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C3b Receptor Activity on Transfected Cells Expressing Glycoprotein C of Herpes Simplex Virus Types 1 and 2

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Glycoprotein C from herpes simplex virus type 1 (gC-1 from HSV-1) acts as a receptor for the C3b fragment of the third component of complement on HSV-1-infected cell surfaces. Direct binding assays with purified gC-1 and C3b demonstrate that other viral and cellular proteins are not required for this interaction. Although C3b receptor activity is not expressed on HSV-2-infected cell surfaces, purified gC-2 specifically binds C3b in direct binding assays, suggesting that gC-1 and gC-2 are functionally similar. Here, we used a transient transfection system to further characterize the role of gC-1 and gC-2 as C3b receptors and to localize the site(s) on gC involved in C3b binding. The genes for gC-1 and gC-2 were each cloned into a eucaryotic expression vector containing the Rous sarcoma virus long terminal repeat as the promoter and transfected into NIH 3T3 cells. The expressed proteins were similar in molecular size, extent of carbohydrate processing, and antigenic properties to gC-1 and gC-2 purified from infected cells. Using a double-label immunofluorescence assay, we found that both gC-1 and gC-2 were expressed on the surfaces of transfected cells and bound C3b. These results suggest that other proteins expressed during HSV-2 infection prevent receptor activity. We constructed three in-frame deletion mutants of gC-2 to identify domains on the protein important for C3b receptor activity. These mutants lacked amino acids 26 to 73, 219 to 244, or 318 to 346. The mutant protein lacking residues 26 to 73 was reactive with two monoclonal antibodies recognizing distinct epitopes, showed a wild-type pattern of carbohydrate processing, and bound C3b on the transfected cell surface. These results suggest that residues 26 to 73 are not involved in C3b binding. The other two mutant proteins were present on the cell surface, but did not bind C3b. In addition, these mutant proteins showed altered patterns of carbohydrate processing, formed aggregates, and were no longer recognized by the monoclonal antibodies. These properties indicate that removal of residues 219 to 244 or 318 to 346 disrupted the native conformation of gC-2, possibly owing to an alteration in the spacing between critical cysteine residues.

Herpes simplex viruses (HSV) encode a number of glycoproteins which are found on the virion envelope as well as on the surface of infected cells (52, 53). Although their functions are not completely defined, these glycoproteins are likely to be involved in virus attachment (17) and penetration (18, 21, 33, 49), envelopment and egress from the infected cell, and membrane fusion (20, 38, 41). Because of their location on the surface of infected cells, HSV glycoproteins act as major antigenic determinants for the cellular and humoral immune responses of the host (39, 42, 53). Three of these glycoproteins have functions which may modulate the immune response. Glycoprotein E (gE) and glycoprotein I (gI) function as a complex to bind the Fc portion of immunoglobulin G (3, 27, 28, 43). Glycoprotein C (gC) binds the C3b fragment of the third component of complement (11, 13, 37). Although gE and gC are not required for infection in cell culture (9, 22, 23, 25, 29, 34, 40, 47, 58), they are both present in clinical isolates (14, 44), suggesting that Fc and C3b receptor activities are important for viral pathogenesis in vivo.

HSV type 1 (HSV-1) (13, 32) and equine herpesvirus type 1 (4) are the only viral agents known to induce C3b receptor activity on infected cells. This activity can be detected on a variety of cell types following infection (4, 13, 32, 51), but other viruses tested, including other herpesviruses, do not induce detectable expression of the receptor on the surface of infected cells (4, 13, 51). Monoclonal antibodies (MAbs) against gC of HSV-1 (gC-1) block receptor activity, whereas MAbs against other HSV glycoproteins have no effect (13, 14). In addition, cells infected with gC-1-negative mutants do not bind C3b (13, 14, 50). These studies implicate gC-1 in C3b receptor activity. Neuraminidase treatment of the infected cells enhances receptor activity, suggesting that sialic acid residues on gC-1 interfere with C3b binding (50).

We recently showed that purified gC-1 binds directly to purified C3b (11), suggesting that other viral or cellular proteins are not required for this interaction. Surprisingly, gC from HSV type 2 (HSV-2)-infected cells (gC-2) also specifically binds C3b (11, 37) even though no receptor activity can be detected on HSV-2-infected cell surfaces. Removal of sialic acid residues from purified gC-1 or gC-2 enhances C3b binding (11). Although gC-1 and gC-2 share amino acid homology (8, 16, 54) and are antigenically related (11, 57, 59, 60), this was the first indication that gC-2 is functionally related to gC-1. However, there are a number of differences in C3b-binding activity. First, enzymatic removal of N-linked oligosaccharides from gC-2 adversely affects C3b binding; removal of these oligosaccharides from gC-1 has little effect (11). In addition, purified gC-2 differs from gC-1 in its influence on the stability of the C3 convertase and its ability to reduce the efficiency of complement-mediated lysis (11, 15). These differences may have implications for

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understanding the pathogenicity of HSV-1 and HSV-2 infections and the role of gC in modulating the immune response.

