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Generation and characterization of a large panel of murine monoclonal antibodies against vaccinia virus

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ABSTRACT

Vaccinia virus (VACV), the vaccine for smallpox, induces an antibody response that is largely responsible for conferring protection. Here, we studied the antibody response to VACV by generating and characterizing B cell hybridomas from a mouse immunized with VACV. Antibodies from 66 hybridomas were found to recognize 11 VACV antigens (D8, A14, WR148, D13, H3, A56, A33, C3, B5, A10 and F13), 10 of which were previously recognized as major antigens in smallpox vaccine by a microarray of VACV proteins produced with a prokaryotic expression system. VACV C3 protein, which was not detected as a target of antibody response by the proteome array, was recognized by two hybridomas, suggesting that selection of hybridomas based on immune recognition of infected cells has the advantage of detecting additional antibody response to native VACV antigens. In addition, these monoclonal antibodies are valuable reagents for studying poxvirus biology and protective mechanism of smallpox vaccine.

Introduction

Vaccinia virus (VACV), a member of the *Orthopoxvirus* genus of the *Poxviridae* family (Moss, 2007), serves as the live vaccine against smallpox, which is caused by another orthopoxvirus, variola virus (Damon, 2007). As a vaccine, VACV is one of the most successful in human history, responsible for eradicating smallpox from nature. Live VACV immunization elicits robust antibody and cytotoxic T cell responses that persist for decades in humans (Crotty et al., 2003; Hammarlund et al., 2003; Putz et al., 2005; Viner and Isaacs, 2005). In animal models, the antibody response alone is sufficient to protect against diseases caused by pathogenic orthopoxviruses, although the cytotoxic T cell response also contributes to the immune protection (Belyakov et al., 2003; Panchanathan et al., 2008).

VACV produces two different infectious virion forms (Condit et al., 2006; Smith et al., 2002), both of which are targets of antibody response in smallpox vaccine. The majority of the infectious VACV are the intracellular mature viruses (MV), which remain inside cells until cell lysis. MV has a membrane that is associated with at least 19 different viral proteins (Condit et al., 2006). Among them, A27 (Rodriguez et al., 1985), L1 (Ichihashi and Oie, 1996; Wolffe et al., 1995), D8 (Hsiao et al., 1999), H3 (Davies et al., 2005b) and A28 (Nelson et al., 2008) are known to be the targets of neutralizing antibodies. A small fraction of MV in the cells gain additional membranes through wrapping with Golgi cisternae (Smith et al., 2002). They are eventually released through exocytosis as the extracellular enveloped viruses (EV), which are responsible for long-range spread of the virus within the host. EV has one additional outer membrane than MV, which is associated with at least 6 different viral proteins (Smith et al., 2002). Among them, B5 is the major target of neutralization antibodies (Bell et al., 2004; Benhnia et al., 2009; Putz et al., 2006), while A33 is known to elicit protective antibody response (Galmiche et al., 1999). For optimal immune protection against smallpox, antibodies against both MV and EV are required (Smith et al., 2002).

In response to a renewed interest in developing a safer smallpox vaccine, studies were recently carried out to systematically characterize the immune responses to VACV following VACV immunization. A large number of $CD4^+$ and $CD8^+$ T cell epitopes were discovered in VACV (Moutaftsi et al., 2006; Oseroff et al., 2005; Sette et al., 2008; Tscharke et al., 2005; Tscharke et al., 2006). In addition, the antibody response to VACV was profiled with a proteome microarray consisting of recombinant VACV proteins that were produced with a prokaryotic expression system (Davies et al., 2005a, 2007, 2008). The array consistently detected antibodies to 25 VACV proteins, the majority of which are virion components and belong to the late class of viral proteins (Davies et al., 2007). In our current studies, we developed and characterized a large panel of B cell hybridomas from a mouse immunized with VACV. The spectrum of the monoclonal antibodies that we generated matched nicely with the polyclonal antibody profile obtained with the proteome microarray. In addition, we found antibodies to a VACV antigen that was not previously found with the microarray. More importantly, our study resulted in monoclonal antibodies against a wide variety of VACV antigens, which could be used to study B cell epitopes in smallpox vaccine. These antibodies are also valuable research reagents for studying VACV biology, as some

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represent the first-ever monoclonal antibodies against several important VACV membrane and core proteins.

Results

Generation and selection of B cell hybridomas specific for VACV

A BALB/c mouse was infected intranasally with an attenuated VACV mutant, eliciting an immune response that was able to protect the mouse against a subsequent high dose intranasal challenge of the wild type (WT) VACV WR. As we were interested in developing some monoclonal antibodies specific for VACV, this hyperimmune mouse was then boosted with an intravenous injection of UV-inactivated WT VACV and, three days later, its spleen was harvested for hybridoma generation. The hybridomas were screened for their specificity for VACV with an immunofluorescence assay, in which WR-infected HeLa cells were stained with culture supernatants of the hybridomas. The HeLa cells had been infected for 8 h at a multiplicity of infection (MOI)

