously characterized proteins - VACV L1, A27, A33 and HSV glycoproteins—The production and purification of recombinant VACV L1(185t), A27, A33 (58-185), HSV-1 gD(306t) and gC(457t) were described elsewhere (Aldaz-Carroll et al., 2005; Rux et al., 2002; Rux et al., 1998; Xiao et al., 2007). The purified soluble proteins were stored frozen at − 80 °C in PBS.

Inhibition of virus entry by antibodies

Purified complete IgG or Fab fragments were serially diluted in DMEM containing 5% heat-inactivated FCS and then mixed with an equal volume of reporter virus VACV vSIJC-20. The virus-antibody mixture was incubated for 1 h at 37 °C and then used to infect BSC-1, parental L, gro2C or sog9 cells at a multiplicity of infection (MOI) of 0.5 PFU/cell in 96-well plates. After 6 h at 37 °C, β-galactosidase expression was determined in a β-galactosidase entry assay as described previously (Whitbeck et al., 1997). Cells were lysed by the addition of an equal volume of PBS containing NP-40 (1.5% final concentration). Lysates were then mixed with the β-galactosidase substrate (chlorophenol red-ß-D-galactopyranoside; Roche) in a separate 96-well plate and absorbance was measured at 570 nm in a microtiter plate reader. Data were collected at 5-min intervals over 50 min and the amount of entry was calculated from the rate of substrate conversion over time. After subtraction of background values, net values from wells where antibody was not added (control) was set to 100% and virus entry was presented as a percent of control. For experiments using Fab, the concentration of the Fab antibodies were normalized to reflect the equivalence of two moles of Fab subunit to one mole of complete IgG. Results presented are representative of a series of at least two independent experiments.

Inhibition of virus entry by recombinant proteins

(i) β-galactosidase entry assay—Purified recombinant proteins (soluble L1, A27, A28, F9 or M1) were serially diluted in DMEM containing 10% FCS and 30 mM HEPES (DMEM-H) and then added to prechilled cell monolayers grown in 96-well plates. After 1 h at 4 °C, an equal volume of reporter virus VACV vSIJC-20 (diluted in DMEM-H) or control DMEM-H without virus was added to the cells at a MOI of 2.5 PFU/cell and incubated for another 1 h at 4 °C to allow adsorption. Cells were washed with cold DMEM-H to remove unbound virions, refed with warm media and then transferred to 37 °C to allow synchronous penetration to proceed. After 6 h at 37 °C, β-galactosidase expression as a measure of entry was assessed as described above. In experiments designed to address the possibility that soluble proteins inhibited *lacZ* expression by affecting post-entry steps, the standard protocol described above was also modified. Unlike the standard protocol, VACV vSIJC-20 (diluted in DMEM-H) was first allowed to adsorb to prechilled BSC-1 cell monolayers at a MOI of 2.5 in 96-well plates. After 1 h at 4 °C, cells were washed to remove unbound virions, refed with warm media and then transferred to 37 °C. An equal volume of purified proteins [20 μM] diluted in DMEM-H at RT were added to the cells either shortly before transfer to 37°C, or at 30 min post transfer to 37°C. β-galactosidase activity was measured at 6 h post transfer to 37°C in both alternative protocols. Unless otherwise stated, results presented are representative of a series of at least three independent experiments.

(ii) Immunofluorescence microscopy of intracellular cores—20 μM of purified proteins (soluble L1, A27 or A28) were serially diluted in DMEM-H and then added to prechilled HeLa cell monolayers grown on glass coverslips in 24-well plates. After 1 h at 4 °C, VACV vSIJC-20 (diluted in DMEM-H) or control DMEM-H without virus was added to

the cells at a MOI of 50 PFU/cell and incubated for another 1 h at 4 °C to allow adsorption. Cells were washed with cold DMEM-H to remove unbound virions, refed using warm media with 100 μg/ml of cycloheximide and then transferred to 37 °C to allow synchronous penetration to proceed. After 2 h at 37 °C, cells were rinsed with ice-cold DMEM-H, fixed with cold 3% paraformaldehyde and washed again with PBS. Subsequent steps were performed at room temperature. Cells were quenched with 50 mM NH_4Cl for 10 min, permeabilized using 0.1% saponin dissolved in PBS and incubated with blocking solution (10% goat serum in PBS) for 1 h. Cells were incubated for 45 min with 0.25 μg/ml of anti-A4 rabbit antibody IgG (R236) which was diluted in blocking solution. Following rinses with PBS, cells were further incubated for 30 min with fluorescent-tagged anti-rabbit antibodies (GAR-488) and TO-PRO®-3 iodide (Molecular Probes) diluted 1:1000 and 1:10000 respectively in blocking solution. After the final washing step with PBS, cells were rinsed once with distilled water. Coverslips were extracted from the plates and mounted onto microscope slides in ProLong Antifade mounting solution (Molecular Probes). Cells were observed under a Nikon TE2000-U inverted microscope coupled to a PerkinElmer confocal imaging system. A two-line argon krypton laser emitting at 488 and 568 nm was used. Images from multiple optical sections perpendicular to the z-axis were captured for each field at 1000 × magnification. The extent of viral penetration was determined by counting all intracellular cores present in an average of 30 cells per coverslip.

Binding of soluble proteins to the cell surface

(i) CELISA—We used a modification of a cellular enzyme-linked immunosorbent assay (CELISA) (Connolly et al., 2002). Purified recombinant proteins (soluble L1, A27, A28, gD or gC) were serially diluted in DMEM-H and then added to prechilled, confluent BSC-1 cell monolayers grown in 96-well plates. After 1 h at 4 °C, cells were rinsed with ice-cold DMEM-H, fixed with cold 3% paraformaldehyde and washed again with PBS supplemented with Ca^{++} and Mg^{++} (PBS+). Subsequent steps were next performed at room temperature. Cells were incubated for 1 h with 2.5 μg/ml of R180, R193, R204 antibody IgGs or 1:2000 of R8 rabbit sera which were diluted in PBS+ containing 3% bovine serum albumin (PBS-B) (BSA Fraction V; Roche). Following more rinses with PBS+, cells were further incubated for 45 min with HRP-conjugated anti-rabbit antibodies diluted 1:1000 in PBS-B. After the final washing step with PBS+ containing 0.1% Tween-20, cells were rinsed once with 20 mM citrate buffer, pH 4.5. 2,2'-Azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) peroxidase substrate (Moss, Inc.) was added, and the absorbance at 405 nm was measured at multiple time points with a micro titer plate reader. Unless otherwise stated, results presented are representative of a series of at least three independent experiments.