At present, it is not clear why HSV-2-infected cells do not express C3b receptor activity on their surfaces. The simplest explanation is that there is less gC-2 expressed on infected cell membranes than gC-1. A second explanation is that viral proteins expressed on the HSV-2-infected cell surface might interfere with the receptor activity of gC-2. If true, gC-2 expressed on the surface of uninfected cells should bind C3b. Third, gC-2 might assume a conformation on the cell surface which precludes C3b binding. In that case, gC-2 on uninfected cells would not bind C3b. The present study utilized a transient transfection assay to address this question. We constructed eucaryotic expression vectors to direct the synthesis of wild-type gC-1 and gC-2 in uninfected cells. Both gC-1 and gC-2 expressed on the surface of transfected cells bound C3b, suggesting that other proteins expressed during HSV-2 infection prevent receptor activity. In addition, we used site-directed mutagenesis of the gC-2 gene to begin to identify domains on the protein important for C3b receptor activity. Using in-frame deletion mutants of gC-2, we found that removal of residues 26 to 73 had no effect on C3b receptor activity, while removal of residues 219 to 244 or 318 to 346 completely abolished this activity.

MATERIALS AND METHODS

Cell cultures and virus strains. Conditions for the growth and maintenance of BHK cells (11) and for the propagation of HSV-1 (NS) and HSV-2 (333) viral stocks have been previously described (11). NIH 3T3 cells were propagated in Eagle minimum essential medium with 10% fetal bovine serum.

Purified glycoproteins and preparation of polyclonal antibodies and MAbs. gC-1 and gC-2 were purified from cytoplasmic extracts of HSV-1 (NS)- or HSV-2 (333)-infected cells as described previously (11). The preparation of rabbit polyclonal anti-gC-1 serum (R46) and anti-gC-2 serum (R64) used in this study has also been described elsewhere (11). MAbs MP-1 and MP-5 used to characterize the deletion mutants were prepared as described previously for anti-gD MAbs (10), using immunosorbent-purified gC-2 as the immunogen. These antibodies are type specific and react with native but not denatured gC-2. Blocking studies (10) showed that these MAbs bind to different antigenic sites (M. Ponce de Leon, C. Seidel-Dugan, G. H. Cohen, and R. J. Eisenberg, unpublished observations).

Construction of recombinant plasmids for expression of gC-1 and gC-2 and construction of gC-2 in-frame deletion mutants. Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England BioLabs, Inc. (Beverly, Mass.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as directed by the manufacturer. Plasmid DNAs were propagated in *Escherichia coli* HB101 or DH5 supplied as competent cells by Bethesda Research Laboratories. Standard methods (35) were used to prepare plasmid DNAs for subcloning the gC-1 and gC-2 genes and for preparing the deletion mutants. Each of the predicted in-frame deletion mutants was confirmed by double-stranded sequencing of the plasmid DNA across the region of the mutation (5, 48).

(i) Plasmid pCD14. Plasmid pCD14 was designed to express full-length gC-1 when transfected into eucaryotic cells (Fig. 1A). A plasmid containing the *Sal*I-*Bam*HI fragment T-I (0.621 to 0.643) of HSV-1 (KOS) was kindly provided by E. Wagner. Digestion with *Nhe*I and *Mst*II yielded a 1.6-kilobase fragment containing the complete gC-1-coding region (9, 16) with an additional 35 base pairs upstream of the initiation codon and 100 base pairs downstream of the termination codon. The 5′ overhangs were filled in with the Klenow fragment of *E. coli* DNA polymerase, *Hin*dIII linkers were added, and the gene was cloned into the expression vector pRSVntEPA (7). The protein expressed after transfection of NIH 3T3 cells is designated gC-1-(pCD14).

(ii) Plasmid pCD45. Plasmid pCD45 was designed to express full-length gC-2 when transfected into eucaryotic cells (Fig. 1B). A plasmid containing the *Sal*I fragment (0.610 to 0.639) of HSV-2 (333) was kindly provided by D. Galloway. Digestion with *Bss*HII yielded a 1.9-kilobase fragment containing the complete gC-2-coding region (8, 54) with an additional 43 base pairs upstream of the initiation codon and 494 base pairs downstream of the termination codon. The 5′ overhangs were filled in with the Klenow fragment of *E. coli* DNA polymerase, *Hin*dIII linkers were added, and the gene was cloned into pRSVntEPA. The protein expressed after transfection of NIH 3T3 cells is designated gC-2-(pCD45).