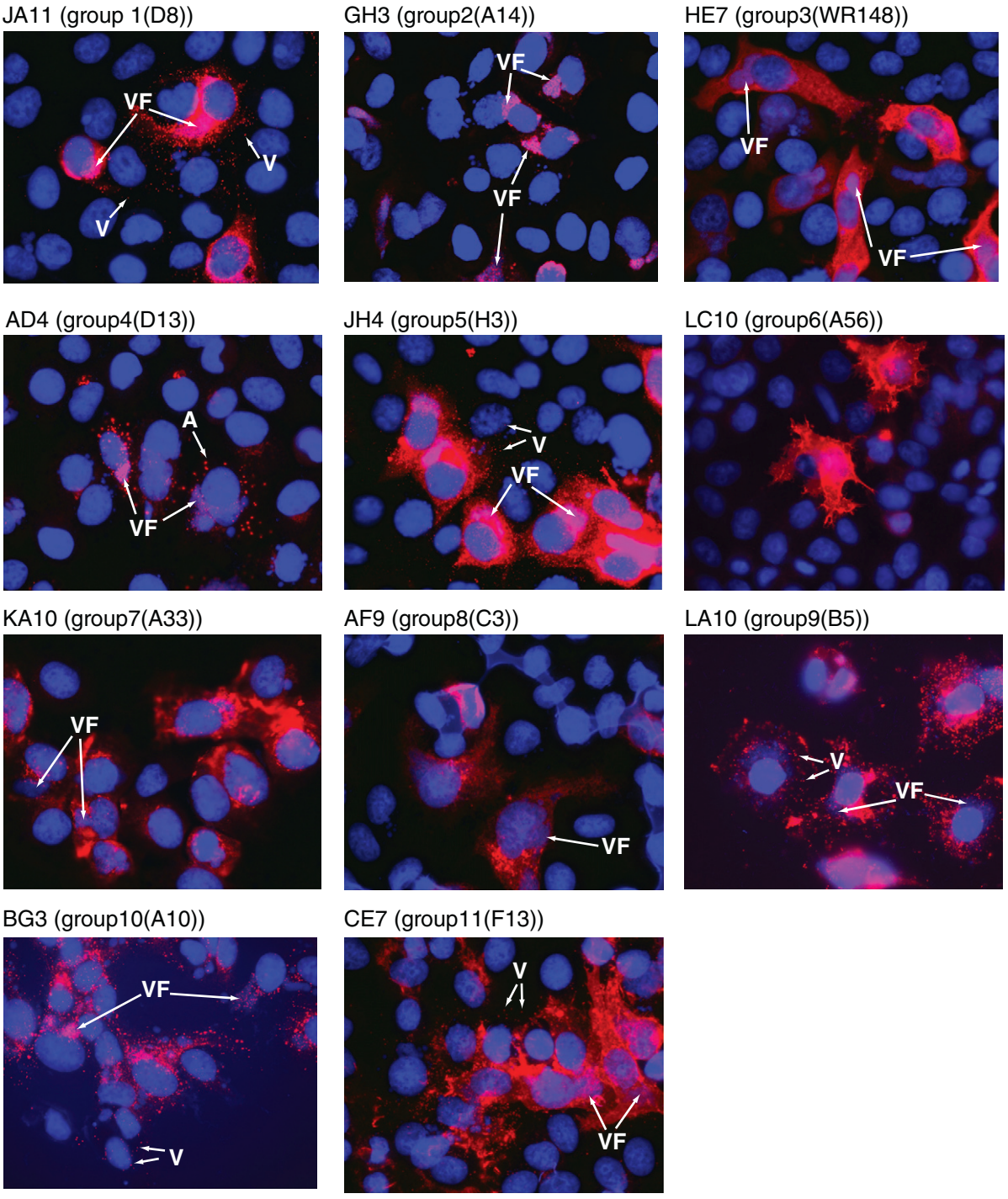


Fig. 1. Screen for murine hybridomas that secret anti-VACV antibodies with an immunofluorescence assay. HeLa cells grown on cover-slips were infected with VACV WR at a MOI of 0.1 to 0.5 PFU/cell for 8 h and then analyzed by immunofluorescence with hybridoma culture supernatants as the primary antibodies. The primary antibodies were stained with a Cy3-conjugated goat anti-mouse secondary antibody (red), and the DNA was stained with DAPI (blue). Specificity of the antibody to VACV is indicated by staining of only WR-infected cells, which display viral DNA factories as areas of cytoplasmic DNA staining. Images obtained with 11 representative hybridomas are shown with the hybridoma clone name. Shown in parenthesis is the group that the hybridoma was subsequently categorized into. Arrows point to viral factories (VF), virion-size particles (V) and aggregates (A).

of 0.1 to 0.5 plaque-forming unit (PFU)/cell, so uninfected cells as well as infected cells with all temporal classes of VACV proteins could be stained together with the antibodies. Hybridomas were deemed specific for VACV if their culture supernatant stained only WR-infected cells, which displayed viral DNA factories as areas of cytoplasmic DNA staining (Fig. 1). Approximately 100 hybridomas that were repeatedly positive for VACV by immunofluorescence assay were kept. Around 25 ml culture supernatant was collected from each hybridoma for subsequent analysis.

Several distinct immunofluorescence patterns were observed during the initial screening (Fig. 1), and they were used later to categorize the hybridomas and corroborate with antigen identification (Table 1). Immunofluorescence with JA11 (group 1) and JH4 (group 5) stained viral factories, additional areas outside viral factories and many virion-size particles, indicating that their targets may be abundant proteins on the surface of MV (Fig. 1). GH3 (group 2), AD4 (group 4) and BG3 (group 10) also stained the viral factories, indicating that their targets are proteins involved in MV assembly. While GH3 (group 2) exclusively stained viral factories, BG3 (group 10) also stained some virion-size particles, and AD4 (group 4) also stained some aggregates that were significantly bigger than virion particles. The nature and biological significance of the aggregates are unclear. The relatively smaller factories in some cells were not stained with the above-mentioned antibodies, perhaps because they were newly formed factories in cells that had been infected for a shorter period of time. KA10 (group 7), AF9 (group 8), LA10 (group 9) and CE7 (group 11) predominantly stained areas outside the viral factories, suggesting that their targets are not involved in MV assembly. In addition, LA10 (group 9) and CE7 (group 11) also stained virion-size particles, indicating that their targets may be on the surface of EV. HE7 (group3) stained the entire cytoplasm very evenly, while LC10 (group6) appeared to stain the outline of the infected cells.