(ii) Immunofluorescence microscopy—10 μM of purified proteins (soluble L1, A27, A28 or gD) diluted in DMEM-H or control DMEM-H without protein were added to prechilled, confluent BSC-1 cell monolayers seeded on glass coverslips in 24-well plates. After 1 h at 4 °C, cells were rinsed with ice-cold DMEM-H, fixed with cold 3% paraformaldehyde and washed again with PBS+. Cells were quenched with 50 mM NH_4Cl for 15 min and incubated with blocking solution (10% goat serum in PBS+) overnight at 4 °C. Subsequent steps were performed at room temperature. Cells were incubated for 45 min with 5 μg/ml of R180, R193, R204 antibody IgGs or 1:1000 of R8 rabbit sera which were diluted in blocking solution. Following rinses with PBS+, cells were further incubated for 30 min with fluorophore-tagged anti-rabbit antibodies diluted 1:1000 in blocking solution. After the final washing step with PBS+, cells were rinsed once with distilled water. Coverslips were extracted from the plates and mounted onto microscope slides in ProLong Antifade mounting solution with DAPI (4', 6'-diamidino-2-phenylindole) (Molecular Probes). Cells were observed under a Nikon Eclipse E600 microscope and images were captured at 20 × magnification.

Glycosaminoglycan inhibition studies

Serially diluted heparin or chondroitin sulfate A (Sigma) or both were incubated with 3.75 μM of purified soluble L1 or 0.375 μM of soluble A27 or gC in DMEM-H for 1 h at 4 °C. The protein-glycosaminoglycan (GAG) mix was then added to prechilled, confluent cell monolayers cultured in 96-well plates. After another 1 h at 4 °C to allow binding of proteins to cells, cells were rinsed with ice-cold DMEM-H, fixed with cold 3% paraformaldehyde and examined by CELISA for bound recombinant proteins.

Binding of VACV to the cell surface (virus-CELISA)

A cellular enzyme-linked immunosorbent assay (CELISA) was adapted for the quantification of cell-bound VACV (Connolly et al., 2002). Recombinant proteins (soluble L1, A27 or A28) were serially diluted in DMEM-H and then added to prechilled, confluent parental L or derivative sog9 cell monolayers grown in 96-well plates. After 1 h at 4 °C, an equal volume of VACV vSIJC-20 (diluted in DMEM-H) or control DMEM-H without virus was added to the cells at a MOI of 25 PFU/cell and incubated for another 1 h at 4 °C to allow adsorption. Cells were rinsed with ice-cold DMEM-H, fixed with cold 3% paraformaldehyde and washed again with PBS+. Subsequent steps were next performed at room temperature. Cells were incubated for 1 h with 2.5 μg/ml of either anti-A27 monoclonal antibody IgG (VMC-38) (J. C. Whitbeck et al., unpublished data) for wells added with soluble L1 or A28 proteins, or anti-L1 monoclonal antibody IgG (VMC-2) for wells added with soluble A27 proteins. Antibodies were diluted in PBS-B. Following more rinses with PBS+, cells were further incubated for 45 min with HRP-conjugated anti-mouse antibodies diluted 1:1000 in PBS-B. After the final washing step with PBS+ containing 0.1% Tween-20, cells were rinsed once with 20 mM citrate buffer, pH 4.5. ABTS peroxidase substrate (Moss, Inc.) was added, and the absorbance at 405 nm was quantified using a micro titer plate reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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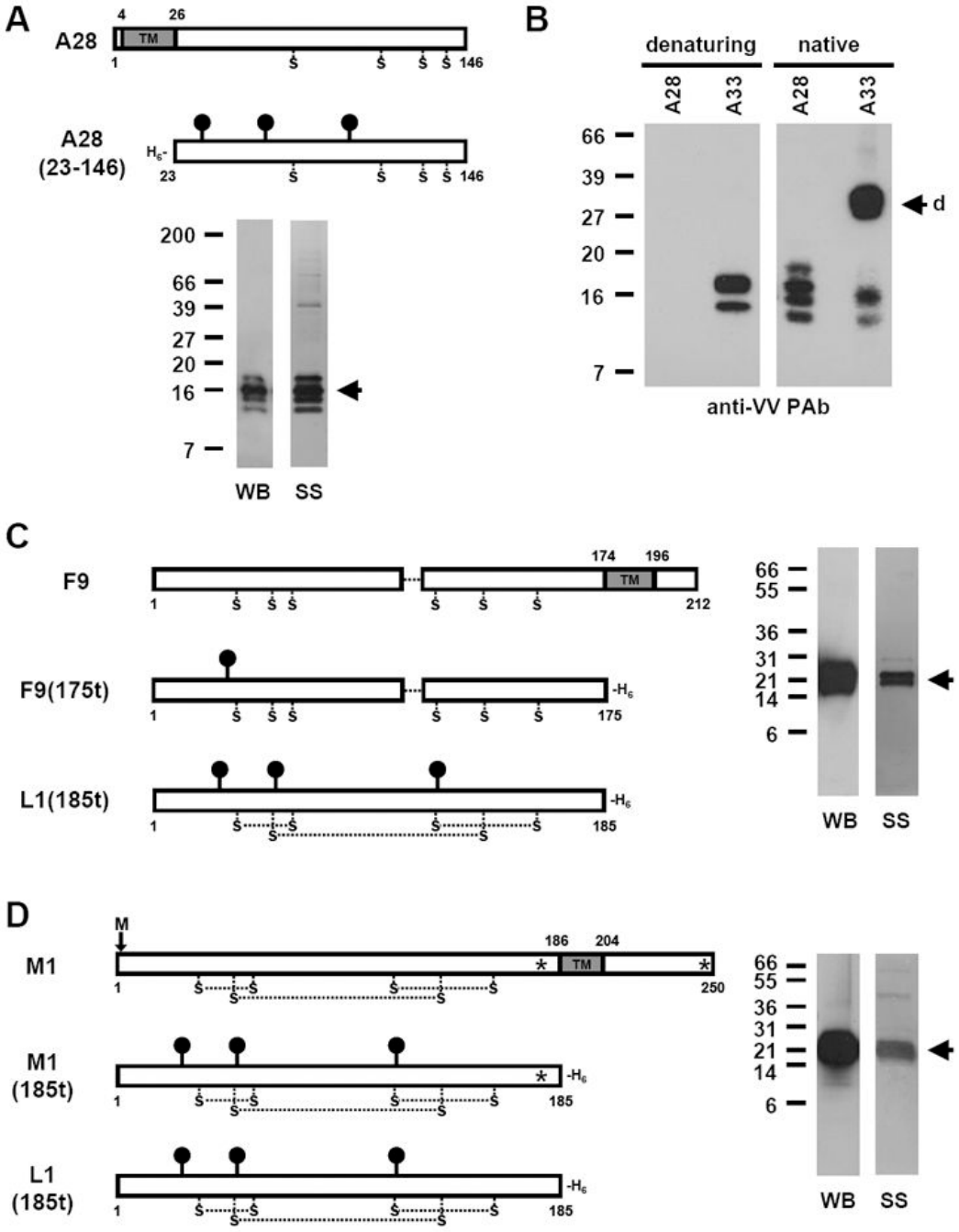

