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Construction of Recombinant Mouse IgG1 Antibody Directed Against Varicella Zoster Virus Immediate Early Protein 63

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Abstract

Five varicella zoster virus (VZV) genes are known to be transcribed in latently infected human ganglia. Transcripts from VZV gene 63, which encodes an immediate early (IE) protein, are the most prevalent and abundant. To obtain a reagent that might facilitate studies of the role of the IE63 protein in latency and reactivation, we selected an IE63-specific Fab fragment from a phage library and used it to prepare a recombinant mouse IgG1 antibody that detects IE63 and functions in Western blot, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence assays.

INTRODUCTION

Analysis of human ganglia latently infected with varicella zoster virus (VZV) reveals transcription of no less than five VZV genes (open reading frames [ORF] 21, 29, 62, 63, and 66).(1) VZV gene 63 transcripts are the most prevalent and abundant.(2) VZV ORF 63 encodes immediate-early 63 (IE63), a 278 amino acid protein expressed early after VZV infection. IE63 is detected primarily in the nucleus of productively infected cells, where it is modified by phosphorylation(3,4); while in latently-infected neurons, IE63 is located in the cytoplasm.(5, 6) Studies of IE63 in VZV-infected cells have suggested several possible functions, including both viral and cell gene transactivation and inhibition of apoptosis.(7–11)

VZV IE63 has been detected with a rabbit polyclonal antibody(5) and a mouse monoclonal antibody (MAb) 9A12.(12) Polyclonal antibodies target a wide range of epitopes, each with different affinities, while the MAb has an affinity for a single epitope near the C-terminus of the protein; however the MAb does not detect IE63 truncation deletions.(13) Thus, additional monoclonal antibodies directed against VZV IE63 are needed to identify different IE63 epitopes that are likely to play a role in VZV latency and gene regulation.

Herein, we constructed a phage library displaying a random assortment of Fabs present in mouse spleens after immunization with purified recombinant VZV IE63. Phage displaying Fabs that recognize IE63 were selected by panning against the antigen expressed in bacteria. The selected Fab was shuttled into plasmids, which synthesized functional bivalent mouse IgG1. Analysis of the recombinant mouse antibody for IE63 reactivity in Western blot, immunoprecipitation, ELISA, and immunofluorescence assays indicates the promise of this reagent in molecular analysis of VZV latency, reactivation, and gene regulation.

MATERIALS AND METHODS

Virus, cells, plasmids, and bacteria

VZV was isolated from a zoster lesion and propagated in MeWo cells, a continuous cell line derived from human malignant melanoma,(14) by co-cultivation of virus-infected cells with uninfected cells in Dulbecco modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 9% heat-inactivated fetal bovine serum (Gemini Bio-Products, West Sacramento, CA).(4) The plasmids pFlag-MAC (Sigma, St. Louis, MO) and pCEP-4 (Invitrogen) were used to express proteins with an N-terminal Flag epitope. The plasmid pCI-Neo (Invitrogen) was used to shuttle various inserted DNA fragments. Plasmids were maintained in DH5 α *Escherichia coli* (Invitrogen). Bacteria containing the plasmid-derived ampicillin resistance marker were propagated in Luria-Bertani broth (LB) supplemented with 50 μ g/mL carbenicillin (LB/carb).

Construction of VZV IE63 expression vectors

The eukaryotic IE63 expression vector pF63-CEP was constructed as follows: first, pCI-Neo was modified to introduce unique EcoRI and PciI restriction sites after which sense and antisense adaptors (Table 1, primers 1 and 2) encoding a Flag epitope (DYKDDDDKG) were annealed and inserted between the NheI and XhoI restriction sites. The full-length ORF 63 was shuttled from p63AltMut(4) into the Flag-modified pCI-Neo through the unique PciI and XbaI sites, resulting in plasmid pF63-Neo (Fig. 1A). The Flag-ORF 63 cassette was then excised with NheI and XbaI from pF63-Neo and inserted in the NheI site of pCEP4. To construct pF63-MAC, the ORF 63 gene along with 9 bp of upstream DNA was excised from pF63-Neo and directionally inserted into pFlag-MAC at unique EcoRI and XbaI sites. The fidelity of all constructs was confirmed by DNA sequencing.