Categorization of the hybridomas according to molecular weight (MW) of the immunoprecipitated proteins

We further characterized the hybridomas by using their culture supernatants to precipitate proteins from VACV-infected cells. HeLa cells were infected with VACV WR at a MOI of 10 for 8 h and then metabolically-labeled with ^{35}S-methionine and -cysteine for another 8 h. Cell lysates were prepared and subjected to immunoprecipitation with the hybridoma supernatants. SDS-PAGE of the precipitates showed that the supernatants of 66 hybridomas pulled down specific proteins from VACV-infected cells (Fig. 2 and data not shown). The rest of the hybridomas did not pull down any protein (data not shown), and they were not characterized further. Hybridomas that pulled down VACV proteins can be categorized into 11 groups according to the apparent MW of the precipitated protein and the immunofluorescence pattern (Table 1). For example, group 1 of 18 hybridomas, including JA11, precipitated a single protein with an apparent MW of 30 kDa by SDS-PAGE (Fig. 2). In addition, they all stained virion-size particles in immunofluorescence (Fig. 1), indicating they might recognize the same antigen.

Identification of the antigens recognized by the hybridomas

We identified the antigens that were recognized by the hybridomas with a variety of methods, which are described separately for each hybridoma group.

Identification of the antigen with antigen-specific rabbit antiserum

Group 1(D8). 18 hybridomas of group 1 precipitated a protein with an apparent MW of 30 kDa (Fig. 2), and stained viral factories and virion-size particles in immunofluorescence (Fig. 1). As MV structural proteins usually localize to viral DNA factories during VACV infection, the immunofluorescence of group 1 hybridomas suggested that the antigen might be a MV membrane protein such as D8 (theoretical MW of 35 kDa) or L1 (theoretical MW of 27 kDa). We therefore used anti-D8 and anti-L1 rabbit antisera to test whether the precipitated antigen was D8 or L1. In a Western blot analysis, D8 antiserum (Sakhatskyy et al., 2006), but not L1 antiserum (not shown), specifically recognized the antigen that was precipitated by these 18 hybridomas and resolved by SDS-PAGE in the absence of any reducing agent (Fig. 3A and data not shown). The D8 antiserum also recognized a protein with an apparent MW of 67 kDa, which disappeared upon the

Table 1
A panel of murine monoclonal antibodies against vaccinia virus.

Group #	Clone name[a]	Immunofluorescence pattern[b]	MW by SDS-PAGE[c]	Antigen
1	*IgG1:* AB12(WB++),CC7, JF11 *IgG2a*:JE10,JE11,EE11,BF1,EB2,JA11,FH4,HG12,LA5,JE4 *IgG2b*:BH7,BG9,CF10,BD6 *Nd:* KF4	Factory; some outside factory; virion-size particles	30	D8(WR113)
2	*IgG1:* BF8,HE6 *IgG2b:* FE11 *IgG3:* LB8,GH3 (WB++), DF8,AD10 *Nd:* KD8,LE11,TH6,BB5, CE11,JG8, BF9,AD5,JE1, AG12	Factory	15,23	A14(WR133)
3	*IgG2a:* HE7(WB++),DF5,BA7 *IgG2b:* CB9 *Nd:*AG6,AF10,BB1,GB10,CG3,CG6,CA2, FD4,CF3	Entire cytoplasm	87	WR148
4	*IgG2b:* AD4 (WB−) *Nd:* AB10, AE5,KB1,HD9,FG5,GB11	Factory, aggregates	61	D13(WR118)
5	*IgG1:* AE11 *IgG2a:* JH4 (WB−)	Factory; some outside factory; virion-size particles	32	H3(WR101)
6	*IgG1:* LC10 (WB+) *Nd:* BE2	Cell surface	79	A56(WR181)
7	*IgG2a:*KA10 (WB++) *Nd:* LA11	Outside factory	22	A33(WR156)
8	*IgG1:*AF9 (WB−) *Nd:* JB11	Outside factory	27	C3(WR025)
9	*IgG2b:* LA10 (WB−)	Outside factory, virion-size particles	40	B5(WR187)
10	*IgG1:* BG3 (WB+)	Factory, particles	93	A10(WR129)
11	*IgG2a:* CE7 (WB−)	Outside factory, virion-size particles	38	F13(WR052)

[a] The isotypes of the clones are shown in italics and underlining. Nd: isotype not determined. A representative clone of each group was tested for its ability to recognize antigen in Western blot (WB) and the results are shown in parenthesis. WB++: positive in WB; WB+: weak positive in WB; and WB−: negative in WB.
[b] Factory: staining predominantly of viral DNA factory; and outside factory: staining predominantly of areas outside viral DNA factory.
[c] Molecular weight (MW) was calculated from mobility of the protein relative to molecular weight standards on SDS-PAGE by using the Bio-rad quantity one program.