(iii) Plasmid pCD51 (*Bal*I-*Stu*I deletion). The in-frame deletion mutant pCD51 was constructed by digestion of the gC-2-coding region with *Bal*I and *Stu*I, the resulting blunt ends were religated, and the gene was cloned back into pRSVntEPA. This deletion (see Fig. 6) removed residues 26 to 73, leaving the predicted signal sequence (8, 54) intact, so that the expressed protein would be lacking the first 48 amino acids of gC-2 if the signal sequence is removed during processing. Three potential N-linked glycosylation sites (8, 54) were removed, but cysteine residues remained unchanged in number and relative position. The expressed protein is designated gC-2-(Δ26-73).

(iv) Plasmid pCD57 (*Rsr*II-*Dra*III deletion). The pCD57 mutant was constructed by digestion of pCD45 with *Rsr*II and *Dra*III, removal of the protruding ends with mung bean nuclease, and religation of the ends. The resulting in-frame deletion removed 26 amino acids between residues 219 and 244 inclusive (see Fig. 6). No cysteines or potential N-linked glycosylation sites (8, 54) were removed, although cysteines 2 and 3 were moved closer together. The expressed protein is designated gC-2-(Δ219-244).

(v) Plasmid pCD55 (*Pvu*II-*Nco*I deletion). The pCD55 mutant was constructed by digestion of pCD45 with *Pvu*II and *Nco*I, removal of the protruding ends with mung bean nuclease, and religation of the ends to put the sequence back into frame. The deletion removed 29 amino acids between residues 318 and 346 inclusive (see Fig. 6). One potential N-linked glycosylation site (8, 54) was removed, and cysteine residues 5 and 6 were brought within nine amino acids of each other. The expressed protein is designated gC-2-(Δ318-346).

DNA transfection. Transfection assays were performed as previously described (7), using NIH 3T3 cells and conditions optimized for gC expression. Plasmid DNA (10 μg) was added to the cells (60-mm dish) as a calcium phosphate precipitate and incubated for 18 h at 37°C. Mock-transfected cells received precipitate not containing DNA. The precipitate was removed, and the cells were incubated for an additional 24 h. Cytoplasmic extracts were prepared (11) or the cells were harvested for immunofluorescence (described below).

SDS-PAGE and Western blot analysis. Cytoplasmic extracts were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing or