Expression of IE63 from pF63-MAC in *E. coli*

A 10 mL overnight culture of *E. coli* DH5 α containing pF63-MAC was diluted with 850 mL of LB/carb containing 20 mM glucose and incubated at 37°C to OD₆₀₀ = 0.43. Cultures were induced with IPTG (0.5 mM final concentration) at 30°C for an additional 2–3 h. Bacteria were centrifuged at 5000 *g* for 12 min at 4°C, and pellets were flash-frozen and stored at –80°C. Cell pellets were thawed in 20 mL TBS (20 mM Tris-HCl [pH 7.9], 150 mM NaCl) containing EDTA-free protease inhibitors (Roche Diagnostics, Mannheim, Germany) disrupted by sonication at 4°C for 5 min and clarified by centrifugation at 40,000 *g* for 30 min at 4°C. Triton X-100 (0.1% final concentration) and 1 mL anti-Flag M2 affinity gel (Sigma) were added to the clarified lysate, and the suspension was mixed at 4°C for 2 h. The slurry was packed into a 9.0 $\times$ 0.4 cm column and washed with 20 mL TBS at room temperature. Immunoabsorbed proteins were eluted in six 1 mL fractions of 0.1 M glycine (pH 3.5), and immediately neutralized with 25 μ L of 1M Tris-HCl (pH 8.0). Column fractions containing Flag-IE63 fusion protein were pooled, diluted 1:1 with TBS, and concentrated at 4°C by centrifugation at 2000 *g* for 10 min in a spin-filtration column (30 kDa exclusion, Millipore, Billerica, MA). The purity of the eluted Flag-IE63 fusion protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue-R staining (Fig. 1B). The Flag-IE63 fusion protein expressed in *E. coli* resolved as a single band on SDS-PAGE corresponding to its expected molecular weight (~35 kDa). The Flag-IE63 fusion protein expressed in HEK 293 cells resolved at the expected ~35 kDa and also as a homodimer with apparent molecular weight ~70 kDa. The presence of the Flag-IE63 homodimer was dependent upon the concentration of 2-mercaptoethanol in the SDS-PAGE sample buffer, and not the result of tandem gene insertion within the expression vector. Increasing the concentration of the reducing agent diminished the ~70 kDa protein band. The molecular weights of the recombinant proteins were estimated based on migration corresponding to protein molecular weight markers (M).

Expression of IE63 from pF63-CEP in human embryonic kidney 293 cells

To express eukaryotic IE63, 30 μ g of pF63-CEP plasmid DNA using 40 μ L 293fectin reagent (Invitrogen) was transfected into 45 mL of human embryonic kidney (HEK) cells (Freestyle 293-F suspension cells, Invitrogen) at 10^6 cells/mL. Cultures were propagated for 72 h, followed by collection of cells by low speed centrifugation. The cell pellet was lysed in 4 mL of 50 mM Tris-HCl (pH 7.4), 350 mM NaCl, 1 mM EDTA, and 1% Triton X-100. Flag-IE63 fusion protein was bound to 1 mL anti-Flag M2 affinity gel (Sigma), and non-specific protein was removed by washing in TBS. Protein bound to the affinity column was eluted with 0.1 M glycine (pH 3.5) into 6×1 mL and immediately neutralized with 25 μ L 1M Tris-HCl (pH 8.5). SDS-PAGE and Western blots were used to detect column fractions that contained Flag-IE63. These IE63 positive fractions were pooled, concentrated, and buffer exchanged into 1 mL of TBS using a spin-filtration column (30 kDa exclusion, Millipore). Flag-IE63 fusion protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL), with yields ranging from 100 to 200 μ g.

Immunization of mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center in accordance with regulations developed by the Office of Laboratory Animal Welfare at the National Institutes of Health. Two 4-week-old female BALB/c mice were injected four times over a 2-month period with immunoaffinity-purified Flag-IE63 fusion protein expressed in *E. coli*. Each mouse received 20, 24, 48, and 42 μ g of antigen by intraperitoneal injection on days 1, 13, 35, and 61, respectively. Mice were injected with antigen diluted in 250 μ L PBS, emulsified with 250 μ L Freund's complete adjuvant (day 1) and Freund's incomplete adjuvant (days 13, 35, and 61). On day 64, mice were euthanized with nembutal intraperitoneally and exsanguinated. Spleens were removed, washed in PBS, snap-frozen in liquid nitrogen, and stored at -80°C . Serum samples were obtained by retro-orbital bleeding on days 24 and 45 and a terminal intracardiac puncture.

RNA extraction and cDNA synthesis

Frozen spleens were homogenized in 9.0 mL of TriReagent (Molecular Research Center, Cincinnati, OH), which allowed extraction of RNA from the aqueous phase, as described previously.⁽²⁾ Poly[A⁺] RNA was extracted from 250 μ g of total spleen RNA by oligo-dT-affinity chromatography (Nucleo Trap mini kit, BD Biosciences, San Jose, CA). First-strand cDNA was synthesized from 500 ng of poly[A⁺] RNA in an 80 μ L reaction consisting of 50 mM Tris-HCl (pH 8.5), 30 mM KCl, 8 mM MgCl₂, 80 U RNase inhibitor, 1 mM of deoxynucleotide triphosphates (dNTP), 2.5 μ M oligo-dT[18], and 40 U reverse transcriptase (Transcriptor, Roche Diagnostics, Mannheim, Germany). Reaction mixtures were incubated at 65°C for 10 min, quenched on ice for 5 min, incubated at 50°C for 100 min and at 85°C for 5 min, and either used immediately or stored at -80°C .