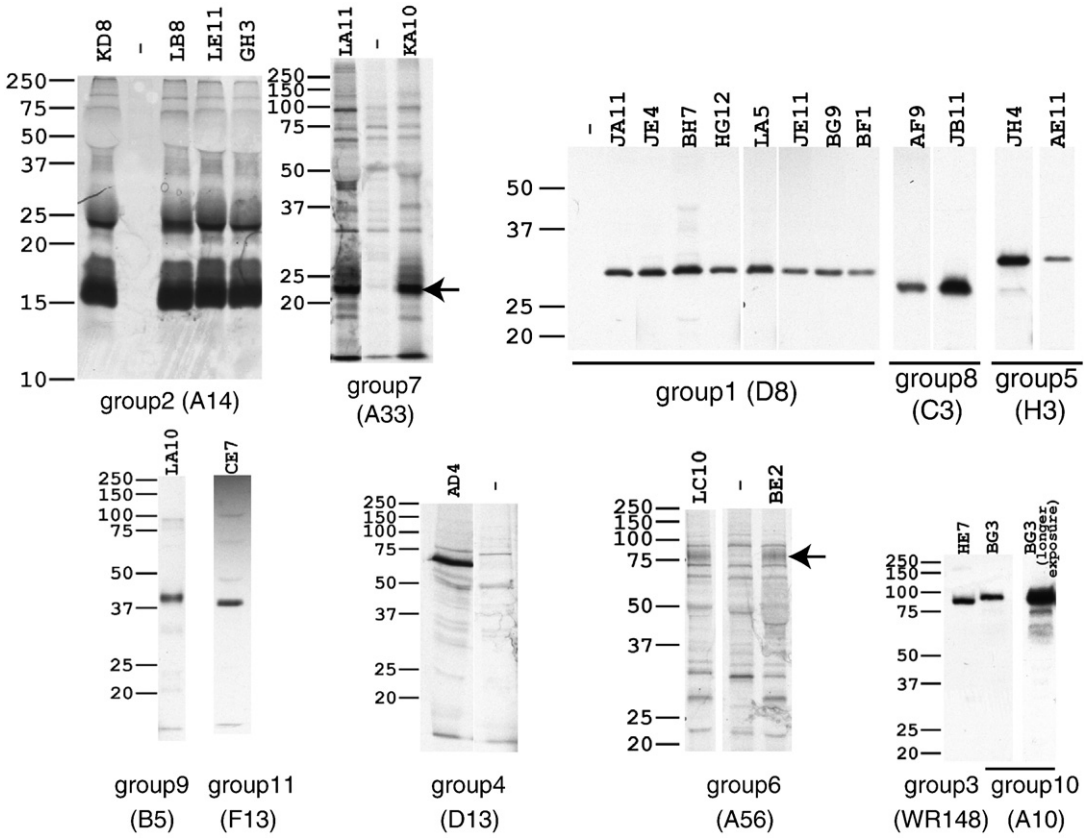


Fig. 2. Categorization of the hybridomas into 11 groups according to the apparent molecular weight of the precipitated protein. HeLa cells were infected with VACV WR at a MOI of 10 and metabolically-labeled with ^{35}S-methionine and -cysteine from 8 to 16 hpi. The cells were lyzed and immunoprecipitated with the hybridoma supernatants and Protein G sepharose. The precipitated proteins were analyzed by SDS-PAGE, and the autoradiographs of representative samples are shown. The results are grouped together according to the size of the precipitated proteins and are shown from the smallest to the largest precipitated proteins. Shown above each lane is the clone name of the hybridoma whose supernatant was used for the immunoprecipitation. The control immunoprecipitation with DMEM-10 medium is indicated by "–". The group number of the hybridomas is indicated below the lanes.

addition of a reducing agent and thus is presumed to be the disulfide-linked D8 dimer. Protein finger-printing with mass spectrometry also confirmed that the antigen precipitated by this group of hybridomas is indeed D8 protein (data not shown).

To further confirm if group 1 hybridomas are specific for D8, we performed Western blot on recombinant GST fusion protein of D8. As shown in Fig. 4A, AB12 specifically recognized GST-D8 fusion protein among the bacterial cell lysate, indicating that its target is D8. AB12 also recognized the D8 protein in infected cells and in purified MV virions in a Western blot (Fig. 4B).

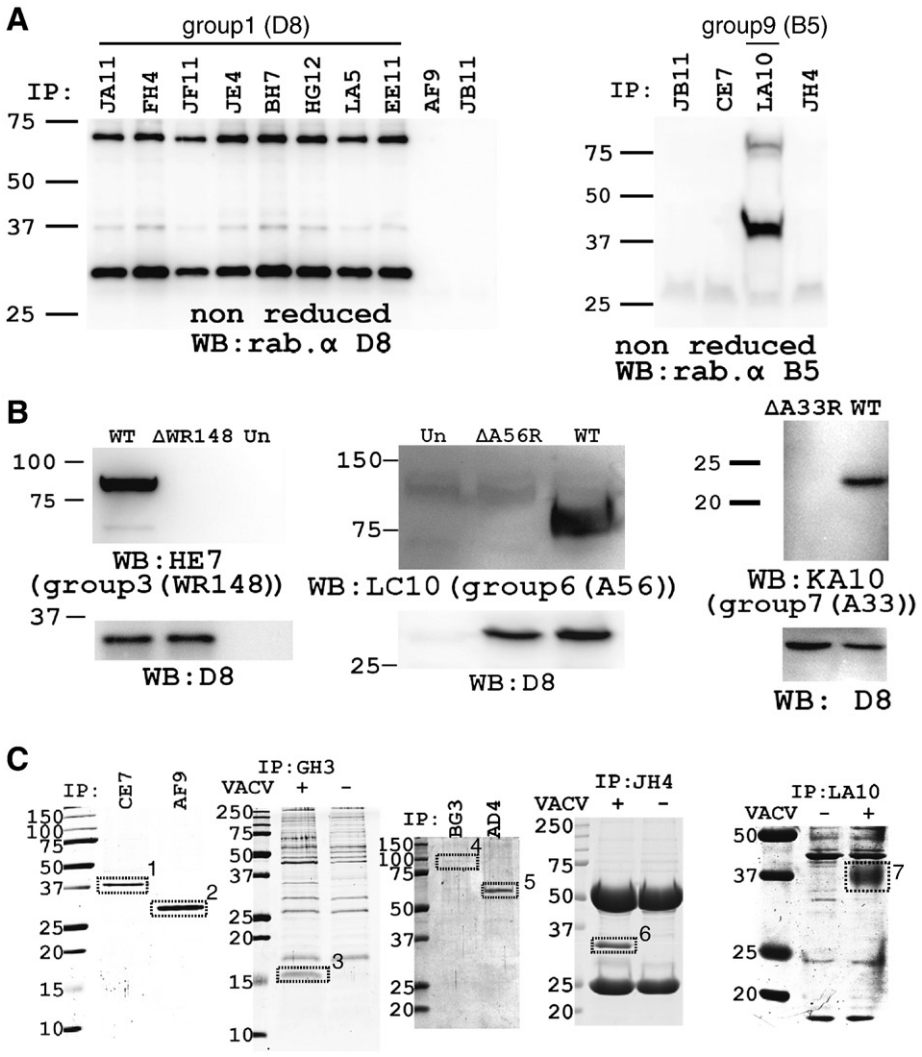
Group 9(B5). LA10 precipitated a protein with an apparent MW of 40 kDa (Fig. 2). Immunofluorescence with LA10 showed strong staining of virion-size particles as well as areas outside the viral DNA factories (Fig. 1). As EV membrane proteins usually localize to Golgi instead of the viral factories, the immunofluorescence of LA10 indicated that the target of LA10 may be an EV membrane protein such as B5 (theoretical MW of 42 kDa). Rabbit anti-B5 antiserum (Sakhatskyy et al., 2006) recognized the antigen that was precipitated by LA10 in a Western blot (Fig. 3A). As a control, B5 antiserum did not recognize the antigens that were precipitated by three other hybridomas of groups 5, 8 and 13. Mass spectrometry analysis of the antigen precipitated by LA10 confirmed that the antigen was indeed B5 protein (Fig. 3C). LC10 failed to recognize B5 in infected cells in Western blot (data not shown), suggesting that it recognizes a conformational epitope.

