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Characterization of Domains of Herpes Simplex Virus Type 1 Glycoprotein E Involved in Fc Binding Activity for Immunoglobulin G Aggregates

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Herpes simplex virus type 1 glycoproteins gE and gI form receptors for the Fc domain of immunoglobulin G (IgG) which are expressed on the surface of infected cells and on the virion envelope and which protect the virus from immune attack. Glycoprotein gE-1 is a low-affinity Fc receptor (FcR) that binds IgG aggregates, while gE-1 and gI-1 form a complex which serves as a higher-affinity FcR capable of binding IgG monomers. In this study, we describe two approaches used to map an Fc binding domain on gE-1 for IgG aggregates. First, we constructed nine plasmids encoding gE-1/gD-1 fusions proteins, each containing a large gE-1 peptide inserted into the ectodomain of gD-1. Fusion proteins were tested for FcR activity with IgG-sensitized erythrocytes in a rosetting assay. Three of the fusion proteins containing overlapping gE-1 peptides demonstrated FcR activity; the smallest peptide that retained Fc binding activity includes gE-1 amino acids 183 to 402. These results indicate that an Fc binding domain is located between gE-1 amino acids 183 and 402. To more precisely map the Fc binding domain, we tested a panel of 21 gE-1 linker insertion mutants. Ten mutants with insertions between gE-1 amino acids 235 and 380 failed to bind IgG-sensitized erythrocytes, while each of the remaining mutants demonstrated wild-type Fc binding activity. Taken together, these results indicate that the region of gE-1 between amino acids 235 and 380 forms an FcR domain. A computer-assisted analysis of the amino acid sequence of gE-1 demonstrates an immunoglobulin-like domain contained within this region (residues 322 to 359) which shares homology with mammalian FcRs.

Viruses have evolved mechanisms that modify the effectiveness of the host immune response and interfere with viral clearance. Although strategies for immune evasion are varied, several viruses encode proteins that mimic host immunoregulatory molecules. The BCRF1 protein of Epstein-Barr virus is a homolog of interleukin-10 and inhibits synthesis of cytokines by activated T-helper cells (21). The E3/19K protein of adenovirus forms a molecular complex with major histocompatibility complex class I antigens and downregulates major histocompatibility complex expression at the cell surface (7). Herpes simplex virus type 1 (HSV-1) encodes a receptor for complement components C3b and iC3b, formed by glycoprotein C (gC) (9, 16, 41), as well as a receptor for the Fc domain of immunoglobulin G (IgG), formed by glycoproteins E (gE) and I (gI) (5, 25, 26, 43). These HSV receptors are expressed on the viral envelope and on the surface of infected cells and, in vitro, protect from antibody- and complement-mediated attack (13, 15, 19). Other herpesviruses have been shown to express similar immune receptors, such as the gC homolog of pseudorabies virus (glycoprotein III), which binds complement component C3b (22), and the gE homolog of varicella-zoster virus (gpI), which has Fc binding activity (29, 30).

Early studies of the HSV Fc receptor (FcR) focused on its ability to protect the virus and virus-infected cells by binding nonimmune IgG. Dowler and Veltri demonstrated that nonimmune IgG or purified Fc fragments protect HSV-2 from neutralization by HSV-2-specific antibody (11). Aggregated IgG protects HSV-1-infected cells from antibody- and complement-mediated cytolysis or destruction by sensitized lymphocytes (1). More recently, Frank and Friedman demonstrated that anti-HSV IgG also binds to the HSV-1 FcR, as part of a process termed antibody bipolar bridging (15). This refers to the binding of an antiviral IgG molecule to its antigenic target by its Fab domain while concomitantly binding to the HSV FcR by its Fc domain. Antibody bipolar bridging has been shown to protect HSV from host immune defenses mediated by the Fc domain of antiviral IgG. Specifically, it protects the virus from antibody- and complement-mediated neutralization and virus-infected cells from antibody-dependent cellular cytotoxicity (13, 15). In addition, antibody bipolar bridging blocks the binding of complement component C1q to antiviral IgG on infected cells, suggesting that it protects from complement-mediated cytolysis (13).

The HSV-1 FcR is formed by a complex of gE-1 and gI-1 and binds both IgG monomers and aggregates (6, 12, 18, 25, 26). When expressed individually, gE-1 is an FcR for IgG aggregates but not monomers, while gI-1 binds neither IgG aggregates nor monomers (12). These observations suggest that gE-1 has intrinsic IgG Fc binding activity which is modified by its interaction with gI-1 (6, 12, 18). Using a panel of synthetic gE-1 peptides, Williams et al. identified several regions of gE-1 that have Fc binding activity (45). In this report, we describe two alternative approaches used to define a domain on gE-1 required for the binding of IgG aggregates. In addition, we show that this domain contains a region which shares sequence homology with mammalian FcRs, suggesting

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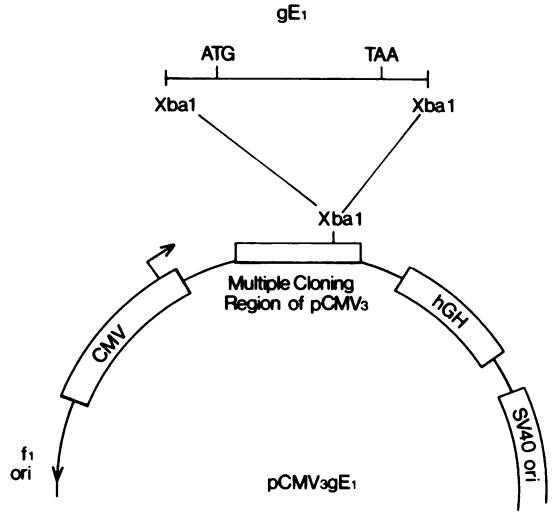

FIG. 1. Construction of pCMV3gE-1. Abbreviations: CMV, cytomegalovirus major immediate-early promoter; hGH, human growth hormone transcription termination and polyadenylation signals; SV40 ori, simian virus 40 origin of replication and promoter-enhancer sequences.