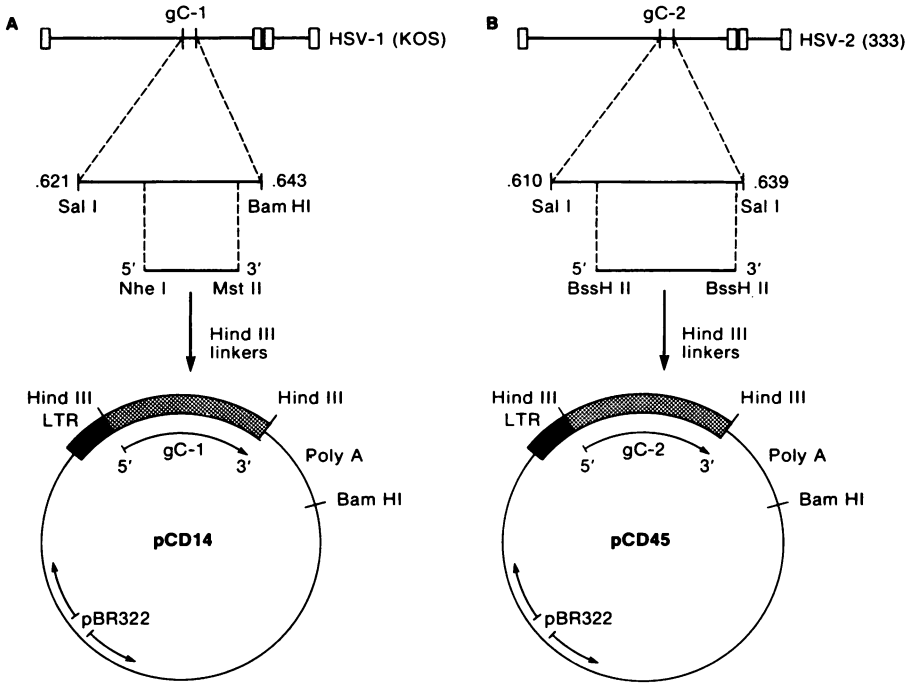

FIG. 1. Schematic representation of the HSV-1 and HSV-2 genomes (prototype arrangement) and the cloning strategy used for construction of the gC expression vectors pCD14 and pCD45. (A) A 1.6-kilobase *Nhe*I-*Mst*II fragment containing the entire coding region of gC-1 but lacking the promoter was removed from the *Sal*I-*Bam*HI fragment T-I of HSV-1 (KOS) (16). *Hin*dIII linkers were added, and the fragment was cloned into the expression vector pRSVntEPA (7). This vector contains the long terminal repeat (LTR) of Rous sarcoma virus as a promoter, and downstream of the cloned gene is a simian virus 40 early polyadenylation site. (B) A 1.9-kilobase *Bss*HII fragment containing the entire coding region of gC-2 but lacking the promoter was removed from a *Sal*I fragment of HSV-2 (333) (8, 54). *Hin*dIII linkers were added, and the fragment was cloned into pRSVntEPA (7).

nondenaturing ("native") conditions (6) followed by Western blot (immunoblot) analysis as previously described (6, 7).

Double-label immunofluorescence. NIH 3T3 cells were transfected or infected with HSV-1 or HSV-2 (each at a multiplicity of infection of 0.5) and used for the immunofluorescence assay 18 h postinfection. The cells were washed once with phosphate-buffered saline and gently scraped into 0.5 ml of phosphate-buffered saline, transferred to a conical glass tube, and incubated with 0.25 U of type X neuraminidase from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 37°C. Enzyme was removed by washing the cells once in phosphate-buffered saline. The cells were reacted with 2.5 μg of purified human C3b (11) for 30 min at 37°C. Control cells received no C3b. The cells were washed free of C3b and then reacted with rhodamine-conjugated $F(ab')_2$ goat anti-human C3 (Organon Teknika, Malvern, Pa.) at a 1:40 dilution for 30 min at room temperature. The cells were washed and reacted with polyclonal anti-gC-1 (R46) or anti-gC-2 (R64) serum at a 1:100 dilution. After removal of excess antibody, the cells were reacted with fluorescein-conjugated goat anti-rabbit immunoglobulin (Boehringer Mannheim) at a 1:100 dilution. The cells were viewed with a Leitz epifluorescence microscope.

Enzyme digestions. For each enzyme, 20 μl of cytoplasmic extract (representing approximately 2×10^4 transfected NIH 3T3 cells) in lysing buffer (20 mM Tris hydrochloride [pH 7.5] containing 50 mM NaCl, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) was digested for 6 h at 37°C. The enzymes used per digestion were as follows (milliunits): type X neuraminidase from *C. perfringens*, 150; endo-β-*N*-acetylglucosaminidase F (endo F) (Boehringer Mannheim), 80; endo-β-*N*-acetylglucosaminidase H (endo H) (Boehringer Mannheim), 2. After digestion, the extracts were evaluated by Western blot under denaturing conditions.

RESULTS

Expression of gC-1 and gC-2 in transfected cells. Figure 1 shows the strategy used to construct the expression vectors pCD14 and pCD45, designed to express full-length gC-1 and gC-2, respectively. In both cases, the coding regions for the glycoproteins lacking their natural promoter sequences (1, 8, 16, 24, 54) were cloned into the unique *Hin*dIII site of the eucaryotic expression vector pRSVntEPA. NIH 3T3 cells were transfected with plasmid DNA, and cytoplasmic extracts were prepared at 42 h posttransfection. Control extracts were prepared from mock-transfected cells. These extracts were electrophoresed by SDS-PAGE under native conditions, and the proteins were transferred to nitrocellulose and reacted with anti-gC-1 or anti-gC-2 polyclonal serum. gC-1 and gC-2 purified from infected cell extracts were used as markers.

Polyclonal anti-gC-1 serum reacted with a single polypeptide (molecular weight, 120,000) in pCD14-transfected cell extracts (Fig. 2, lane 2) and comigrated with the product form of gC-1 purified from infected cells (lane 1). Polyclonal anti-gC-2 serum reacted with two polypeptides in an extract of pCD45-transfected cells (Fig. 2, lane 4). These proteins, with apparent molecular weights of 68,000 and 78,000, comigrated with gC-2 purified from infected cells (Fig. 2, lane 3). No antigenic reaction was found with extracts of mock-transfected cells (data not shown).