Fig 1.

Recombinant VACV proteins produced in the baculovirus expression system. Schematic diagrams depict the full length (top) or the truncated, recombinant form (second from top) of VACV A28 (A), F9 (C) and variola virus M1 (D). In C and D, the diagram of soluble L1 (bottom) was also shown for comparison. Grey rectangles indicate putative transmembrane domains (TM), black lollipops represent sites for N-linked glycosylation potentially used in the secreted recombinant proteins, dotted line in (C) depicts the region of L1 which shares no homology with F9 (C), M points to the myristoylation site (D), S symbolizes conserved cysteines or intramolecular disulfide bonds (C and D) and H6 represent the six histidine tail used for affinity purification. Asterisks in (D) point to the two residues in variola virus M1 that

are different in VACV L1 (R177 and I248 in M1 versus K177 and M248 in L1). Secreted, recombinant A28 (A), F9 (C) or M1 (D) were purified by nickel chelate affinity chromatography, separated by SDS-PAGE under reducing conditions and stained with silver nitrate (SS), or transferred to a nitrocellulose membrane and then probed with a monoclonal antibody against the His-tag (WB). Migration positions of the recombinant proteins are indicated with arrows. The molecular weights of the markers are shown in kilodaltons. Note that recombinant L1 and M1 are not expected to be myristoylated since the sequence for a melittin signal peptide was appended to the 5' end of their ORFs (Aldaz-Carroll et al., 2005). (B) Recombinant A28 shares conformational epitopes with the authentic VACV A28. Purified soluble A28 or control A33 (Xiao et al., 2007) was resolved by SDS-PAGE under denaturing or native conditions, transferred to membranes and probed with a rabbit hyper-immune serum raised against VACV. Arrow points to the putative A33 dimer.

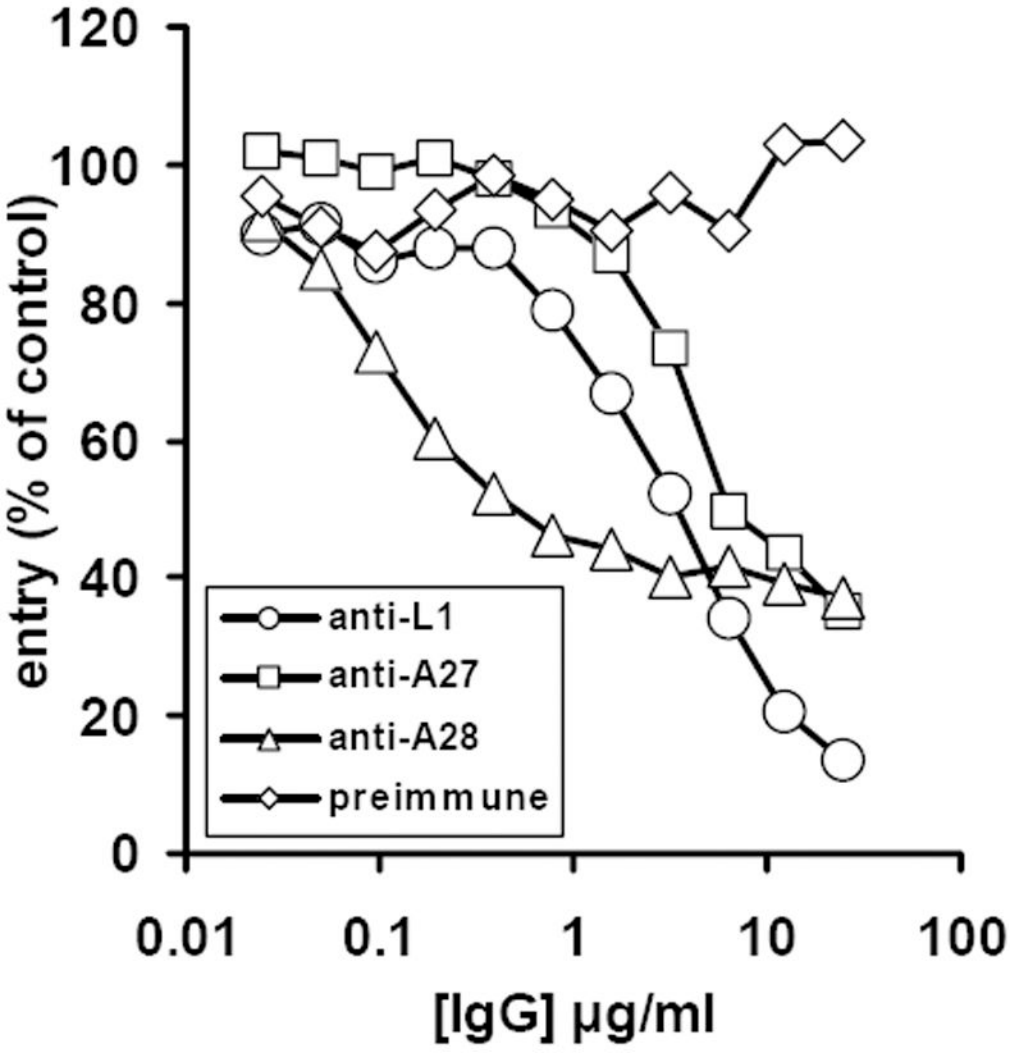

Fig 2.