Construction of phage-displayed Fab library

Mouse IgG kappa light chain (LC) variable (V_k) and constant (C_k) regions, along with heavy chain (HC) variable (V_H) and constant framework region 1 (C_{H1}) regions, were amplified by polymerase chain reaction (PCR) from mouse spleen cDNA and cloned into the pComb3H vector.⁽¹⁵⁾ Oligonucleotide primers (Table 1) included a V_k degenerate sense primer (primer 11), which is specific for LC sequences from 10 of 17 mouse IgG classes,⁽¹⁶⁾ and a common LC antisense primer (primer 12) that binds to the C region of all mouse V_k chains. LC sequences were amplified in a 100 μ L reaction containing 4 μ L cDNA, 50 mM KCl, 15 mM Tris-HCl (pH 8.0), 1.5 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M each LC primer, and 2.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA). PCR conditions consisted of initial denaturing for 10 min at 94°C , followed by 35 cycles of 20 s at 94°C , 20 s at 50°C , 1 min at

72°C, and a final extension cycle of 7 min at 72°C. The ~680 bp LC PCR products were extracted from a 2% agarose in Tris-acetate EDTA preparative gel by affinity chromatography on silica matrix (Qiagen, Valencia, CA). Purified PCR products were digested with SacI and XbaI restriction endonucleases, gel-purified and inserted into the unique SacI and XbaI restriction sites in pCOMB3H. The ligated plasmids were transformed in 0.3 mL of ultra competent XL-2 Blue *E. coli* (Stratagene, La Jolla, CA) and propagated for 1 h at 37°C in 7 mL of Superbroth (3% Bacto-tryptone, 2% yeast extract, 1% MOPS [pH 7.0]) containing 10 µg/mL tetracycline and 20 µg/mL carbenicillin; thereafter, the carbenicillin concentration was increased to 50 µg/mL. Cultures were incubated 1 h at 37°C and diluted with 90 mL Superbroth (SB) containing 10 µg/mL tetracycline, 50 µg/mL carbenicillin, and 20 mM glucose. Bacteria were propagated overnight at 37°C. The library of LC sequences inserted into pComb3H was designated pLC-COMB (Fig. 2A). The library plasmid DNA was gently extracted using a silica vacuum column kit (Sigma).

Similarly, V_H and C_{H1} were PCR amplified using a common antisense primer specific for the H chain constant region (primer 10) along with one of seven IgG1 class-specific sense primers in seven separate reactions (primers 3–9). These PCR reactions consisted of 4 µL cDNA, 50 mM KCl, 15 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 200 µM dNTPs, 0.5 µM each sense primer and the common antisense primer, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a 100 µL volume. PCR reaction conditions were an initial denaturing step for 10 min at 94°C, followed by 37 cycles of a denaturing step of 20 s at 94°C, annealing for 20 s at 50°C, extending for 1 min at 72°C, and a final extension for 7 min at 72°C. PCR products were gel purified as above, digested with XhoI and SpeI, and again gel purified. Samples were quantitated and 200 ng of each of the PCR products were pooled and inserted into the unique XhoI and SpeI in pLC-COMB. Like the LC procedure, ligation products were transformed in 0.3 mL of XL-2 Blue *E. coli*, except that after an addition of 90 mL complete SB, half of the culture was grown overnight for plasmid DNA extraction, while the remaining culture was infected with 1.2×10^{12} pfu VCSM13 helper phage (Stratagene) by absorption (15 min, 25°C) followed by 1 h at 37°C in SB, and overnight incubation at 37°C in SB containing 70 µg/mL kanamycin, 10 µg/mL tetracycline, and 50 µg/mL carbenicillin. The library of phage-displayed Fabs made from HC and LC fragments was designated pIE63-COMB (Fig. 2A). Cultures were clarified by centrifugation (15 min at 5000 g) and recombinant phage were precipitated at 4°C for 30 min upon addition of PEG 8000 and NaCl to final concentrations of 4% and 0.5 M, respectively. Precipitated phage were collected by centrifugation for 20 min at 20,000 g and stored at 4°C in 1.5 mL PBS containing 1% bovine serum albumin (BSA).