Sugar-modifying enzymes were used to determine the extent of processing of gC-1-(pCD14) and gC-2-(pCD45)

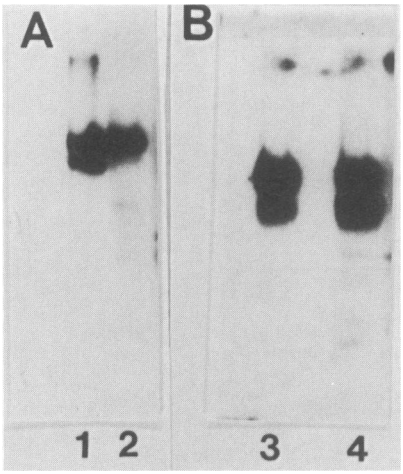

FIG. 2. Western blot analysis of gC-1-(pCD14) and gC-2-(pCD45). (A) Purified gC-1 (lane 1) or a cytoplasmic extract prepared from pCD14-transfected NIH 3T3 cells (lane 2) was electrophoresed under nondenaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gC-1 serum, followed by iodinated protein A. The blot was exposed to X-ray film two to three times longer than the blot in panel B. (B) Purified gC-2 (lane 3) or a cytoplasmic extract prepared from pCD45-transfected NIH 3T3 cells (lane 4) was Western blotted as above and reacted with polyclonal anti-gC-2 serum, followed by iodinated protein A.

found in cells at 42 h posttransfection. Three enzymes were used: (i) neuraminidase, which removes sialic acid residues from complex N-linked and O-linked oligosaccharides; (ii) endo H (55), which removes N-linked high-mannose oligosaccharides (found in the precursor form of the glycoproteins); and (iii) endo F (12), which removes N-linked oligosaccharides of both the high-mannose and complex type (found in the product form of the glycoproteins).

Extracts treated with these enzymes were separated by SDS-PAGE under denaturing conditions, transferred to nitrocellulose, and probed with polyclonal anti-gC-1 serum (Fig. 3). Susceptibility to any of the enzymes is reflected by a change in electrophoretic mobility of the proteins compared with that of the untreated control (Fig. 3, lane 1 in each panel). gC-1-(pCD14) was not susceptible to digestion with endo H (Fig. 3A, lane 3), but was susceptible to endo F (lane 4). Although neuraminidase had little effect on the mobility of gC-1-(pCD14) (lane 2), a significant change in mobility did occur when the same extract was electrophoresed in a native gel (data not shown). The results indicate that the gC-1-(pCD14) accumulating in transfected cells was the product form of gC-1 and contained complex N-linked oligosaccharides as well as sialic acid residues. The 78,000-molecular-weight form of gC-2-(pCD45) (78K protein) was susceptible to neuraminidase (Fig. 3B, lane 2) and endo F (lane 4), but resistant to endo H (lane 3). These results indicated that the 78K polypeptide contained complex sugars and sialic acid residues. The 68K polypeptide contained neuraminidase- and endo F-sensitive oligosaccharides (lanes 2 and 4), but also contained some endo H-sensitive sugars (lane 3). This pattern of glycosylation of gC-2-(pCD45) is nearly identical to that of gC-2 purified from infected cells. These results indicate that pCD14 and pCD45 direct the synthesis of full-length gC-1 and gC-2 which are similar to gC produced during viral infection.

C3b receptor activity on the surfaces of transfected cells. Previous results showed that HSV-1- but not HSV-2-infected cells expressed a C3b receptor on the cell surface (4, 13, 14). A double-label immunofluorescence assay was used to determine whether gC-1 and gC-2 were expressed on the surface of transfected cells and whether the expressed proteins had C3b receptor activity.

NIH 3T3 cells infected with HSV-1 and HSV-2 or transfected with pCD14 and pCD45 were treated with neuraminidase to enhance C3b binding (11, 50) and reacted with purified human C3b. After removal of excess C3b, cells were reacted with goat anti-human C3b-rhodamine conjugate. The cells were washed and reacted with polyclonal gC-1 or gC-2 antiserum, followed by fluorescein-labeled goat anti-rabbit immunoglobulin G conjugate. The order of addition was critical to avoid blocking of C3b binding by antibody. The cells were viewed with a fluorescein filter to detect binding of