Identification of the antigen with gene-specific deletion mutants of VACV

Group 3(WR148). A large group of 13 hybridomas, including HE7, precipitated a protein with an apparent MW of 87 kDa and strongly stained the entire cytoplasm in immunofluorescence (Figs. 1 and 2). WR148 (theoretical MW of 84 kDa) was recently reported to be an immunodominant antigen in smallpox vaccine (Davies et al., 2007), so we tested whether WR148 is the target of these hybridomas by using a WR mutant that is specifically deleted of WR148 (Jones-Trower et al., 2005). As shown by a Western blot in Fig. 3B, HE7 recognized an antigen that was present in WT WR-infected cells by not in ΔWR148-infected cells, indicating that its target is WR148. The cells were infected equally by the two viruses, as a similar amount of D8 was detected in both samples. HE7 also specifically recognized a recombinant GST fusion protein of WR148 among the bacterial cell lysates in Western blot (Fig. 4A).

Group 6(A56). LC10 and BE2 precipitated a protein that ran as a broad band with an apparent MW of 79 kDa (Fig. 2), suggesting that these two hybridomas recognize a viral glycoprotein. We considered A56 as the most likely target among the few glycoproteins expressed by VACV, as A56 was reported to migrate as 85/89 kDa on SDS-PAGE (Payne, 1979; Shida and Dales, 1981). Indeed, LC10 recognized an antigen that was present in WT WR-infected cells but not in cells infected by an A56 deletion mutant (Fig. 3B). In addition, LC10 specifically recognized a recombinant GST fusion protein of A56 among the bacterial cell lysates in Western blot (Fig. 4A). LC10 and BE2 stained the outline of the infected cells in immunofluorescence (Fig. 1), which is consistent with previous reports that A56 is predominantly distributed on cell surface (Lorenzo et al., 2000).

Group 7(A33). Immunofluorescence with KA10 and LA11 stained areas outside viral factories (Fig. 1), suggesting that the target might be an EV membrane protein. KA10 and LA11 precipitated a protein



Band number	Mab used	Protein Matched	# of fragments matched	sequence coverage
1	CE7	F13	10	31%
2	AF9	C3	9	44%
3	GH3	A14	1	17%
4	BG3	A10	17	19%
5	AD4	D13	24	39%
6	JH4	H3	17	63%
7	LA10	B5	7	15%

Fig. 3. Identification of the antigenic targets of the hybridomas. (A) Identification of hybridoma specific for VACV D8 or B5 with rabbit antisera against D8 or B5. Culture supernatants from the indicated hybridomas were used to immunoprecipitate proteins from WR-infected HeLa cells. The precipitated proteins were resolved by SDS-PAGE in the absence of any reducing agent and analyzed by Western blot with the indicated rabbit antisera (D8 or B5). (B) Identification of hybridoma specific for WR148, A56 or A33 with WR mutant deleted of the specific gene. Proteins from uninfected (Un) cells or cells infected with the indicated WR virus (WT, ΔWR148, ΔA56R or ΔA33R) were resolved by SDS-PAGE and analyzed by Western blot using supernatants from the indicated hybridomas. The same membranes were also blotted with rabbit antiserum against D8 as a control for loading of viral proteins. (C) Identification of the antigen by mass spectrometry analysis. Proteins from uninfected cells (−) or WR-infected cells (+ or not indicated) were immunoprecipitated with the indicated antibodies. Except for JH4 and LA10, all other antibodies were conjugated to Protein G sepharose with a crosslinker. Coomassie stains of the precipitated proteins after SDS-PAGE separation are shown. Specific bands (boxed and numbered from 1 to 7) were cut off the gel, digested with trypsin and analyzed with mass spectrometry, the result of which is shown in the table below.

that ran as a broad band with an apparent MW of 22 kDa (Fig. 2), further suggesting that they may recognize a viral glycoprotein of EV membrane such as A33 (theoretical MW of 21 kDa) or A34 (theoretical MW of 20 kDa). They recognized an antigen that was present in WT WR-infected cells but not in cells infected by an A33 deletion mutant (Roper et al., 1998) in a Western blot (Fig. 3B and