Antibodies to L1, A27 and A28 inhibit entry of VACV. Rabbits were immunized with purified recombinant proteins L1, A27 or A28 to generate rabbit PAbs. VACV vSIJC-20 was incubated for 1 h with increasing concentrations of the PAbs (R180 for anti-L1, R194 for anti-A27 and R203 for anti-A28) or control antibody (preimmune). The virus-antibody mixture was used to infect BSC-1 cells at an MOI of 0.5 PFU/cell. β-galactosidase activity was assessed at 6 h p.i. as a measure of entry.

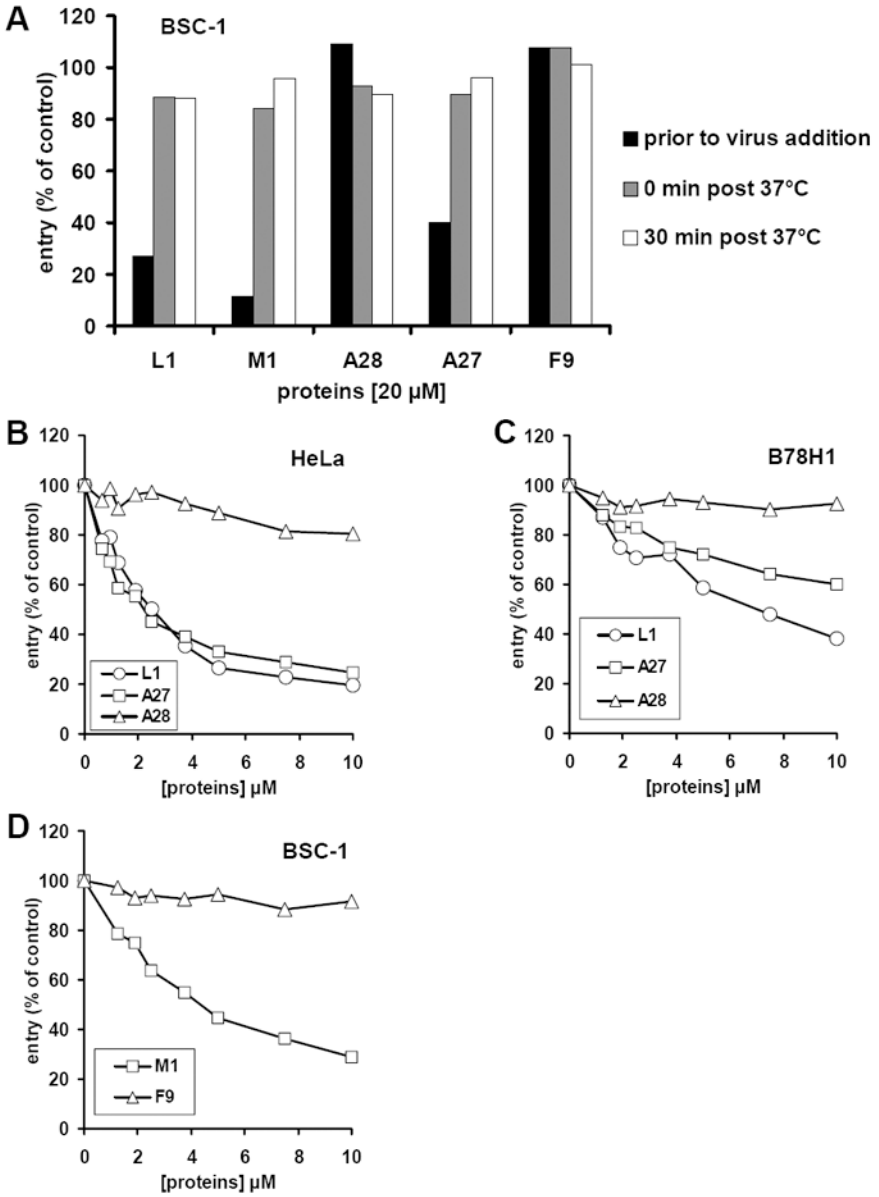

Fig 3.

Soluble L1 and A27, but not A28, block entry of VACV into several cell types. (A) Recombinant proteins inhibit *lacZ* expression of vSIJC-20 only if added to cells prior to virus adsorption, indicating the blocking effect is at the stage of entry. A single concentration [20 μM] of soluble L1, M1, A28, A27, or F9 was either added to BSC-1 cells for 1 h at 4°C prior to virus binding and then cells were treated exactly as described in (B) below (black bars), added to virus-bound cells at time of transfer to 37°C (grey bars), or added to virus-bound cells at 30 min post transfer to 37°C (white bars). β-galactosidase activity was subsequently measured at 6 h post transfer to 37°C. (B, C and D) Increasing multiple concentrations of recombinant L1, A27 or A28 were incubated with HeLa (B) or B78H1 (C) cells seeded in 96-well plates for 1 h at 4°C. VACV vSIJC-20 was then added to the cells at an MOI of 2.5 PFU/cell to allow adsorption. After 1 h at 4°C, cells were washed, transferred to 37°C, and β-galactosidase activity was measured at 6 h p.i. All data depicted are average values from at

least two independent experiments. (D) Soluble M1 and F9, which are homologous to L1, were also tested for their ability to block VACV entry using the same protocol.