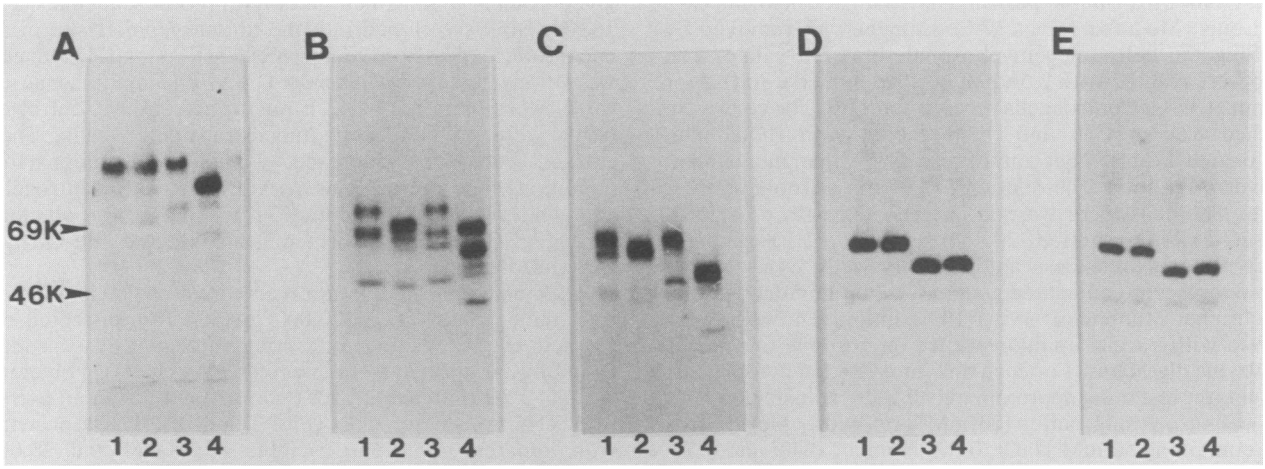

FIG. 3. Effect of sugar-modifying enzymes on gC-1-(pCD14), gC-2-(pCD45), and the gC-2 deletion mutants synthesized in transfected NIH 3T3 cells. Cytoplasmic extracts were treated with enzyme, electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gC-1 serum. This serum is type common (11) and reacts well with denatured gC-2. (A) gC-1-(pCD14); (B) gC-2-(pCD45); (C) gC-2-(Δ26-73); (D) gC-2-(Δ219-244); (E) gC-2-(Δ318-346). The enzymes used were: lane 1, mock-digested control; lane 2, neuraminidase; lane 3, endo H; lane 4, endo F. The arrows indicate the position of markers of 69,000 (69K) and 46,000 molecular weight.

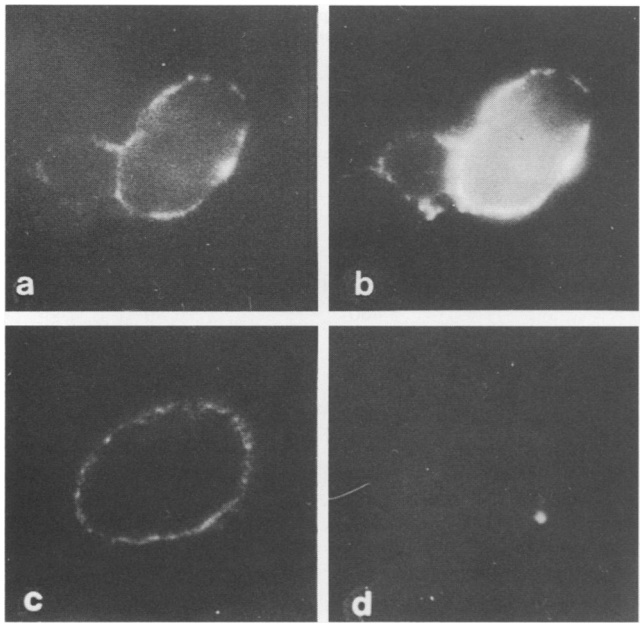

FIG. 4. Double-label immunofluorescence of HSV-1 (NS)- and HSV-2 (333)-infected NIH 3T3 cells. Infected cells were treated with neuraminidase and reacted with purified human C3b. After removal of the C3b, cells were reacted with goat anti-human C3b-rhodamine conjugate, followed by polyclonal anti-gC-1 or anti-gC-2 serum and fluorescein-labeled goat anti-rabbit immunoglobulin G conjugate. Cells were viewed with either a fluorescein filter to detect binding of anti-gC serum (a and c) or a rhodamine filter to detect binding of C3b (b and d). (a and b) HSV-1-infected cell; (c and d) HSV-2-infected cell.