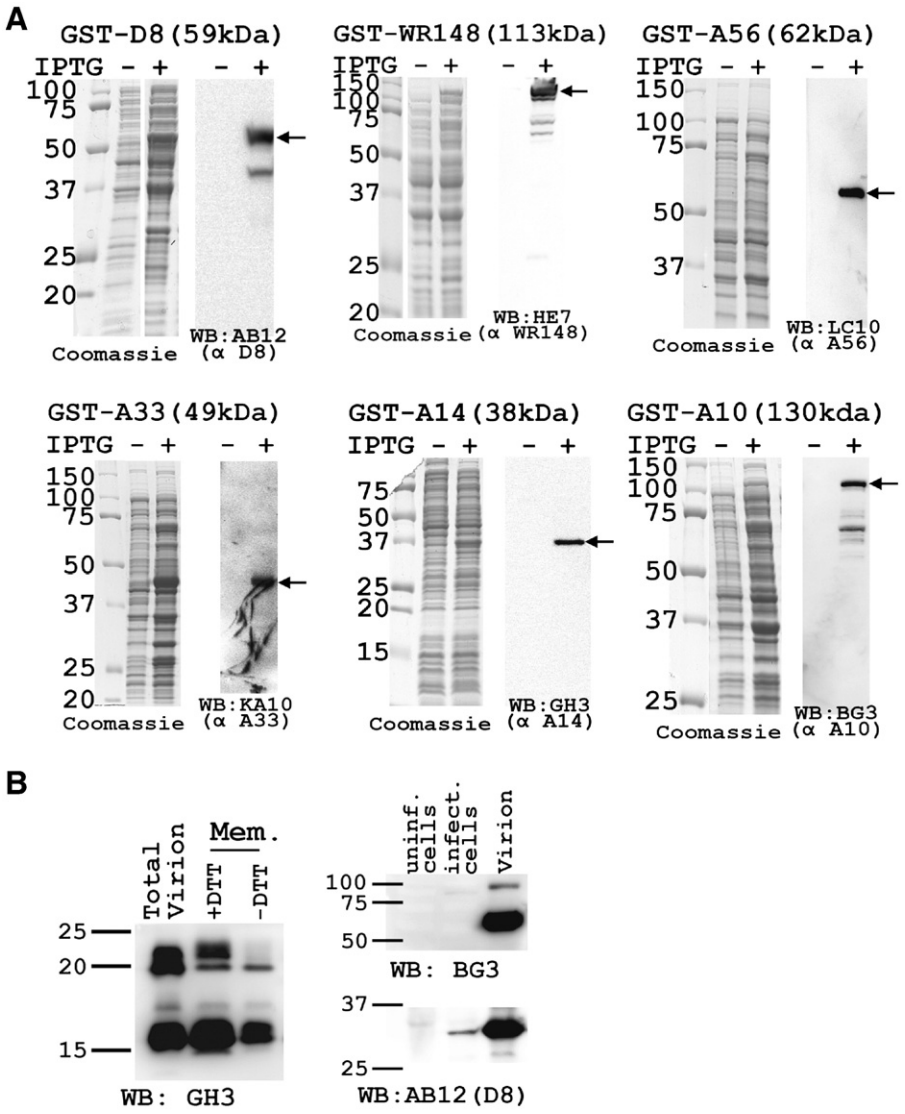


Fig. 4. Confirmation of the antigenic targets of the hybridomas. (A) Western blot of recombinant GST fusion proteins. *E. coli.* strains were either not induced (−) or induced with IPTG (+) to express fusion of GST with the indicated VACV antigens. Proteins from the whole cell lysates were resolved by SDS-PAGE and analyzed by either Coomassie staining or by Western blot with the indicated antibody. (B) Western blot of purified WR mature virions. Mature virions were purified according to the standard protocol (Earl et al., 1998). Membrane-associated virion proteins (Mem.) were extracted with 0.5% NP-40 in the absence (−) or presence (+) of 50 mM DTT. Infected cells, whole virions or the fractions were analyzed by SDS-PAGE and Western blot with the indicated antibody.

data not shown). In addition, KA10 specifically recognized in Western blot a recombinant GST fusion protein of A33 among the bacterial cell lysates (Fig. 4A), indicating that its target is A33.

Identification of the antigen by protein finger-printing with mass spectrometry

For the remaining hybridomas, we did not have the reagents for testing our specific hypothesis, so we cross-linked the antibodies to protein G sepharose, precipitated the antigens from infected cells and identified them by mass spectrometry analysis.

Group 11(F13). CE7 precipitated a protein with an apparent MW of 38 kDa (Figs. 2 and 3C). The protein band shown in Fig. 3C was cut off the gel, digested with trypsin, and analyzed with mass spectrometry. 10 peptide fragments of the protein matched that of F13, an EV membrane protein. This identification is consistent with immunofluorescence pattern of CE7 (Fig. 1), which showed extensive staining of areas outside viral factories and virion-size particles.

Group 8(C3). Both AF9 and JB11 precipitated a protein with an apparent MW of 27 kDa by SDS-PAGE (Figs. 2 and 3C). Mass spectrometry finger-printing of the precipitated protein showed that the protein is C3, the complement binding protein (Kotwal and Moss, 1988).

Group 2(A14). A large group (group 2) of 17 hybridomas, including GH3, precipitated a 15-kDa and a 23-kDa protein from metabolically-labeled, WR-infected cells (Fig. 2). Coomassie staining of the proteins that were precipitated by GH3 also detected the 15-kDa protein along with additional other proteins (Fig. 3C). Most of the additional proteins were also precipitated from uninfected cells, indicating that they were host cell proteins, which were not metabolically-labeled in VACV-infected cells but were nonspecifically precipitated. The 15-kDa protein was initially tested for A27 (theoretical MW of 13 kDa), but it was not recognized by anti-A27 rabbit antiserum (data not shown). Protein finger-printing with mass spectrometry suggested that the protein was A14, as a peptide fragment of this protein matched that of A14. Indeed, GH3 specifically recognized the recombinant GST fusion

protein of A14 among the bacterial cell lysate in a Western blot (Fig. 4A), indicating that its target is A14. This identification is consistent with the fact that group 2 hybridomas precipitated the additional 23-kDa protein and stained the viral DNA factory in immunofluorescence (Figs. 1 and 2), as A14 is a MV membrane protein that forms a disulfide-bonded dimer even under the denaturing condition of SDS-PAGE (Mercer and Traktman, 2003). In a Western blot of MV virion, GH3 detected both the 15-kDa and the 23-kDa proteins, which was most efficiently extracted from the virion by the detergent NP-40 in combination with the reducing agent DTT (Fig. 4B), consistent with the localization of A14 as a disulfide-bonded dimer in MV membrane.