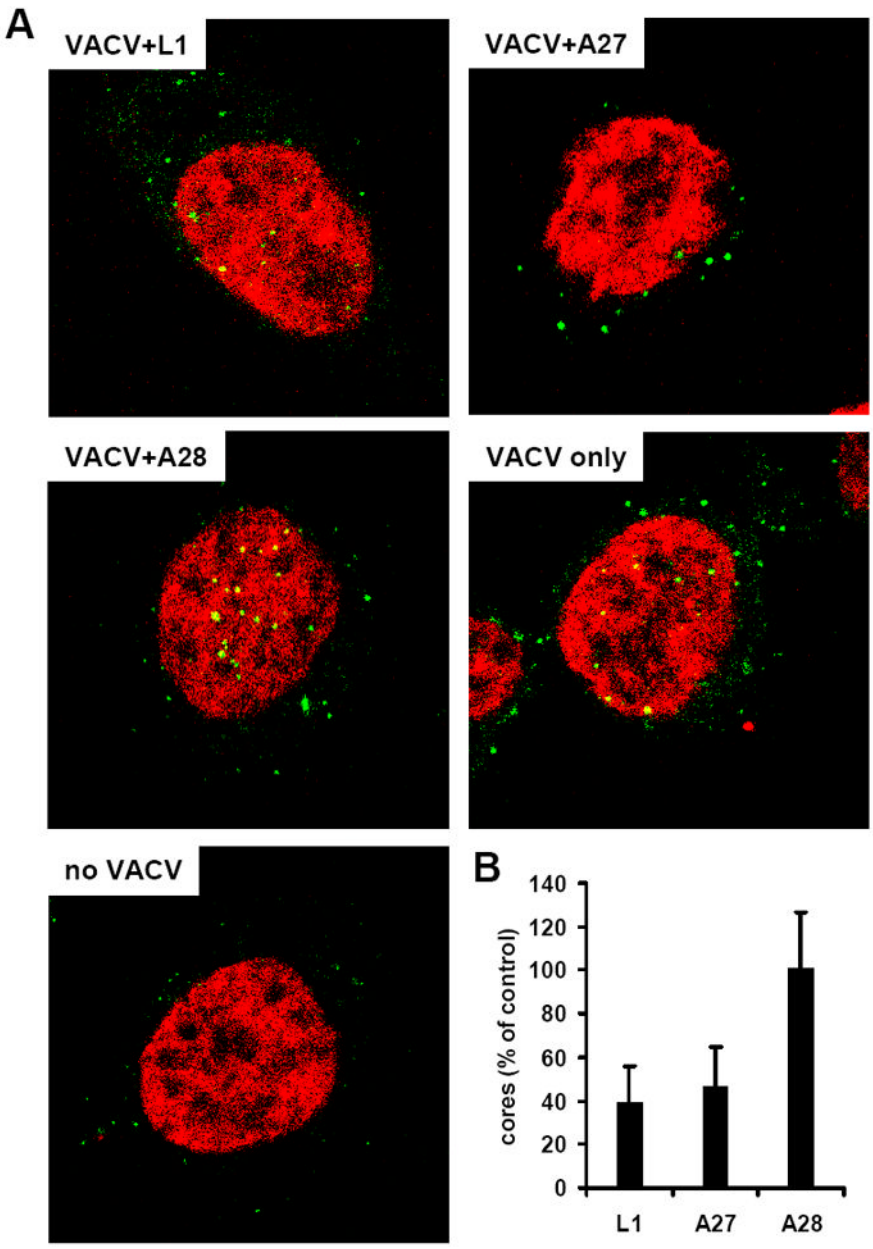

Fig 4.

Soluble L1 and A27, but not A28, inhibit the penetration of VACV cores into cells. 20 μM of soluble L1, A27, A28 or culture medium only (VACV only control) were incubated with HeLa cells seeded on coverslips for 1 h at 4°C. VACV vSIJC-20 was then added to the cells at an MOI of 50 PFU/cell to allow adsorption. After 1 h at 4°C, cells were washed, transferred to 37°C and incubated for 2 h in the presence of cycloheximide. The cells were then fixed with 3% paraformaldehyde, permeabilized using 0.1% saponin and incubated with an anti-core rabbit polyclonal antibody. Intracellular cores were visualized at 1000 × magnification (A) and quantified (expressed as % of control without protein in B) under a fluorescence confocal microscope using fluorophore-conjugated goat-anti-rabbit antibodies (green in A). Cell nuclei were stained with TO-PRO®-3 iodide (red in A). The images represent single optical sections perpendicular to the z-axis.