Panning the Fab library on IE63

Phage were selected and enriched for particles displaying Fabs recognizing IE63 by panning, as described previously.⁽¹⁷⁾ Briefly, four wells of a 96-well plate were coated with 1 µg of purified IE63-Flag in 50 µL of 0.1 M NaCO₃ (pH 8.6), and incubated overnight at 4°C. Wells were washed twice with PBS and blocked with 50 µL of 3% BSA in PBS for 1 h at 37°C. After removal of the blocking solution, 50 µL of PBS with 10^{11} phage from the pIE63-COMB library were added to each well and incubated for 2 h at 37°C. Unbound phage were removed with five 5-min washes in 0.5% Tween-20 in PBS (PBST). Bound phage were eluted in 50 µL/well of 0.1 M glycine, (pH 2.2) for 10 min at room temperature, and 24 µL of 1 M Tris-HCl (pH 8.0) were added. Eluted phage were used to infect log-phase *E. coli* XL1-Blue cells in 2 mL. After 15 min at room temperature, several aliquots of infected *E. coli* were removed to titer the eluted phage, while the remaining infected *E. coli* culture was propagated with the addition of LB containing antibiotics and helper phage as described above. The resulting phage library was reapplied to four wells of a microtiter plate freshly coated with IE63-Flag to initiate a subsequent round of panning. This was repeated three times for a total of four pannings.

Construction of plasmids to express recombinant mouse IgG1

Specific HC and LC sequences were shuttled from pIE63-COMB into two separate vectors for production of full-length mouse IgG1. To facilitate protein expression and secretion, LC and HC leader sequences were amplified from the antibody expression plasmid pDR12(18) using primer pairs 13/14 and 17/18, respectively (Table 1). Purified phage DNA of the Fab recognizing IE63 was used to amplify the full-length LC sequence (V_k and C_k) using primer pair 15/16. The V_H and a portion of the mouse C_{H1} region were amplified with primer pair 19/20. The leader segments and pIE63-COMB inserts were PCR amplified in 100 μ L reaction volumes containing 1X Expand High Fidelity buffer (Roche), 1.75 mM $MgCl_2$, 200 μ M dNTPs, 100 pmoles of each primer, and 3.5 U of Expand High Fidelity polymerase mix (Roche Applied Science, Penzberg, Germany). The cycle conditions were: initial denaturation at 95°C for 4 min followed by 38 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension cycle at 72°C for 7 min. The LC and HC inserts from pIE63-COMB were joined to their respective leader sequences by crossover PCR, which consisted of 120 ng of gel-purified insert and 20 ng of the appropriate purified leader sequence in a 100 μ L PCR reaction containing 1X High Fidelity buffer (Roche), 1 mM $MgCl_2$, 200 μ M dNTPs, and 0.9 U of Expand High Fidelity polymerase mix. After denaturation at 95°C for 5 min, 9 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, 100 pmol of each outside primer (primers 13 and 16 for LC construction and primers 17 and 20 for HC construction) and an additional 2.6 U of Expand High Fidelity polymerase mix were added to respective tubes, and samples were amplified for an additional 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension cycle at 72°C for 7 min.

The LC PCR product was inserted into pCEP-4 at unique HindIII and XhoI sites. The HC product was inserted at unique KpnI and NarI sites in murine pIgG1-Flag, which is a modified pCEP4 plasmid that contains the complete mouse HC constant domains 2 and 3 fused to a Flag epitope. The C_{H1} domain in this vector was engineered to contain a single silent mutation yielding the unique NarI cloning site. The resultant plasmids anti-IE63P4 LC-CEP4 and anti-IE63P4 HC-mIgG1 (Fig. 2B) were used to express full-length mouse recombinant IgG.

Expression of recombinant mouse IgG1 in HEK 293 cells

HEK cells (Freestyle 293-F cells, Invitrogen) were grown as suspension cultures in serum-free 293FS medium (Invitrogen). For recombinant protein expression, replicate transfections were performed, each consisting of 45 mL (10^6 cells/mL) HEK 293 cells, 25 μ g anti-IE63P4 LC-CEP4 and 25 μ g anti-IE63 HC-mIgG1 plasmid DNA, and 60 μ L of lipid-based transfection reagent (293fectin, Invitrogen). Four days after transfection, cells were centrifuged for 15 min at 2000 g, and the tissue culture fluid (TCF) was pooled. Mouse IgG1 was purified from the TCF by affinity chromatography on protein A/G-coated beads (ImmunoPure A/G, Pierce). Briefly, equal volumes of binding buffer (Pierce) and TCF were mixed and applied to columns of 0.4 mL protein A/G-agarose. The gravity-assisted flow-through was reapplied, and columns were washed with 10 mL (25 column volumes) binding buffer. Protein was eluted with 3 mL of IgG elution buffer (pH 2.8, Pierce) directly into 300 μ L of 1 M Tris-HCl (pH 8.5). The eluted IgG was concentrated and buffer exchanged to PBS by centrifugal filtration (Amicon Ultra-4 centrifugal filter [30 kDa cut-off], Millipore). Affinity-purified recombinant mouse anti-IE63 IgG1 was quantitated and stored at 4°C in PBS in 0.1% BSA and 0.02% sodium azide. The purified antibody was designated anti-63P4 IgG1.