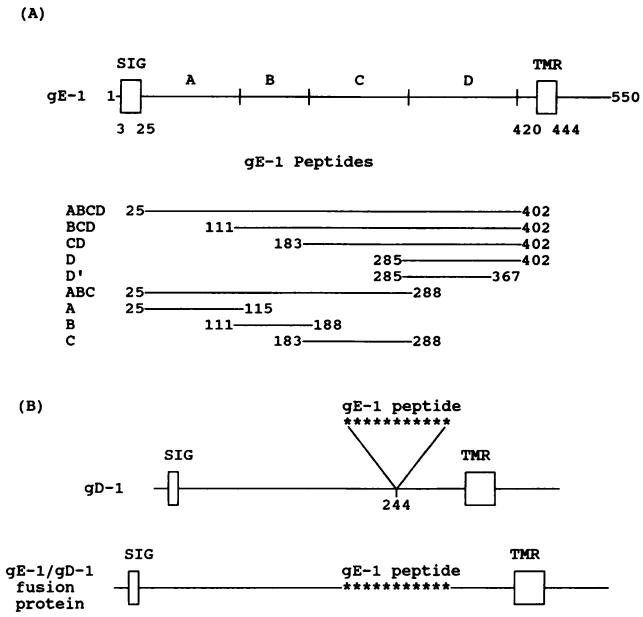

FIG. 2. Expression of gE-1 peptides as gD-1/gE-1 fusion proteins. (A) PCR was used to amplify nine gE-1 DNA fragments from plasmid DNA. These DNA fragments encode a set of overlapping peptides that contain regions of the gE-1 ectodomain (denoted by the letters A, B, C, and D). (B) Each gE-1 DNA fragment was inserted into pCMV3gD-1 at a unique *Apa*I restriction site corresponding to gD-1 amino acid position 244 to create a panel of plasmids encoding gE-1/gD-1 fusion proteins. Abbreviations: SIG, signal sequence; TMR, transmembrane region.

that HSV-1 evades host immune defenses by mimicking the mammalian receptors.

MATERIALS AND METHODS

Cell cultures. Mouse Ltk− cells (L cells) were grown in α-minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Inc., Logan, Utah), gentamicin, amphotericin B, vitamins, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer solution.

Antibodies. Anti-gE-1 monoclonal antibodies (MAb) used in these studies included 1BA10 (16), Fd.172 (kindly provided by S. Chatterjee) (32), II-481 (kindly provided by P. G. Spear) (27), and 18-81-A2 (kindly provided by K. E. van Vliet). Anti-gD-1 MAbs used included ID3 (16) and DL6 (kindly provided by G. Cohen and R. Eisenberg) (14).

Construction of a recombinant plasmid for expression of gE-1. To construct the gE-1 mammalian expression plasmid pCMV3gE-1, the gE-1 coding region from pMMTV-gE (12) was excised with *Xba*I and subcloned into pCMV3 (3). This plasmid places gE-1 transcription under the control of the immediate-early promoter of cytomegalovirus (Fig. 1).

Construction of recombinant plasmids to express gE-1 peptides in gD-1 fusion proteins. Fragments of the gE-1 gene were amplified from plasmid DNA with PCR and cloned into the coding region of gD-1 to create a panel of recombinant plasmids encoding gE-1–gD-1 fusion proteins. Each fusion protein has a gE-1 peptide inserted into the ectodomain of gD-1 and allows expression of the gE-1 peptides at the surface of transfected cells.

(i) Amplification of gE-1 DNA fragments by PCR. Separate PCRs with different pairs of synthetic oligonucleotide primers were used to amplify nine gE-1 DNA fragments. For each reaction, 10 ng of pCMV3gE-1 was mixed with 2.5 U of *Taq*I DNA polymerase (Perkin-Elmer Cetus) in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, gelatin (0.001% [wt/vol]), a 200 μM concentration of each deoxynucleotide triphosphate, and a 1.0 μM concentration of each primer. The PCR mixtures were subjected to 26 cycles of amplification (94°C for 1 min, 56°C for 15 s, and 72°C for 3 min) and extracted with phenol-chloroform, and the amplified DNA product was purified from low-melting-point agarose by gel electrophoresis.