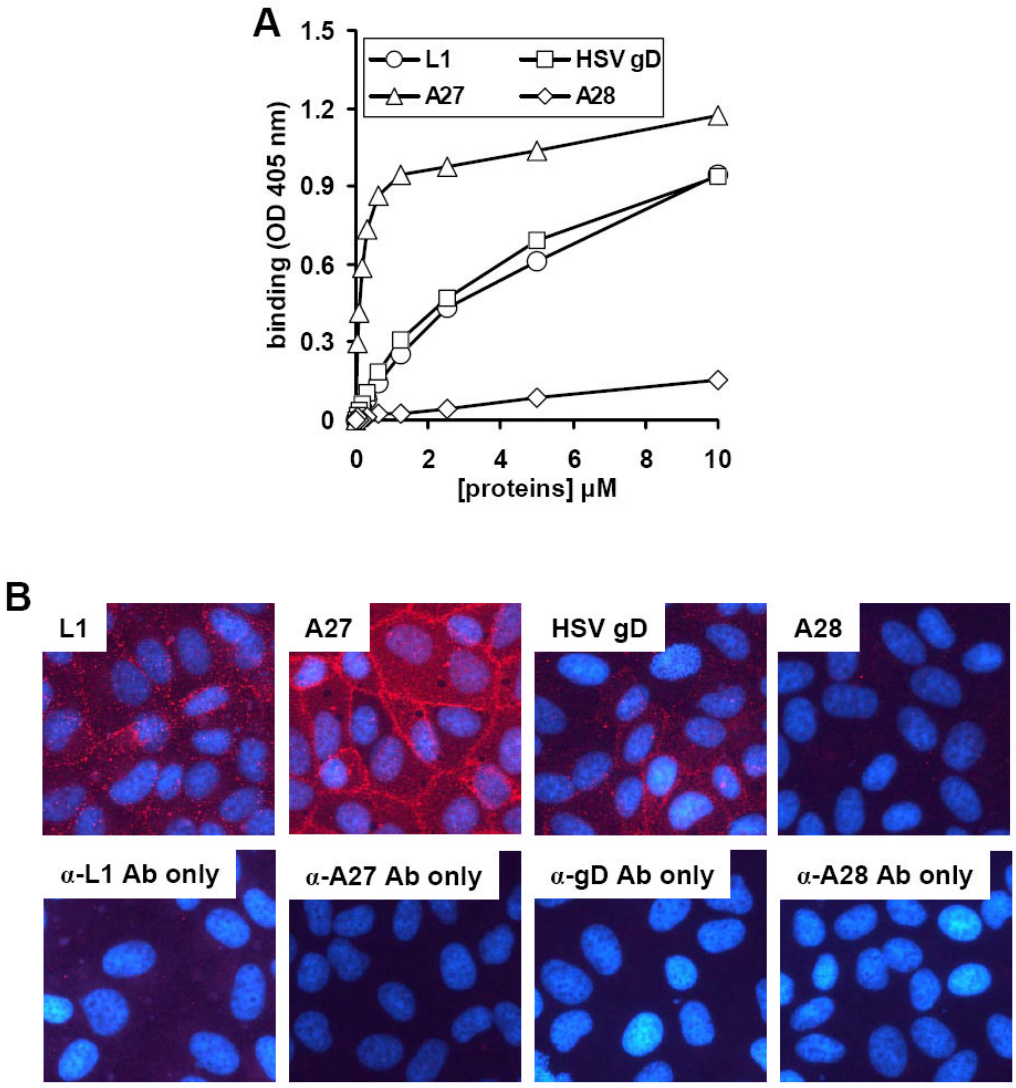

Fig 5.

Soluble L1 and A27, but not A28, bind to surfaces of cells. Binding of proteins to cell surfaces were assessed by CELISA (A) and indirect immunofluorescence (B). Increasing concentrations (A) or 10 μM (B) of soluble L1, A27, gD or A28 were incubated with BSC-1 cells seeded in either 96-well plates (A) or on coverslips in 24-well plates (B). After 1 h at 4°C, cells were washed, fixed with 3% paraformaldehyde and then incubated with rabbit polyclonal antibodies to the respective proteins. The amount of protein bound was quantitated enzymatically using HRP-conjugated anti-rabbit antibodies (A) or visualized under an epifluorescence microscope using fluorescent-tagged goat-anti-rabbit antibodies (red in B). Cell nuclei were stained with DAPI (blue in B).

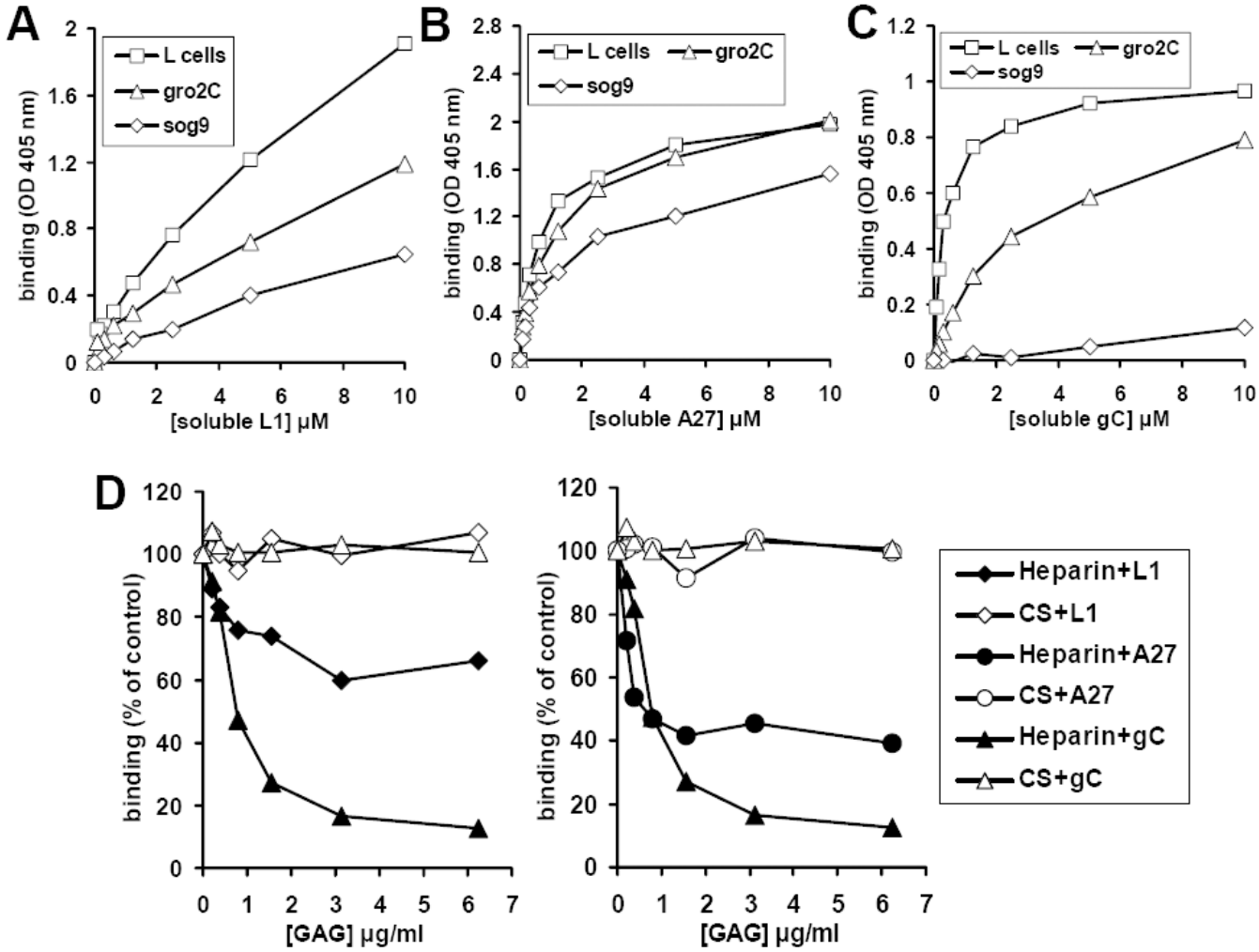

Fig 6.