Group 10(A10). BG3 precipitated a protein that was close to 100 kDa (Figs. 2 and 3C), which was identified by mass spectrometry to be VACV A10 protein (theoretical MW of 102 kDa). Indeed, BG3 specifically recognized a recombinant GST fusion protein of A10 among the bacterial cell lysates in Western blot (Fig. 4A), confirming that its target is A10. During virion morphogenesis, the full-length A10, also known as p4a, is proteolytically processed into three fragments, and the N-terminal 62-kDa fragment, known as 4a, is packaged into virion core as a major virion structural protein (Vanslyke et al., 1991). In Western blot of purified virion, BG3 recognized the 62-kDa 4a protein (Fig. 4B), indicating that its epitope is present within 4a. It was therefore surprising that BG3 mainly precipitated p4a instead of 4a from infected cells. Upon examining a longer exposure of the autoradiograph, we found that BG3 also precipitated several smaller proteins of 60–75 kDa (Fig. 2), among which could be the 4a protein. The reason why BG3 predominantly precipitated p4a may be that 4a was tightly packaged into the virion core and thus was less accessible for immunoprecipitation. Immunofluorescence with BG3 stained the viral factories and some virion-size particles (Fig. 1), the latter of which could be uncoated virions in newly infected cells. BG3 failed to recognize any protein in Western blot of infected cell lysates (Fig. 4B), so its epitope may be conformational, which was recognizable only when a large amount of A10 protein was analyzed in Western blot.

Group 4(D13). Seven hybridomas, including AD4, precipitated a protein of approximately 61 kDa (Figs. 2 and 3C), which was identified by mass spectrometry to be D13 (theoretical MW of 62 kDa). Typical immunofluorescence with this group of hybridomas stained some viral factory as well as some aggregates in the cytoplasm (Fig. 1).

Group 5(H3). JH4 and AE11 precipitated a protein with an apparent MW of 32 kDa by SDS-PAGE (Figs. 2 and 3C), which was identified by mass spectrometry to be the VACV MV membrane protein H3 (theoretical MW of 37 kDa). Immunofluorescence with these hybridomas stained the viral factories and virion-size particles (Fig. 1), consistent with MV membrane protein staining.

Discussion

We initiated the current study with the simple goal of generating some monoclonal antibodies (mAbs) against VACV as reagents for studying VACV biology. In the end, 66 mAbs against 11 different VACV antigens were developed from a single immunized mouse. To our knowledge, it is the first time since 1980s that such a large number of mAbs against such a diverse group of VACV proteins have been reported. Several reports published decades ago had described a large number of mAbs against VACV (Ichihashi and Oie, 1988; Wilton et al., 1986). However, the antigenic targets of these mAbs were largely unknown, and the mAbs are no longer available. Ten years ago, a panel of VACV-specific mAbs were generated by Alan Schmaljohn's group (Hooper et al., 2000), but, unfortunately, they have never been fully described in a publication. Nevertheless, some of the mAbs, including a neutralizing antibody against L1, were made available to a few groups and have been very useful in a number of studies (Lustig et al., 2005). More recently, multiple mAbs against recombinant L1 and B5 proteins were generated from recombinant proteins by Cohen, Eisenberg and co-workers (Aldaz-Carroll et al., 2005a,b), and these mAbs have been very usefully in studies of MV and EV neutralization. Here, we report for the first time mAbs against A14, A10 and D13, which are all proteins playing critical role in VACV morphogenesis. The mAbs we generated in this study target nonstructural proteins (WR148, D13L, and C3) as well as structural proteins in the virion core (A10) or on the membranes of MV (D8, H3, and A14) or EV (B5, A33, F13, and A56). Collectively, they make up an excellent molecular "toolkit" for studying the life cycle of VACV.

We are pleasantly surprised with the number and the diversity of the antibodies that we obtained from a single immunized mouse. This success could perhaps be attributed to our immunization and screening methodology. The mouse was initially infected with a live attenuated VACV, which elicited an immune response that was strong enough to protect against a subsequent high dose VACV challenge. The mouse was finally given an intravenous dose of UV-inactivated VACV three days before the harvest of the spleen. This immunization scheme resulted in high titer of serum antibody against VACV and ultimately led to a large number of hybridomas that are specific for VACV. For screening the hybridomas, we performed immunofluorescence assay of cells infected with VACV at low MOI. This method was more labor-intensive than other commonly used methods such as ELISA, but we found it to be highly specific and very sensitive. In our hands, ELISA with infected cell lysate has a high percentage of false positive, while ELISA with purified virus appears to only detect antibody against major virion membrane proteins. In contrast, immunofluorescence of infected and uninfected cells in the same slide unambiguously identified hybridomas that are specific for VACV. In addition, cellular localization of the target proteins showed by immunofluorescence aided subsequent target identification.