Nine DNA fragments encoding overlapping gE-1 peptides were amplified with the following PCR oligonucleotide primer pairs (shown as 5′ and 3′ primers, respectively [Fig. 2A]): fragment A (amino acids 25 to 115), primers 5′-AAACGTC CTGGAGACGGGT-3′ and 5′-CACACGAAGTCCGTTCG T-3′; fragment B (amino acids 111 to 188), primers 5′-GTCTACGAACGGACTTCGT-3′ and 5′-AGACTTTCGTC CTCGCCCT-3′; fragment C (amino acids 183 to 288), primers 5′-AGGGCGAGGACGAAAGTCT-3′ and 5′-CATTCTGG GAGCTGCGGGT-3′; fragment D (amino acids 285 to 402), primers 5′-TTAGGGCCCCAGCTCCCAGAGTGTCTGT-3′ and 5′-TAAGGGCCCGTGGGTGGGCTCGGCCAAAT-3′; fragment D′ (amino acids 285 to 367), primers 5′-CGC AGCTCCCAGAATGTCTGT-3′ and 5′-TGAATATGGTC GTTGACGT-3′; fragment ABDC (amino acids 25 to 402), primers 5′-TTAGGGCCCACGTCCTGGAGACGGGTGAG T-3′ and the 3′ primer of fragment D; fragment ABC (amino acids 25 to 288), the 5′ primer of fragment ABCD and 5′-TTTAGGGCCCACATTCTGGGAGCTGCGGGTGAT-3′; fragment BCD (amino acids 111 to 402), primers 5′-TT TAGGGCCCGGTCTACGAACGGACTTCGT-3′ and the 3′ primer of fragment D; and fragment CD (amino acids 183 to 402), primers 5′-TTTAGGGCCCGAGGGCGAGGACGA AAGTCT-3′) and the 3′ primer of fragment D. Several of the primers contain an *Apa*I restriction site (underlined) to facilitate cloning.

(ii) Construction of recombinant plasmids for expression of gE-1 peptides in gD-1 fusion proteins. Each gE-1 DNA fragment was inserted, in-frame, into the gD-1 coding region (44) of pCMV3-gD-1 at the unique *Apa*I site at amino acid position 244 located in the ectodomain of the protein (Fig. 2B). pCMV3gD-1 (kindly provided by M. Muggeridge, R. J. Eisenberg, and G. H. Cohen) has the gD-1 gene (obtained as a *Hin*dIII fragment from pRE4) (33) cloned into the pCMV3 expression vector at the *Hin*dIII site in the multiple cloning region. gE-1 DNA fragments amplified with PCR primers containing *Apa*I restriction sites (ABCD, ABC, BCD, CD, and D) were digested with *Apa*I to generate cohesive ends and were cloned directly into the *Apa*I site of pCMV3-gD-1. The remaining gE-1 DNA fragments (A, B, C, and D′) were ligated into the *Apa*I site of pCMV3-gD-1 after the fragments and the linearized plasmid were blunt ended with the Klenow fragment of *Escherichia coli* DNA polymerase. Proper construction of each fusion plasmid was verified by DNA sequencing.

Construction of recombinant plasmids to express in-frame gE-1 linker insertion mutants. Linker insertion mutagenesis was performed as previously described to prepare a panel of 21 gE-1 mutants (37, 42). Details of the construction of these mutants will be described elsewhere (4). Briefly, pCMV3gE-1 was partially digested with either *Hae*III, *Sma*I, *Xma*III, *Aha*II, *Nae*I, *Alu*I, *Rsa*I, or *Taq*I, which cut at multiple sites in the plasmid, or digested to completion with *Bst*EII, which cuts at a single site. Single-cut linear plasmid DNAs were separated from other digestion products by agarose gel electrophoresis, eluted from the gel, blunt ended with the Klenow fragment of DNA polymerase (except in the case of *Hae*III and *Sma*I, which generate blunt ends), and recircularized by ligation of 8-mer, 10-mer, or 12-mer phosphorylated *Xho*I linkers (New England Biolabs, Beverly, Mass.), which were selected to restore the original reading frame of gE-1. The religated DNAs were used to transform *E. coli* DH5α, and plasmids isolated from individual colonies were screened for the presence and location of the linker insertion in gE-1 by restriction enzyme analysis. To ensure that a single linker was inserted, each of the mutant plasmids was digested with *Xho*I, separated from any digested linkers by agarose gel electrophoresis, and religated. As described below, gE-1 mutants were tested for cell surface expression in transfected cells and only mutant plasmids encoding surface-expressed proteins were further studied. Each of these plasmids was sequenced across the region of the insertion to verify proper construction. The sequencing identified an in-frame gE-1 deletion mutant, designated H(Δ), which has a 17-amino-acid deletion between two adjacent *Hae*III sites substituted by a 4-amino-acid insert.

DNA transfection. Transient-transfection assays were performed as previously described (17), using L cells on 6-well plates. At 42 h posttransfection, cytoplasmic extracts of cells were prepared (10) or cells were suspended in phosphate-buffered saline (PBS) containing 0.005 M EDTA, washed twice, and resuspended in PBS containing 1.0% bovine serum albumin for use in rosetting or immunofluorescence assays (see below).

Rosetting assay to detect FcR activity for IgG aggregates. Sheep erythrocytes were sensitized with subagglutinating concentrations of goat anti-sheep erythrocyte IgG (Cordis Laboratories, Miami, Fla.) as previously described (15). Transfected cells, either in suspension or as monolayers, were incubated with IgG-coated erythrocytes (EAIgG) for 2 h at 37°C (ratio of EAIgG to transfected cells, 100:1) and observed for erythrocyte rosette formation by light microscopy. A rosette was defined as a cell with ≥four bound erythrocytes bordering its perimeter. Each rosetting assay was performed on at least two separate occasions, and only plasmids that induced consistent rosette formation were reported as positive for FcR expression.

Immunofluorescence. To evaluate cell surface expression of gD-1/gE-1 fusion proteins or gE-1 linker insertion mutants, unfixed cells were incubated with the gD-1 MAb 1D3 or a gE-1 MAb for 30 min at 4°C, washed with PBS, incubated with a 1:40 dilution of goat anti-mouse IgG $F(ab')_2$ fluorescein-labeled conjugate (Organon Teknika, West Chester, Pa.) for 30 min at 4°C, washed, and analyzed by fluorescence microscopy. Each immunofluorescence assay was performed on at least two separate occasions, and the intensity of immunofluorescence was scored on a scale of 0 to 4. A score of 0 was defined as the intensity of fluorescence observed with untransfected cells. Cells achieving an immunofluorescence score of ≥2 on each assay were considered positive for reactivity with the antibody tested.