Soluble L1 and A27 bind to cell surfaces independently of glycosaminoglycans. (A, B and C) Unlike soluble HSV gC, soluble L1 and A27 attach to surfaces of GAG-deficient cells in a dose-dependent manner. Increasing concentrations of soluble L1 (A), A27 (B) or gC (C) were incubated at 4°C with parental L (L cells) or mutant L cells deficient in HSPG (gro2C), or HSPG and CSPG (sog9). After 1 h, cells were washed, fixed, and the quantity of proteins attached was probed using rabbit polyclonal antibodies to L1 (R180), A27 (R193) or gC (R47) in a CELISA format. (D) Soluble heparin only partially inhibits attachment of soluble L1 and A27 to surfaces of cells. Increasing concentrations of soluble heparin (filled symbols) or chondroitin sulfate (open symbols) were preincubated with 3.75 μM soluble L1 (diamonds), 0.375 μM soluble A27 (circles) or 0.375 μM soluble gC (triangles) for 1 h at 4°C. The soluble protein-GAG mix was then incubated at 4°C with L cells. After 1 h, cells were washed, fixed, and CELISA was performed as described above. Heparin abrogates attachment of gC to cells. All data depicted in (D) were obtained from the same experiment but are presented in separate figures for clarity.

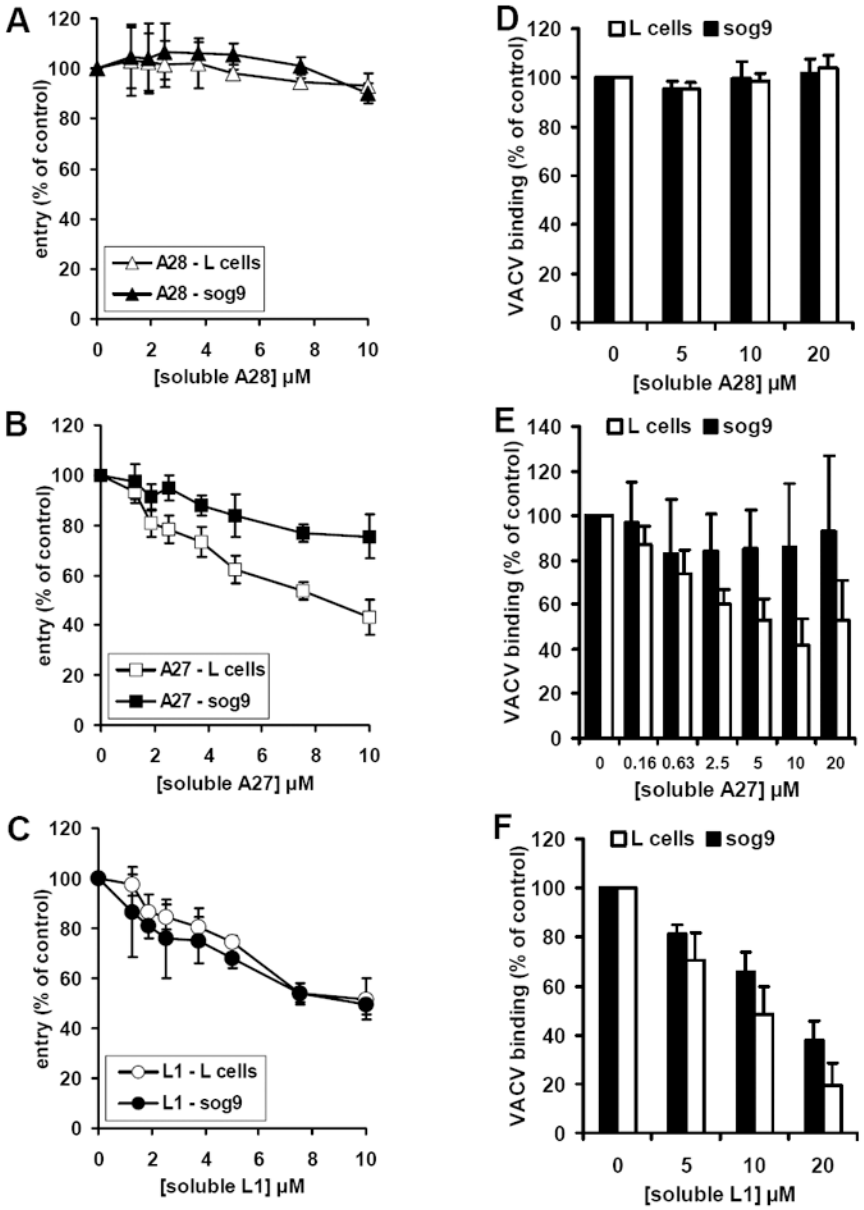

Fig 7.

Soluble L1, but not A27, blocks entry of VACV independently of cell surface proteoglycans at the cellular binding stage. Increasing concentrations of recombinant A28 (A and D), A27 (B and E) or L1 (C and F) were incubated with parental L cells (open symbols or bars) or derivative GAG-deficient sog9 cells (filled symbols or bars) for 1 h at 4°C. VACV vSIJC-20 was then added at an MOI of 2.5 PFU/cell (A, B and C) or 25 PFU/cell (D, E and F). The abilities of the proteins to block entry of VACV (A, B and C) were then tested as described in Fig. 3 while their abilities to block cell-surface binding of VACV (D, E and F) were determined using an immunochemical assay (virus-CELISA). All data depicted are average values from at least three independent experiments.