Western blots

Proteins were resolved by 12% SDS-PAGE and transferred using a mini-protein II wet blotter (Bio-Rad, Hercules, CA) to a nitrocellulose membrane in 50 mM Tris, 40 mM glycine, and 0.1% SDS buffer at 200 mA for 2 h at 4°C. Membranes were blocked in TBST containing 3% non-fat milk (TBSTM) for 2 h. Membranes were probed overnight at 4°C with MAb 9A12

(1:1000 dilution) and anti-63P4 antibody (2 $\mu\text{g}/\text{mL}$) in TBSTM, followed by four 5-min washes in TBST. Membranes were incubated for 1 h at room temperature with either a goat anti-rabbit- or a rabbit anti-mouse-IgG (whole molecule) secondary antibody conjugated to horseradish peroxidase (HRP) (1:5000 dilution) in TBSTM. Membranes were washed with TBST, rinsed with distilled water, and developed in 50 mM Tris-HCl (pH 7.6), 10 mM imidazole, 0.015% H_2O_2 , and 0.04% 3'-3'-diaminobenzidine.

Immunoprecipitation

VZV-infected and uninfected MeWo cells were washed twice in TBS and lysed by sonication on ice twice for 30 s each time in RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 5 mM EGTA, 1 mM orthovanadate, 1 mM metavanadate, 50 mM NaF, and EDTA-free protease inhibitor cocktail [Roche]). Lysed cells were clarified by centrifugation at 12,500 g for 10 min at 4°C. Samples were mixed for 1 h at 4°C with 200 μL of 10% protein A-coated agarose beads (Sigma), 1 $\mu\text{g}/\text{mL}$ goat anti-mouse IgG, and either the recombinant anti-63P4 or MAb 9A12. After 1 h at 4°C, immunocomplexes were centrifuged (5 min at 2000 rpm) and washed twice with RIPA buffer, and proteins were released at 95°C for 5 min in SDS loading buffer (0.25 M Tris-HCl [pH 6.8], 0.1% SDS, and 10% glycerol). Proteins were resolved by 12% SDS-PAGE and examined by Western blot analysis using rabbit anti-IE63 polyclonal antibody.

Enzyme-linked immunosorbent assay (ELISA)

Individual wells of a 96-well plate (Costar, Corning, NY) were coated overnight at 4°C with recombinant Flag-IE63 (100 ng per well) in 50 μL of coating buffer (0.1 M NaHCO_3 [pH 8.6]). Wells were washed twice with PBS and blocked with 100 μL of 3% BSA-PBS for 2 h at 37°C. Both antibodies were diluted in 1% BSA-PBS (1:1000 for MAb 9A12 and 1 $\mu\text{g}/\text{mL}$ for recombinant 63P4) and 50 μL was added to each well and incubated for 2 h at 37°C. Wells were washed 10 times with 250 μL PBS. To detect bound antibody, 50 μL alkaline phosphatase-conjugated horse anti-mouse secondary antibody (Vector Labs, Burlingame, CA) diluted to 1 $\mu\text{g}/\text{mL}$ in 1% BSA-PBS was added and incubated for 1 h at 37°C, and wells were washed with PBS. Color was developed by the addition of 50 μL of 5 mM p-nitrophenylphosphate and 1.25 mM levamisole (Vector Labs) for <20 min at 25°C, and OD_{415} was recorded (Benchmark microplate reader, Bio-Rad) and plotted (Microplate Manager software, Bio-Rad).

Similarly, purified prokaryotic-expressed IE63 (0–400 ng) in 50 μL of coating buffer was incubated in 96-well microtiter plates at 4°C overnight. Wells were washed and incubated at 37°C for 1.5 h with 100 μL of 3% BSA-PBS followed by the addition of primary antibody (1 ng/ μL of anti-63P4, or a 1:1000 dilution of MAb 9A12, or BSA control) in 50 μL of 1% BSA-PBS and rocked at 37°C for 2 h. Wells were washed four times for 5 min each time, probed with HRP-conjugated rabbit anti-mouse antibody (1:200 dilution in 1% BSA-PBS) for 1 h at 37°C, washed with PBS as before, and incubated for 4 min at 37°C with 100 μL of the substrate 2-2'-azino-di-(3-ethylbenz-thiazoline sulfonic acid) added to each well. The mean OD_{450} of three wells and standard deviations were determined and graphed using Excel (Microsoft, Seattle, WA).

Immunofluorescence microscopy

VZV-infected and uninfected MeWo cells were propagated on glass cover slips for 72 h, fixed for 20 min in 4% paraformaldehyde-PBS, permeabilized for 20 min in PBS containing 0.3% Triton X-100, and rinsed extensively with PBS. Cells were blocked in 2% BSA-PBS for 45 min before primary antibody application. Recombinant mouse anti-63P4 IgG (1 $\mu\text{g}/\text{mL}$) and mouse MAb 9A12 (1:500 dilution) was applied in blocking buffer for 2 h at room temperature. Cover slips were rinsed with PBS, re-blocked, and incubated 2 h at room temperature in Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen) secondary antibody (1:500 in blocking buffer).