Additional immunofluorescence studies were performed on acetone-fixed cells with gD-1 and gE-1 MAbs. Transfected cells were allowed to adhere to glass microscope slides and were fixed in acetone prior to incubation with MAb and fluorescein-labeled conjugate. Intensity of immunofluorescence was scored and interpreted as described above.

Western immunoblot analysis. Cytoplasmic extracts of cells transfected with gD-1/gE-1 fusion plasmids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and evaluated by Western blot analysis as previously described (10).

Sequence comparisons between gE-1 and mammalian IgG FcRs. Nucleotide sequences of gE-1 (31) and human IgG FcR types I (2) and II (40) were retrieved from GenBank (accession numbers M12354, X14356, and X62572, respectively), and the predicted amino acid sequences were generated with the Genetics Computer Group (GCG) software package, version 7.2. The amino acid sequences of human FcR type III (39) and murine IgG Fcγ2b/1 (36) were manually entered into GCG files. Amino acid sequence comparisons were performed with the GCG Gap program (34). As a control, gE-1 sequences were randomly shuffled with the GCG Shuffle program.

RESULTS

Binding of IgG aggregates to gE-1 expressed at the surface of transfected cells. L cells transiently transfected with pCMV3gE-1 were evaluated for gE-1 expression by immunofluorescence. Approximately 5 to 10% of fixed cells express gE-1 (Fig. 3A), and a similar percentage of unfixed cells demonstrated gE-1 expression at the cell surface (not shown). Transfected cells bind IgG aggregates in the form of EAIgG, as indicated by the formation of EAIgG rosettes (Fig. 3B).

Use of gD-1/gE-1 fusion proteins to map the region of gE-1 involved in the binding of IgG aggregates. We expressed nine overlapping gE-1 peptides in gD-1 fusion proteins and tested each for binding of EAIgG in a rosetting assay. Expression of gE-1 peptides in fusion proteins allows the use of gD-1 antibodies to determine whether the fusion protein is expressed, which is helpful since some gE-1 peptides fail to react with gE-1 MAbs. In addition, by using fusion proteins, gE-1 peptides could be expressed at the cell surface, facilitating FcR detection with an EAIgG rosetting assay.

(i) Reactivity of fusion proteins with anti-gD-1 and anti-gE-1 MAbs. L cells were transiently transfected with each fusion plasmid, and the expressed proteins were tested by immunofluorescence for reactivity with anti-gD-1 MAbs 1D3 and DL6, which recognize sequential (nonconformational) epitopes contained between residues 1 and 23 (14) and 272 and

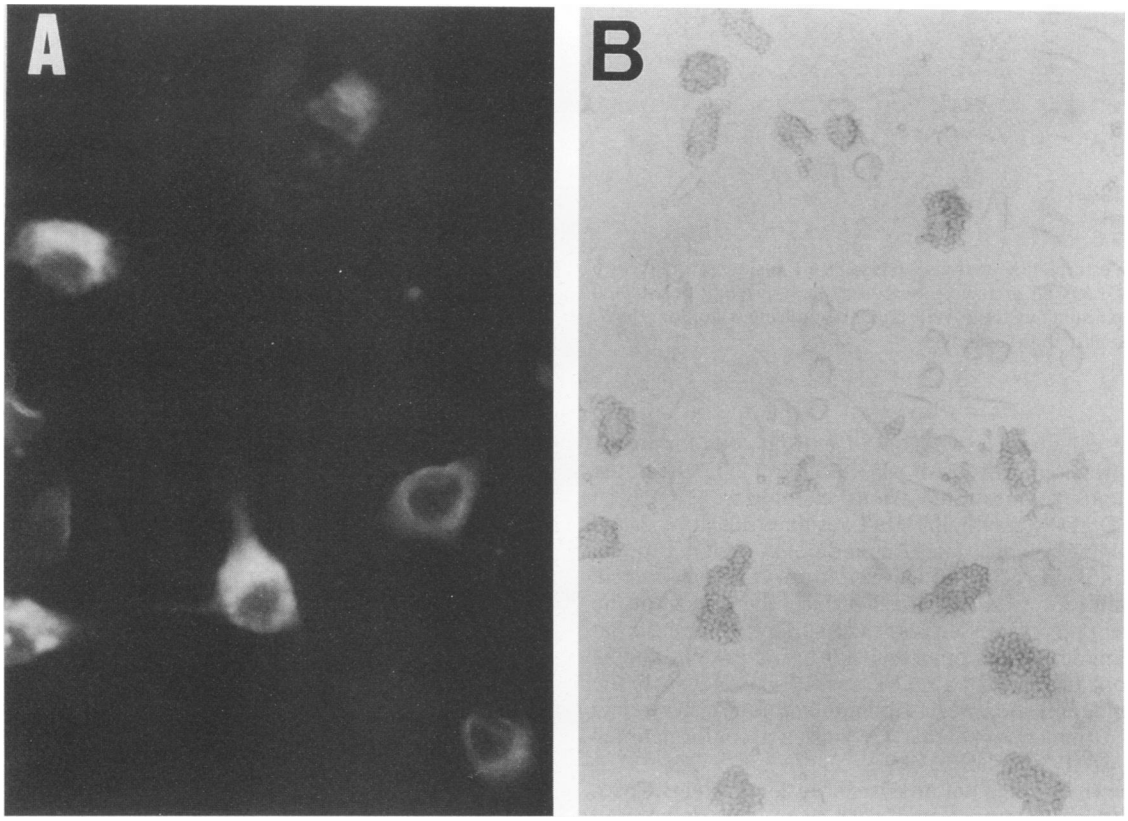

FIG. 3. Expression of wild-type gE-1 in transfected cells. Cells transiently transfected with pCMV3gE-1 were fixed with acetone and incubated with gE-1 MAb 1BA10 and a fluorescein-labeled conjugate to detect gE-1 expression (A) or incubated with EAIgG to detect FcRs with a rosetting assay (B).