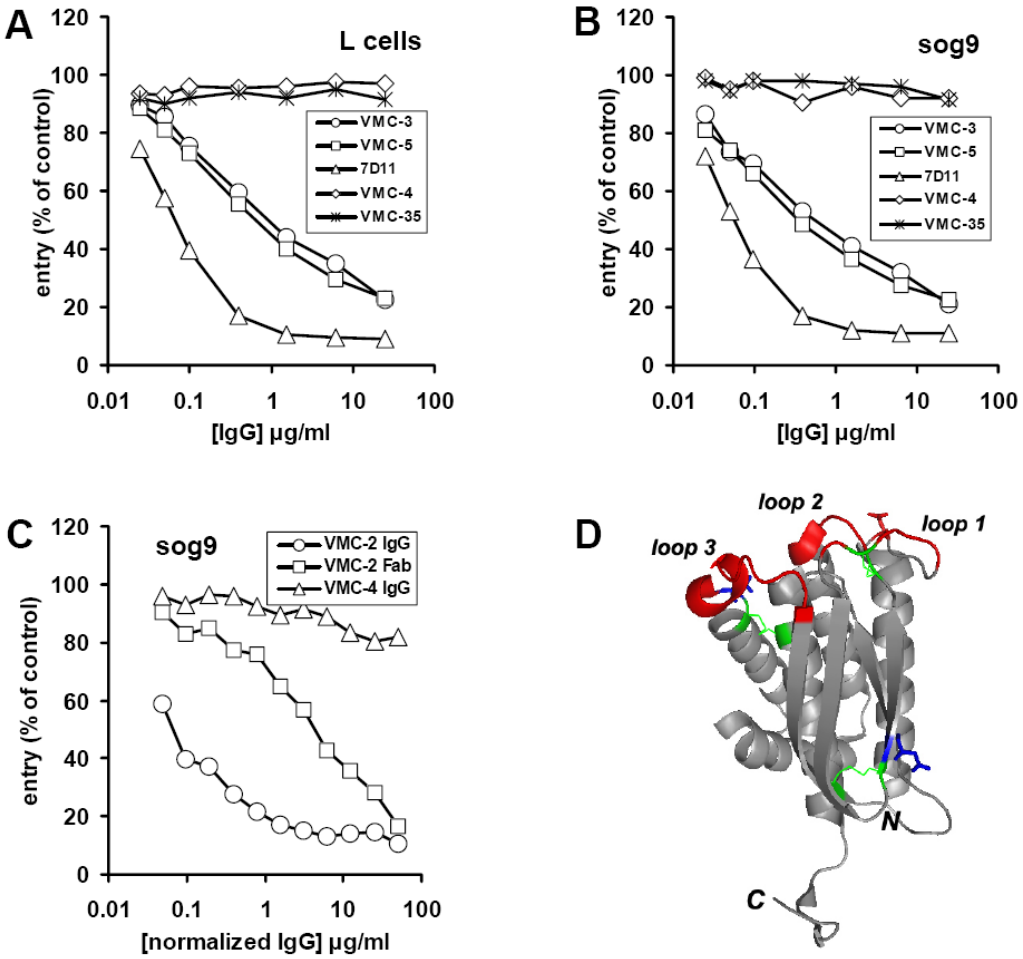

Fig 8.

Neutralizing MAbs to L1 inhibit entry of VACV into cells independently of cell surface glycosaminoglycans. VACV vSIJC-20 was incubated for 1 h with increasing concentrations of MAbs which are neutralizers (VMC-3, VMC-5 and 7D11) or non-neutralizers (VMC-4 and VMC-35). The mixture was then used to infect parental L (A) or sog9 cells (B) and the entry assay was performed as described in Fig. 2. (C) Neutralizing MAbs to L1 do not block entry of VACV by crosslinking virions or steric hindrance. Fab fragments of Group 1 MAbs VMC-2 and VMC-3 (data not shown) were produced and tested for their ability to neutralize VACV entry in sog9 cells. IgG of VMC-4 served as negative control. The concentration of the Fab fragments was normalized to reflect the equivalence of two moles of Fab subunit to one mole of complete IgG unit. (D) Ribbon diagram of the crystal structure of L1 depicting potential receptor-binding domains (Su et al., 2005). The cysteines involved in disulfide bonding (green) and asparagine residues (blue) of the three putative N-linked glycosylation sites are depicted. The neutralizing epitopes (red) of L1 were mapped to three discontinuous loops which are distal from the C-terminus transmembrane region. The neutralizing epitope located near or at Asp-35 of loop 1 is recognized by MAb 2D5 (Ichihashi and Oie, 1996). Multiple residues of the discontinuous loops 1, 2 and 3 form hydrogen bonds or Van der Waals interactions with MAb 7D11-Fab (Su et al., 2007). The linear neutralizing epitope recognized by MAbs VMC-3, 5, 6 and possibly, VMC-2 spanned residues 118-128 of α4’ helix and loop 3 (Aldaz-Carroll et al., 2005). A putative glycosylation site at Asn-27 of loop 1 is situated in the 7D11-Fab epitope.

Table 1

Properties of L1, A27, and A28, MV proteins which are involved in cell entry

		L1	A27	A28
Blocking of entry by antibodies		+	+	+
Binding by soluble proteins to various targets	cells	++	++	−
	heparin [a]	+/−	+	−
	GAG-deficient sog9 cells	+	+	n/a
	cells in presence of soluble GAGs	+	+	n/a
Blocking of entry by soluble proteins	GAG-expressing cells	++	++	−
	GAG-deficient sog9 cells	++	+/−	−
Blocking of virus binding by soluble proteins	GAG-expressing cells	++	++	−
	GAG-deficient sog9 cells	++	−	−
Entry phenotype of null-virus		defective in entry and VACV-induced cell-cell fusion [b] or assembly [c]	reduced infectivity but cell-binding unaffected [d]	defective in entry and VACV-induced cell-cell fusion [e]

[a] As determined by affinity chromatography using agarose beads conjugated to heparin (Supplementary Fig. 2)

[b] (Bisht, Weisberg, and Moss, 2008)

[c] (Ravanello and Hruby, 1994)

[d] Due to the existence of other MV attachment proteins which can compensate for the loss in HSPG-binding by A27 (e.g. D8 or L1) (Hsiao, Chung, and Chang, 1999)

[e] (Senkevich, Ward, and Moss, 2004)