Cover slips were rinsed with PBS and mounted in DAPI-containing mounting medium (Vectashield Hard-Set, Vector Labs). Cells were viewed under ultraviolet light and photographed with a Nikon Eclipse E800 (Tokyo, Japan) fluorescence microscope at 400 × magnification.

RESULTS

Phage-displayed Fab library and anti-VZV-IE63 recombinant mouse IgG1

The titer of the initial, unamplified pIE63-COMB phagemid library was 3.2×10^5 colony-forming units (cfu). During four rounds of amplification, the titer of IE63-bound phage was 3.17×10^5 cfu, 1.1×10^4 cfu, 3.04×10^6 cfu, and 8.0×10^7 cfu, respectively. Complementarity-determining region 3 (CDR3) sequences were determined for pIE63-COMB isolates. After four cycles of panning, one heavy (ARDFIYYGR-RGYAMDY) and light (QQGSSIPLT) chain CDR3 sequence predominated. Phage with these two sequences were propagated; DNA was extracted and digested with SpeI and NheI to remove the gene III fusion protein from the H chain C-terminus, which yielded a soluble Fab.(19) The non-reduced Fab was seen as a 47.0 kDa band and as two heterogeneous monomers at 24.9 kDa after treatment with 2-mercaptoethanol in SDS-PAGE (Fig. 2A).

After Western blotting confirmed reactivity of the soluble Fab with IE63 (data not shown), the IE63-reactive Fab was shuttled into plasmids, which allowed isolation of fully assembled mouse IgG1 from culture supernatants of transfected HEK 293 Freestyle cells. Under non-reducing conditions, anti-IE63 IgG1 migrated as a large 193.9 kDa complex, while after treatment with 2-mercaptoethanol, the antibody was seen as a 53.8 kDa full-length H chain and a 25.9 kDa L chain fragment (Fig. 2B). The yield of anti-IE63 IgG1 from three independent preparations was 0.4–0.6 mg/L of culture (0.4–0.6 μg anti-IE63 IgG1 per 1×10^6 transfected cells).

Anti-63P4 antibody detects IE63 in Western blots

Western blot analysis using recombinant anti-63P4 IgG1 (Fig. 3A) detected a single band corresponding to IE63 in lysates from VZV-infected MeWo cells (lane 3) but not uninfected cells (lane 4), and detected recombinant IE63 purified from *E. coli* (lane 1) and HEK 293 cells (lane 2). Immunoblotting with MAb 9A12 revealed the same IE63 pattern (Fig. 3B). The slower migration of the recombinant IE63 compared to IE63 in VZV-infected cells likely rests in the cluster of seven N-terminal acidic amino acids in the Flag epitope fused to IE63. In Figure 3B (lane 2), IE63 expressed in HEK 293 cells resolved as a doublet. The higher molecular weight IE63 species corresponds to phosphorylated forms of the protein. Purified IE63 expressed in HEK 293 cells treated with lambda-phosphatase resolved the doublet into a single unmodified IE63 protein species (data not shown).

Anti-63P4 antibody immunoprecipitates IE63

Immunoprecipitation (Fig. 4) revealed the IE63 in VZV-infected MeWo cells using the recombinant anti-63P4 IgG1 (lane 5) or the MAb 9A12 (lane 4), but not in uninfected cells (lanes 2 and 3). The immunoprecipitated IE63 was detected on the Western blot with polyclonal rabbit anti-IE63 antibody.

Anti-63P4 antibody detects IE63 in ELISA

Both anti-63P4 IgG1 and MAb 9A12 detected IE63 in a dose-dependent manner in ELISA (Fig. 5). Both antibodies readily detected small amounts of IE63 (6 ng for anti-63P4 and 12.5 ng for MAb 9A12) and both antibodies reached saturation at 75 ng of IE63, with no change in OD₄₁₅ with increasing amounts of IE63.

Anti-63P4 antibody detects IE63 in immunofluorescence assay

In VZV-infected cells, the anti-63P4 antibody detected IE63 primarily in the cytoplasm (Fig. 6A) whereas MAb 9A12 detected IE63 predominantly in nuclei (Fig. 6C). No fluorescence was seen when uninfected cells were incubated with either antibody (Fig. 6B and D).

DISCUSSION

A phagemid library was constructed that displayed randomly assorted H and L chain sequences present in spleen of mice immunized with IE63. After four cycles of panning, the library against purified IE63 antigen, a near homogeneous population of phage displaying the same Fab, was obtained. The Fab detected IE63 on Western blots. Because the background was high (data not shown), both H and L chain sequences were shuttled into separate vectors and used to obtain a functional mouse IgG1 in HEK 293 cells. This recombinant antibody detected IE63 in Western blot, immunoprecipitation, ELISA, and immunofluorescence assays.