279 (14, 24), respectively (Table 1). These epitopes should not be disrupted by the in-frame insertion of a gE-1 peptide at residue 244. Each of the fusion proteins reacted with 1D3 and DL6, confirming protein expression and indicating that insertion of gE-1 DNA fragments did not prevent transcription or translation of the fusion proteins. Reactivity of fusion proteins with gE-1 MAbs was assayed by immunofluorescence to confirm expression of gE-1 peptides. Many of the fusion proteins reacted with one or more gE-1 MAbs (Table 1). The D, D′, A, and B peptides were not recognized by any of the gE-1 MAbs, suggesting that they do not contain epitopes for these antibodies.

(ii) Cell surface expression of fusion proteins. Previous studies with gD-1 linker insertion mutants show that an insertion in gD-1 that disrupts its conformation often causes protein aggregation and prevents processing and transport to the cell surface (8). To determine whether insertion of gE-1 peptides in gD-1 interferes with transport of the fusion pro-

TABLE 1. gE-1 peptides expressed in gD-1 fusion proteins

Fusion protein[a]	Reactivity[b] with:						Cell surface expression[c]	Rosetting[d]
	Anti-gD-1 MAbs		Anti-gE-1 MAbs					
	1D3	DL6	1BA10	II-481	Fd.172	18-81-A2		
ABCD/gD-1	+	+	+	+	+	+	+	+
BCD/gD-1	+	+	−	+	+	−	+	+
CD/gD-1	+	+	−	+	+	−	+	+
D/gD-1	+	+	−	−	−	−	−	−
D′/gD-1	+	+	−	−	−	−	+	−
ABC/gD-1	+	+	+	+	−	−	+	−
A/gD-1	+	+	−	−	−	−	+	−
B/gD-1	+	+	−	−	−	−	+	−
C/gD-1	+	+	−	+	−	−	+	−

[a] Each fusion protein is designated by the name of the gE-1 peptide inserted, followed by gD-1.
[b] Assayed by immunofluorescence with acetone-fixed cells.
[c] Assayed by immunofluorescence with unfixed cells.
[d] Assayed with EAIgG.

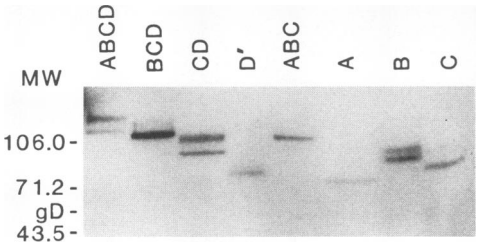

FIG. 4. Western blot analysis of gE-1/gD-1 fusion proteins with gD-1 MAb DL6. Cell extracts prepared from transiently transfected cells were separated by SDS-PAGE under denaturing conditions. MW, molecular weight (in thousands).

teins to the cell surface, we assayed unfixed cells for fusion protein expression with MAb 1D3 (Table 1). Each of the fusion proteins demonstrated strong cell surface expression except for D/gD-1, containing gE-1 residues 285 to 402 and showing minimal expression. An additional fusion protein, D'/gD-1, containing gE-1 residues 285 to 367, was constructed. This was well expressed at the cell surface. Since most of the fusion proteins are expressed at the cell surface, it appears that the gE-1 peptides do not interfere with fusion protein folding and transport. In fact, ABCD/gD-1, the fusion protein with the largest peptide insertion, reacts in immunofluorescence assays with VID, a human MAb that recognizes a conformational epitope on gD-1 (not shown) (38).

(iii) Western immunoblot analysis of fusion proteins. Cytoplasmic extracts of transfected cells were electrophoresed under denaturing conditions (Fig. 4). All of the fusions proteins migrated at rates approximately correlating with the size of the gE-1 peptide insertion. Fusion proteins containing the ABCD, BCD, CD, and B peptides migrated as two distinct bands, indicating the presence of a precursor and a processed form of each protein.

(iv) Binding of IgG aggregates to gE-1/gD-1 fusion proteins. Transfected cells were assayed for FcR activity in a rosetting assay with EAIgG. Cells expressing fusion proteins containing the ABCD, BCD, and CD peptides formed erythrocyte rosettes (Table 1; Fig. 5), while no rosetting was observed on cells expressing other fusion proteins or on control cells transfected with pCMV3gD-1. These results indicate that a binding domain for IgG aggregates is contained within the CD peptide between residues 183 and 402. This finding, however, does not exclude the possibility that portions of gE-1 outside of this region contribute to full FcR activity. Neither the C nor the D peptides expressed individually demonstrated FcR activity, suggesting that formation of the Fc binding domain requires contributions from both the C and the D portions of gE-1. We cannot rule out the possibility, however, that the Fc binding domain is contained exclusively within the C or D peptide but that these peptides are misfolded when expressed as fusion proteins.