Although the immunization protocol that we used here differed from the practice of smallpox vaccination, the spectrum of mAbs that we generated matches nicely with the profile of polyclonal antibody response to smallpox vaccination (Davies et al., 2005a, 2007). A proteome array of recombinant VACV proteins consistently detected polyclonal antibodies to around 25 VACV proteins (Davies et al., 2007), against 10 of which we have found hybridomas in this study. For some VACV proteins that elicited strong polyclonal antibody responses, including D8, A14, WR148 and D13, we found multiple clones of hybridomas against the same protein. Therefore, the spectrum of the hybridomas, which was generated and detected with native VACV proteins, largely supports the polyclonal antibody profiling done with recombinant proteins produced with a prokaryotic system. There are some major targets of polyclonal antibodies, against which we did not find any hybridoma in this study. This may be due partly to inherent limits in hybridoma generation and partly to differences in antibody response to VACV in individual hosts. It was shown that smallpox vaccination in humans could result in different repertoire of antibody responses (Benhnia et al., 2008). On the other hand, we found 2 hybridomas against C3, which was not detected by the proteomic array as a polyclonal antibody target. C3 is the VACV complement control protein (VCP), which is secreted by infected cells and inhibits both the classical and the alternative pathways of complement activation (Kotwal and Moss, 1988). Recently, two studies showed that C3 is a target of vaccinia immune globulin (VIG) (Adamo et al., 2009; Liszewski et al., 2009), which was harvested from individuals vaccinated with smallpox vaccine. It is possible that the proteome array failed to detect antibody response to C3 because the C3 antibodies elicited by VACV only recognize native C3, which forms intramolecular disulfide bonds in eukaryotic cells (Liszewski et al., 2006). In addition to the advantage of detecting additional antibody

response to native VACV proteins, our generation of hybridomas resulted in monoclonal antibodies against a variety of VACV antigens, which could be used to map B cell epitopes in smallpox vaccine that contribute to immune protection.

Material and methods

Cells and viruses

BS-C-1 cells were maintained in minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum (FBS). HeLa 229 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Wild-type (WT) and mutant WR viruses were propagated on BS-C-1 cells. ΔA33R (vA33d) (Roper et al., 1998) and ΔWR148 (v644) (Jones-Trower et al., 2005) mutant viruses were obtained from Bernard Moss and Michael Merchlinsky, respectively. ΔA56R was constructed by homologous recombination of WT WR virus with a plasmid derived from pVote1 (Ward et al., 1995) using standard protocols (Earl et al., 1998).

Hybridoma generation

A 4-week-old BALB/c mouse was infected intranasally with 10^5 PFU of WR.K1L$^-$C7L$^-$ (Meng et al., 2008), a highly attenuated mutant of VACV WR with deletion in host-range genes K1L and C7L. Two weeks later, the mouse was challenged with intranasal inoculation of 4×10^6 PFU of wild type (WT) VACV WR. The mouse survived the challenge. After another 2 weeks, the mouse was injected intravenously with 7×10^7 PFU of UV-inactivated WR virus. Three days later, the spleen of the mouse was harvested, the splenocytes fused with the mouse myeloma SP2/0 cells, and the cells plated into 12 96-well plates for selection, essentially as described before (Zhong et al., 1997). After 14 days of selection, while most of the wells had no colony, some had one or very rarely 2 colonies, which were individually picked out of the wells.

Hybridoma screening

The hybridomas were screened for their ability to secret anti-VACV antibodies with an immunofluorescence assay. Specifically, HeLa cells grown on cover-slips were infected with VACV WR at 0.1 to 0.5 PFU/cell for 8 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with DMEM with 10% FBS, and stained with 200 μl of the hybridoma supernatant followed with a Cy3-conjugated secondary antibody and DAPI dye. The slides were visualized with an AX-70 Olympus fluorescence microscope. The isotypes of the antibodies were determined by immunofluorescence or enzyme-linked immunosorbent assay (ELISA) analysis of WR-infected cells with isotype-specific secondary antibodies (Sigma-Aldrich).

Immunoprecipitation

HeLa cells in 100-mm dishes were infected with WT WR at 10 PFU/cell. At 8 hpi, the culture medium was replaced with 5 ml of methionine- and cysteine-free DMEM plus 100 μCi of ^{35}S-methionine and -cysteine per ml. After another 8 h, the cells were harvested and lysed in a buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals). 80 μl of the cleared cell lysates was mixed with 1 ml of hybridoma supernatant for 1 h, and then added with 30 μl of 50% (vol./vol.) Protein G sepharose (GE Healthcare Life Sciences) and mixed for another 1 h. The beads were washed consecutively with wash buffer (0.1% [wt./vol.] Triton X-100, 50 mM Tris [pH 7.4], 300 mM NaCl, and 5 mM EDTA) and PBS before they were resuspended in SDS sample buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Western blot analysis

For results shown in Fig. 3A, hybridoma supernatants were used to immunoprecipitate proteins as described earlier except that unlabeled cells were used. The precipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk, incubated with rabbit antisera against D8 or B5 (Sakhatskyy et al., 2006) followed with anti-rabbit secondary antibody, and analyzed with chemiluminescence as described before (Meng and Xiang, 2006). For result shown in Figs. 3B and 4, Western blot analysis was performed with undiluted hybridoma supernatants as the primary antibodies.

Mass spectrometry

Antigens that were subjected to mass spectrometry analysis were precipitated from WR-infected cells as described earlier with the exception that, in most experiments, antibodies covalently-linked to protein G sepharose were used for the precipitation. 30 μl of Protein G sepharose was mixed with 1 ml of hybridoma supernatants for 1 h, after which Bis(Sulfosuccinimidyl)-suberate (Pierce) was added to cross-link the antibody to the sepharose. The precipitated proteins were resolved by SDS-PAGE and stained with Coomassie blue dye. Individual bands were cut off the gel, digested with trypsin and analyzed with mass spectrometry by mass spectrometry core facility at UTHSCSA.

Expression of recombinant proteins

The plasmids for expressing the fusion of GST and various viral proteins were constructed by PCR amplifying the viral gene from WR DNA and cloned the PCR fragment into pGEX6P-1 (GE Healthcare Life Sciences) using the NcoI and BamHI sites. The expression of the fusion protein in *E. coli* BL21 strain was induced with isopropyl-beta-D-thiogalactoside (IPTG; Invitrogen). The bacteria were harvested and lyzed via sonication in SDS-PAGE sample buffer. The clarified cell lysates were then used in Western blot analysis.

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