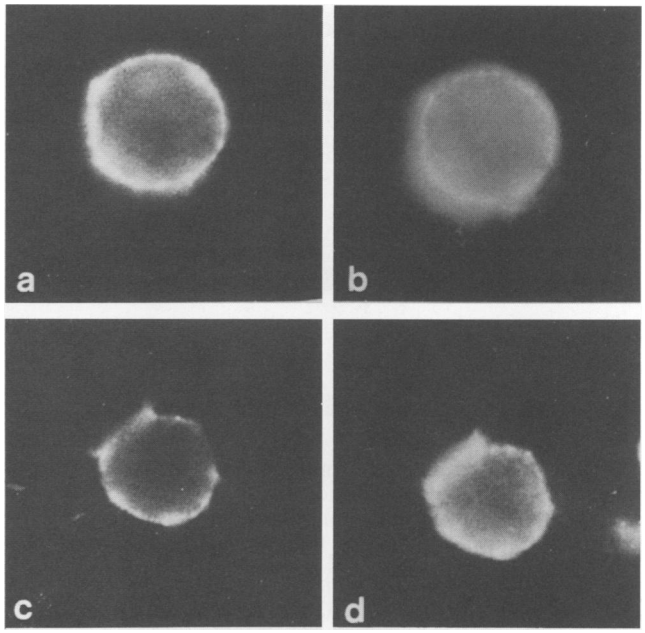

FIG. 5. Double-label immunofluorescence of pCD14- and pCD45-transfected NIH 3T3 cells. Cells were treated as described in the legend to Fig. 4 and viewed with a fluorescein filter to detect binding of anti-gC serum (a and c) or a rhodamine filter to detect binding of C3b (b and d). (a and b) Cell expressing gC-1-(pCD14); (c and d) cell expressing gC-2-(pCD45). Approximately 10% of the transfected cells were expressing gC-1 or gC-2 on the cell surface. All cells which bound anti-gC serum also bound C3b.

anti-gC antibody and a rhodamine filter to detect binding of C3b to the same cell.

HSV-1 (Fig. 4a)- and HSV-2 (Fig. 4c)-infected cells showed bright surface fluorescence when viewed with a fluorescein filter to detect binding of anti-gC sera. In panels b and d are the identical cells shown in panels a and c but viewed with a rhodamine filter to detect binding of C3b to the infected cell. As expected, HSV-1-infected cells did express C3b receptor activity (panel b), but HSV-2-infected cells did not bind C3b (panel d). An exhaustive search of HSV-2-infected cells expressing gC-2 on the membrane did not reveal any which bound C3b, confirming previously published studies employing a rosetting assay to look at C3b receptor activity (4, 13, 14).

pCD14 (Fig. 5a)- and pCD45 (Fig. 5c)-transfected cells showed bright surface fluorescence, indicating that gC-1-(pCD14) and gC-2-(pCD45) were each expressed on the surfaces of transfected cells. In both cases, all cells which expressed gC-1-(pCD14) (panel b) or gC-2-(pCD45) (panel d) on the cell surface bound C3b. Mock-transfected cells did not show any surface fluorescence. Furthermore, control cells expressing gC-1-(pCD14) or gC-2-(pCD45) incubated with phosphate-buffered saline instead of C3b did not show fluorescence (data not shown). The results show that gC-2 can function as a C3b receptor on the cell surface. The possible reasons why this activity is not seen on infected cells are presented in the Discussion.

Binding of C3b to gC-2 deletion mutants. We are interested in localizing domains on gC which are important for C3b receptor activity. We used site-directed mutagenesis as one approach to identify regions of gC important for C3b binding. These initial studies focused on gC-2 since gC-2-(pCD45) is expressed more abundantly than gC-1-(pCD14) in transfected cells. Three in-frame deletion mutants of gC-2 were constructed with pairs of restriction sites in the gC-2 gene (Fig. 6). The *Bal*I-*Stu*I deletion pCD51 removed residues 26 to 73, the first 48 amino acids of the mature protein (minus the signal sequence [8, 54]). The *Rsr*II-*Dra*III deletion (pCD57) removed residues 219 to 244 and the *Pvu*II-*Nco*I deletion (pCD55) removed residues 318 to 346 from gC-2.

Double-label immunofluorescence was used to determine whether the gC-2 mutant proteins were found on the transfected cell surface (Fig. 7a, c, and e) and whether they expressed C3b receptor activity (Fig. 7b, d, and f). For each mutant, the bright surface fluorescence seen with the fluorescein filter indicated that gC-2 was present at the cell surface. However, only cells expressing gC-2-(Δ26-73) bound C3b (Fig. 7b). Cells expressing gC-2-(Δ219-244) (Fig. 7d) and gC-2-(Δ318-346) (Fig. 7f) did not bind C3b even though the mutant proteins were expressed on the transfected cell surface. From these results, we conclude that residues 26 to 73 of gC-2 are not involved in C3b binding but that residues 219 to 244 and 318 to 346 are necessary for receptor activity.