(v) Characterization of gE-1 epitopes with gE-1/gD-1 fusion proteins. Our experiments investigating the reactivity of the gE-1/gD-1 fusion proteins with gE-1 MAbs have allowed us to partially characterize the epitopes recognized by several of the MAbs (Table 1). Previous studies have established that 1BA10 recognizes a sequential gE-1 epitope, since it reacts with denatured gE-1 on Western blots (15). 1BA10 reacted with the ABCD and ABC peptides but not the A, B, or BCD peptides, suggesting that its epitope is within the AB fragment, perhaps overlapping residues 111 to 115 at the junction of the A and B peptides. In support of this is the observation that gE-1 mutant E-H(Δ), which has a deletion of residues 103 to 120, also fails to react with 1BA10. II-481 reacts with the C peptide of gE-1, as well as each of the larger peptides containing the C portion (the ABCD, ABC, BCD, and CD peptides), indicating that it binds to a region of gE-1 between residues 183 and 288.

Binding of 18-81-A2 and Fd.172 to gE-1 requires proper protein conformation, since they do not react with the denatured protein on a Western blot (result not shown). 18-81-A2 reacted with only the ABCD peptide but none of the smaller peptides, further suggesting that it recognizes a conformational epitope. Fd.172 reacts with the ABCD, BCD, and CD peptides, indicating that its epitope is contained within the CD peptide between gE-1 residues 183 and 402 and that this region of each of the gE-1/gD-1 fusion proteins retains a relatively intact conformation resembling native gE-1.

Use of gE-1 linker insertion mutants to fine map the region of gE-1 involved in binding of IgG aggregates. Immunofluorescence experiments have demonstrated that each of the 21 gE-1 linker insertion mutants is expressed at the surface of transfected cells and reacts with one of the conformation-dependent gE-1 MAbs Fd.172 and 18-81-A2, indicating proper protein folding (4). Furthermore, with the exception of the deletion mutant H(Δ), each gE-1 mutant has an electrophoretic mobility identical to that of the wild-type gE-1 protein, as assessed by Western blot (4). To fine map the region of gE-1 between residues 183 and 402 involved in IgG aggregate binding, gE-1 mutants were expressed by transient transfection and assayed for FcR activity in a rosetting assay. Fourteen gE-1 mutants have linker insertions between residues 183 and 402 (Table 2, box), and seven mutants have insertions outside of this region and serve as controls. None of ten gE-1 mutants with insertions between residues 235 and 380 demonstrated rosetting activity, while all remaining mutants bound EAIgG at levels comparable to that of the wild-type gE-1 protein. These results are consistent with those of the gE-1/gD-1 fusion protein experiments and further define the region of gE-1 between residues 235 and 380 as the FcR domain required for binding IgG aggregates.

Sequence homology between gE-1 and mammalian FcRs. Mammalian IgG FcRs are members of the immunoglobulin superfamily and have immunoglobulin-like domains in their extracellular portions. Low-affinity FcRs (types II and III) have two immunoglobulin-like domains, while the high-affinity FcR (type I) has three. Each immunoglobulin-like domain is stabilized by a disulfide bond and contains conserved residues that constitute the structural motif of the immunoglobulin superfamily (reviewed in reference 23). A comparison of the amino acid sequences of gE-1 and several human and murine IgG FcRs revealed a region of gE-1 within the Fc binding domain between residues 322 and 359 that shares homology with domain 2 of human FcRII clone PC23 (40), murine Fcγ2b/1 (36), human FcRIII (CD16) (39), and human FcRI (2). The homology between human and murine FcRs, but not between gE-1 and these mammalian receptors, has previously been reported (2). A computer-assisted amino acid comparison with the GCG Gap program demonstrates that this region of gE-1 has a 46% sequence identity and a 66% sequence similarity compared with domain 2 of human FcRII (Fig. 6). Features of this homology include two conserved cysteine residues, several identical (shaded) and similar (underlined) amino acid residues, and a strongly conserved region at the carboxyl-terminal portion of the domain with the consensus sequence (HSG-Y-C). To determine whether similar alignments between gE-1 amino acids 322 to 359 and mammalian FcRs might occur by chance alone, the sequence of the gE-1 residues was randomly shuffled with the GCG Shuffle program and compared with the