Previous studies have used both polyclonal and monoclonal antibodies directed against VZV IE63 to characterize its presence and localization in cells latently and productively infected with VZV. Like our recombinant anti-IE63 antibody 63P4, polyclonal antibodies raised in rabbits against the purified GST-IE63 fusion protein detect IE63 by immunoprecipitation(4) as well as on Western blots and by immunofluorescence.(20) However, the rabbit polyclonal antibody detects IE63 in both the cytoplasm and nucleus of VZV-infected cells,(20) whereas anti-IE63 antibody 63P4 detects IE63 predominantly in the cytoplasm, the primary site of IE63 during latent VZV infection. The latter may be due to detection of only a single IE63-specific epitope. Using a series of plasmids expressing IE63 truncation deletions and alanine substitutions, we are currently mapping the amino acid epitope in IE63 that is recognized by the antibody 63P4. Mouse MAb 9A12 also detects VZV IE63 by immunoprecipitation, by ELISA (this report), by immunofluorescence,(13) and on Western blots;(12) however, unlike our recombinant antibody, MAb 9A12 detects the protein predominately in the nucleus of VZV-infected cells.(21)

The cytoplasmic detection of IE63 by our recombinant antibody suggests its potential usefulness in studies of VZV latency in human ganglia. One such potential use of the recombinant IE63 antibody is the specific immunoprecipitate of cytoplasmic IE63. During latent VZV infection, IE63 is present in the cytoplasm of neurons.(5,6,22) The ability to specifically immunoprecipitate the cytoplasmic IE63 will permit studies that identify post-translational modifications of the protein that differentiate nuclear from cytoplasmic IE63. In addition, our recombinant IE63 antibody will permit co-immunoprecipitation (Co-IP) studies designed to identify cytoplasmic protein complexes containing IE63. The Co-IP studies will help determine the function of IE63 in both productive and latent infection.

A waning anti-VZV CTL response has been associated with the development of herpes zoster. (23–25) It has been suggested that a subunit vaccine containing IE63 might boost cell-mediated immunity (CMI) to IE63 and thus lessen the occurrence and severity of zoster. An IE63 ELISA, as we have described, can be modified for high-throughput applications and used to monitor levels of circulating anti-IE63 antibody to see if there is any correlation with a declining CMI to VZV IE63. Finally, the strategy and methodology used to derive the recombinant anti-IE63 also promises to ensure an abundant supply of MAbs suitable for analyses of other VZV genes transcribed during latent VZV infection.

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FIG. 1.

Construction, expression, and purification of recombinant IE63. **(A)** Diagrams of the structure of VZV IE63 expression plasmids with restriction enzyme sites used to shuttle VZV ORF 63 from pF63-Neo into pF63-CEP and pF63-Mac. **(B)** SDS-PAGE analysis of recombinant IE63 proteins expressed from the plasmids and purified from either bacteria or mammalian cells by Flag-affinity chromatography. Prokaryotically expressed IE63 (lane P) resolved as a single discrete 38 kDa band, whereas eukaryotically expressed IE63 (lane E) resolved as two discrete 75 and 38 kDa bands. M, molecular weight marker.

FIG. 2.

Construction, expression, and purification of recombinant mouse Fab and IgG1 antibody. (A) Light chain variable and constant regions were PCR amplified from cDNAs using primer 11 and primer 12 (these contain a 5' SacI restriction site followed by a degenerate sequence for 5' V_k sequences and a XbaI restriction site followed by the C_k 3' region, respectively). These PCR products were cloned into pCOMB-3H to make pLC-COMB. Separate PCR reactions for the Fab heavy chain sequences were performed using seven different forward primers (3-9), each of which includes a 5' XhoI site and heavy chain variable region-binding sequence, along with a single reverse primer (10), which contains a SpeI restriction site and encodes the heavy chain constant region 1. The PCR products were pooled and then cloned into the XhoI and SpeI restriction sites of pLC-COMB to make the pIE63-COMB phage library. The Fab proteins corresponding to a single phage were detected by Western blot (right) under non-reducing (N) and reducing (R) conditions as 47.0 and 24.9 kDa bands, respectively. (B) Leader chain sequences were PCR amplified using primers 13 and 14 (light chain leader sequence) or 17 and 18 (heavy chain leader sequence), while the Fab light chain and heavy chain were PCR amplified with primers 15 and 16 or 19 and 20, respectively. PCR amplified leader sequences were mixed with their respective Fab light chain or heavy chain PCR fragments and used for crossover PCR, which resulted in two separate PCR fragments that combine these sequences and add terminal restriction sites for cloning. The PCR fragments for the light chain and the heavy chain were cloned into pCEP-4 and pIgG1 Flag vectors, respectively. The recombinant antibody made from the expression of these clones was detected by Western blot, as shown at right. This purified IgG1 antibody (63P4) directed against bacterially expressed VZV IE63 is resolved on SDS-PAGE under non-reducing (N) conditions as a 194 kDa molecular weight complex, but under reducing (R) conditions, as 54 and 25 kDa bands.