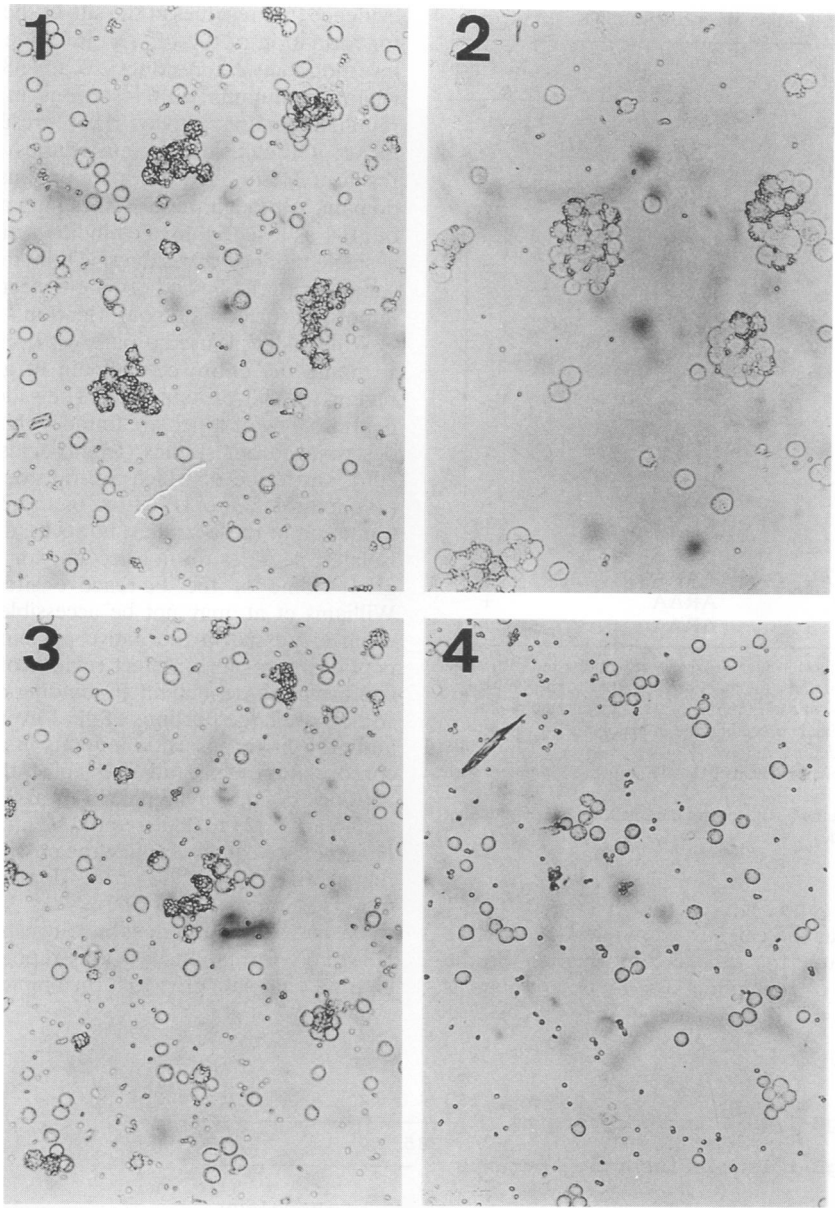

FIG. 5. Binding of EAIgG to cells expressing gE-1/gD-1 fusion proteins. Cells were transiently transfected to express fusion proteins containing the ABCD peptide (1), the BCD peptide (2), the CD peptide (3), or the ABC peptide (4).

sequence of human FcRII domain II. Of 20 randomly shuffled gE-1 sequences that were compared with the mammalian FcR by using the GCG Gap program (34), none achieved an alignment quality score as high as that observed when the unshuffled gE-1 sequence was tested.

DISCUSSION

In this report we describe two complementary approaches used to map a domain of gE-1 involved in the binding of IgG aggregates. First, we expressed nine overlapping gE-1 peptides as gD-1/gE-1 fusion proteins and assayed each for binding of EAIgG in a rosetting assay. The smallest gE-1 peptide that demonstrated FcR activity was the CD peptide containing amino acids 183 to 402. Two larger peptides that overlap this portion of gE-1 (the BCD peptide, containing residues 111 to 402, and the ABCD peptide, containing residues 25 to 402) also demonstrated FcR activity, while smaller peptides did not. These results conclusively establish that an Fc binding domain on gE-1 is located within the CD peptide between residues 183 and 402. Since we cannot determine whether each gE-1 peptide is properly folded when expressed as a fusion protein, it is possible that the Fc binding domain is contained within a smaller peptide (such as the C or D peptide) that is misfolded and, as a result, does not exhibit FcR activity. Another limitation of the fusion protein approach is that it does not allow one to determine whether there are regions of gE-1 between residues 183 and 402 that are dispensable for Fc binding activity.

To further define this Fc binding domain, 21 gE-1 linker insertion mutants were assayed for FcR activity with EAIgG. Each mutant has a single insertion of four or five amino acids

TABLE 2. gE-1 linker insertion mutants

Plasmid[a]	Location of insertion[b]	Amino acids inserted[c]	Rosetting
WT	Wild type	None	+
H55	55	AARA	+
H(Δ)[d]	103–120	AARA	+
X120	120	APRG	+
N190	190	PLER	+[e]
H210	210	PLER	+
B222	222	TPRGV	+
S235	235	PLER	−
T264	264	ALEG	−
A285	285	PLER	−
Ah302	302	PSRA	−
H324	324	ARAA	−
S333	333	RSSG	−
H339	339	ARAA	−
H355	355	RSSG	−
H371	371	RSSG	−
R380	380	SARAD	−
H397	397	ARAA	+
H406	406	ARAA	+
H425(tmr)	425	AARA	+
H455(cyt)	455	ARAA	+
H466(cyt)	466	ARAA	+

[a] The name of each mutant plasmid includes a letter denoting the restriction enzyme used to make the mutant and a number corresponding to the amino acid location of the mutant. tmr, transmembrane region; cyt, cytoplasmic region.
[b] Amino acid residue immediately preceding the insertion.
[c] Single-letter amino acid code.
[d] This mutant has a deletion of amino acids 103 to 120, which are replaced with AARA.
[e] Box, mutants that fall within the boundaries of the smallest gE-1 peptide that retains FcR activity (CD peptide).

at a different site in gE-1, and each reacts with a panel of gE-1 MAbs and is expressed at the cell surface, indicating that the insertion does not disrupt the global conformation of the protein. Therefore, lack of Fc binding activity by a mutant is evidence that residues at the site of the mutation are important for formation of the gE-1 FcR. None of the 10 mutants with insertions between residues 235 and 380 exhibited FcR activity, while all mutants with insertions in other regions of gE-1 retained binding activity. These results are consistent with those of the fusion protein studies and indicate that gE-1 residues 235 to 380 form a continuous IgG aggregate binding domain. Although these studies map a relatively large portion of gE-1 as essential for Fc binding activity, it is likely that only a small number a residues within this domain make direct contact with the Fc end of IgG; the remaining residues may be important for domain conformation.