FIG. 3.

Western blot analysis of VZV-IE63. **(A)** Detection of the 31 kDa recombinant VZV IE63 expressed in prokaryotic (lane 1) and eukaryotic (lane 2) cells, as well as the 27 kDa VZV IE63 in VZV-infected MeWo cell lysate (lane 3) but not in uninfected MeWo cell lysate (lane 4), using recombinant anti-63P4. **(B)** Identical VZV IE63 pattern of detection using MAb 9A12. Location of protein molecular weight standards are shown at right.

FIG. 4.

Immunoprecipitation of VZV IE63. Both MAb 9A12 and recombinant anti-63P4 immunoprecipitate the 27 kDa IE63 protein from VZV-infected cell lysates but not from uninfected cells. Lane 1, VZV-infected MeWo cells without primary antibody; lane 2, uninfected MeWo cells with MAb 9A12; lane 3, uninfected MeWo cells with anti-63P4; lane 4, VZV-infected cells with MAb 9A12; lane 5, VZV-infected MeWo cells with anti-63P4 antibody. The immunoprecipitated IE63 was detected with polyclonal rabbit anti-IE63 antibody followed by coloro-metric development, as described in the Material and Methods section.

FIG. 5.

Detection of IE63 in ELISA. Purified VZV IE63 protein coated on 96-well plates was probed with recombinant anti-63P4 (closed circles) or anti-MAb 9A12 (open circles). A dose-dependent increase in OD₄₁₅ (mean $\pm$ SD) was observed with both antibodies compared to negative control wells coated with BSA (closed, upside-down triangles).

FIG. 6.

VZV IE63 detection by immunofluorescence. (A) VZV-infected MeWo cells incubated with anti-63P4 antibody reveals VZV IE63 predominately in the cytoplasm. (B) No staining is seen when uninfected cells are incubated with anti-63P4. (C) VZV-infected MeWo cells incubated with MAb 9A12 reveals VZV IE63 predominantly in the nucleus. (D) No staining is seen when uninfected cells are incubated with MAb 9A12. After incubation with antibody, Alexa Fluor488 (green) was used to detect the presence of antibody bound to IE63. Cell nuclei were identified by staining with DAPI (blue). Red bar, 50 μm .

Table 1**Primers Used to Generate Plasmids and Phagmids**

Primer	Sequence	Function
1	<u>CTAGCGCCACCATGGACTACAAGGACGATGACGATAAGGGCTC</u>	NheI-Flag sense
2	<u>TCGAGAGCCCTTATCGTCATCGTCCTTGTAGTCCATGGTGGCG</u>	XhoI-Flag antisense
3	SAKGTGCAGCTCGAGSAGTCAGGACCT	Binds Vh sub Ia & Ib
4	GAGGTYCAGCTCGAGCARTCTGGACCT	Binds Vh sub IIa
5	CAGGTCCAACCTCGAGCAGYCTGGGKCT	Binds Vh sub IIb
6	GAGGTTTCAGCTCGAGCAGTCTGGRGCWG	Binds Vh sub IIc&Va
7	GARGTGAAGCTCGAGGAGWCTGGASGA	Binds Vh sub IIIa&c
8	GAGGTGAAGCTTCTCGAGTCTGGAGGT	Binds Vh sub IIIb
9	GAAGTGMAGCTCGAGGAGTCTGGGGGA	Binds Vh sub IIIc
10	AGGCTTACTAGTACAATCCCTGGGCACAAT	Vh Fab constant 3'
11	GGGAGCTCGAYATTGTGMTSACMCARWCTMCA	Vk 5' degenerate
12	GCGCCGTCTAGAATTAACACTCATTCTGTGAA	Vk Fab constant 3'
13	GTGTAAGCTTACCATGGGTGTGCCCACTCAGG	Vk secretor leader - 5'
14	ACATCTGGCATCTGTAAGCCACAG	Vk secretor leader - 3'
15	TGGCTTACAGATGCCAGATGTGATATTGTGATGACACAATCTACA	Join leader to 5' Vk
16	GTGTCTCGAGATTAACACTCTCCCTGTTGAAGC	3' Vk IgG constant
17	GTGTGGTACCGCCGCCACCATGGAATGGAGCTGG	Vh secretor leader - 5'
18	GGAGTGGACACCTGTAGTTACTG	Vh secretor leader - 3'
19	CAGTAACTACAGGTGTCCACTCCGAGGTGCAGCTGCTAGAGCAGTCT	Join leader to 5' Vh
20	CAGCAGATCCAGGCGCCAGTG	3' Vh IgG constant

Degenerative nucleotide bases

K = G or T, S = C or G, M = A or C, W = A or T, R = A or G, Y = C or T.