Williams et al. used a panel of overlapping 7-mer peptides spanning the entire ectodomain of gE-1 to map Fc binding regions of the protein (45). They identified five Fc-reactive peptides, two of which are found within the Fc binding domain described in our studies (amino acids 280 to 286 and 299 to 306), and three of which are outside of the binding domain (amino acids 23 to 31, 64 to 70, and 204 to 210). An inherent limitation of these studies, however, derives from the fact that isolated peptides do not account for the tertiary structure of gE-1. Some of the Fc-reactive sites on gE-1 mapped by Williams et al. may not be accessible for binding to IgG Fc when expressed in the native protein. Furthermore, isolated peptides may fail to detect regions of gE-1 that demonstrate conformation-dependent Fc binding activity. In contrast, our studies use large portions of gE-1 expressed as fusion proteins and gE-1 linker insertion mutants in an effort to retain native conformation. Our studies establish that the three Fc-reactive peptides defined by Williams et al. that fall outside of gE-1 amino acids 235 to 380 are not essential for Fc binding activity. However, we cannot exclude the possibility that these, or other, regions of gE-1 outside of this domain, make some contribution to Fc binding activity.

The present study does not investigate the role of gI-1 in HSV FcR formation. Previous reports have established that gI-1 interacts with gE-1 to form a molecular complex which has

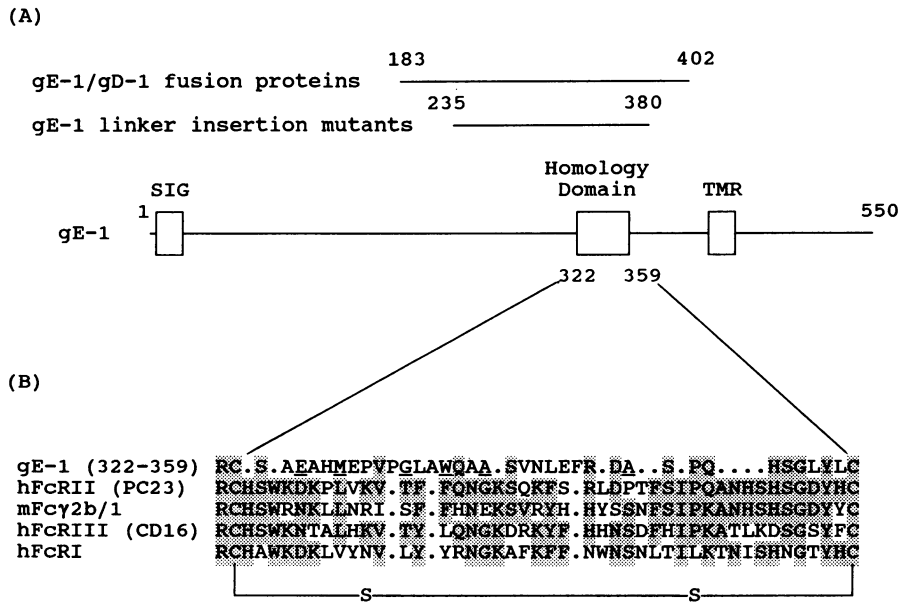

FIG. 6. Region of gE-1 which shares homology with mammalian FcRs. The region of gE-1 shown was compared with domain 2 of several mammalian FcRs by using the GCG Gap program. The shaded amino acids represent identical residues found at the aligned positions in two or more of the proteins. The underlined gE-1 amino acids represent residues that are similar (20) to those of human FcRII. The ——S——S—— symbol indicates the position of the disulfide bond in the mammalian FcRs.

higher affinity for the Fc domain of IgG than gE-1 alone and which binds monomeric IgG (6, 12, 18, 25, 26). In a separate study, we demonstrate that gI-1 may not form part of the Fc binding domain for IgG monomers but may alter the conformation of gE-1 in a way that increases its intrinsic Fc binding affinity (4).

In a computer-assisted amino acid sequence analysis, a region of gE-1 between residues 322 and 359 that shares homology with domain 2 of mammalian FcRs was identified. This region of gE-1 contains an immunoglobulin-like domain motif common to mammalian FcRs and other members of the immunoglobulin superfamily of proteins and is found within the Fc binding domain defined by our studies. The origin of the US8 gene that encodes gE-1 has not been determined. It may be derived from a mammalian FcR gene that was "captured" during evolution of the virus or may have evolved independently because it offers the virus a survival advantage. In either case, gE-1 mimics mammalian FcRs and, as predicted by in vitro studies, may facilitate viral immune evasion (13, 15).

Among the gE-1 homologs of other herpesviruses, only gpI of varicella-zoster virus (VZV) has been shown to form an IgG FcR (29). Comparison of the amino acid sequences of these two proteins demonstrates that the strongest homology is found between gE-1 amino acids 211 to 381 and VZV gpI 328 to 500 (29, 35). This region of gE-1 includes the Fc binding domain mapped in our studies (amino acids 235 to 380), suggesting that the homologous region of gpI may be required for its Fc binding activity. VZV gp1, however, does not share homology with immunoglobulin-like domains of mammalian FcRs (28).

Ultimately, in vivo studies will be necessary to establish a role for the HSV FcR in pathogenesis. Our map of the Fc binding domain of gE-1 will facilitate construction of recombinant viruses that express gE-1 but that have small mutations which inactivate FcR activity. This approach to constructing FcR-negative viruses should help prevent the inadvertent alteration of other gE-mediated activities, and therefore, such mutants will be useful in animal models of HSV infection to study the role of the FcR in pathogenesis.

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