Properties of gC-2 deletion mutants. Although gC-2-(Δ219-244) and gC-2-(Δ318-346) were transported to the cell surface, the proteins no longer bound C3b. This loss of receptor activity may be due to removal of contact residues in the C3b-binding site or, alternatively, may disrupt the native conformation of the mutant proteins. Since an intact tertiary structure is important for C3b binding (11, 14), we used Western blot analysis to determine the characteristics of the expressed proteins, the extent of processing, and the reac-

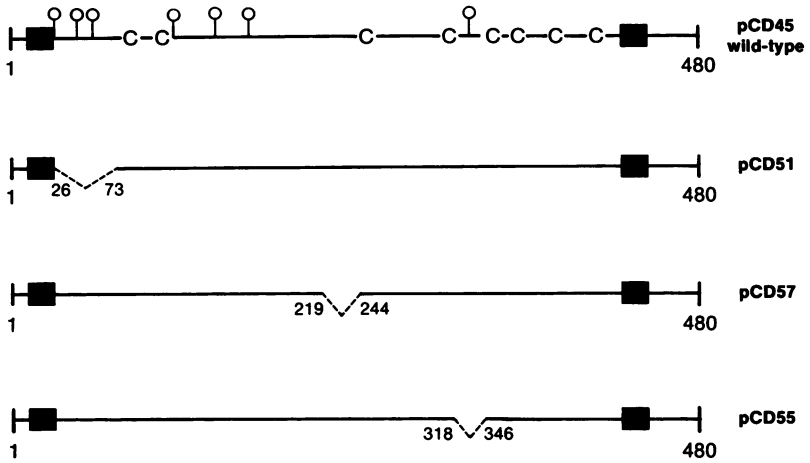

FIG. 6. Stick model of the coding region contained in pCD45 (wild-type gC-2) and the three gC-2 deletion mutants. gC-2 contains 480 amino acids (8, 54) with seven sites (open circles) for the addition of N-linked oligosaccharides and eight cysteine residues (C). Hydrophobic sequences near the amino and carboxy termini (black boxes) are predicted to be the signal peptide and transmembrane anchor, respectively (8, 54). The amino acids deleted from each of the mutants are also shown.

tivity of each protein with MAbs which only recognize a native conformation of gC-2.

Cytoplasmic extracts of transfected cells were evaluated by Western blot analysis under denaturing (Fig. 8A) and nondenaturing (Fig. 8B) conditions. gC-2-(Δ26-73) (lanes 2 and 6) was expressed as two polypeptides of 62,000 and 55,000 molecular weight, both of which migrated more rapidly than gC-2-(pCD45) (lanes 1 and 5). Mutants gC-2-(Δ219-244) (lane 3) and gC-2-(Δ318-346) (lane 4) migrated as a 68,000-molecular-weight polypeptide as well as higher-molecular-weight aggregates when evaluated under native conditions (lanes 7 and 8). Since these aggregates disappeared under reducing conditions (lanes 3 and 4), they could have resulted from intermolecular disulfide bonding of the molecules. Alternatively, they might have resulted from hydrophobic interactions or from a combination of such interactions and disulfide bonding.

Since alterations in protein sequence can result in conformational changes which affect the extent of processing of glycoproteins (7, 19, 45, 56), the mutant proteins gC-2-(Δ26-73), gC-2-(Δ219-244), and gC-2-(Δ318-346) were evaluated for susceptibility to sugar-modifying enzymes as described above. The results are shown in Fig. 3C to E. A fraction of the gC-2-(Δ26-73) polypeptide was sensitive to digestion with endo H (Fig. 3C, lane 3), indicating that some of the N-linked oligosaccharides were high-mannose or precursor type. However, most of the protein which had accumulated in transfected cells was susceptible to digestion with neuraminidase (Fig. 3C, lane 2) and endo F (lane 4) and resistant to digestion with endo H (lane 3), indicating that the oligosaccharides were fully processed to the product form. This pattern is very similar to that seen with the wild-type gC-2-(pCD45). On the other hand, gC-2-(Δ219-244) (Fig. 3D) and gC-2-(Δ318-356) (Fig. 3E) contained oligosaccharides exclusively of the high-mannose type; the proteins were equally sensitive to endo H (lane 3) and endo F (lane 4) digestion and were resistant to digestion with neuraminidase (lane 2). Thus, removal of residues 219 to 244 or 318 to 346 either prevents processing of the oligosaccharides or prevents the accumulation of the product form of the protein in transfected cells.

MAbs MP-1 and MP-5 were used to evaluate the gC-2 deletion mutants. These MAbs are type specific and react

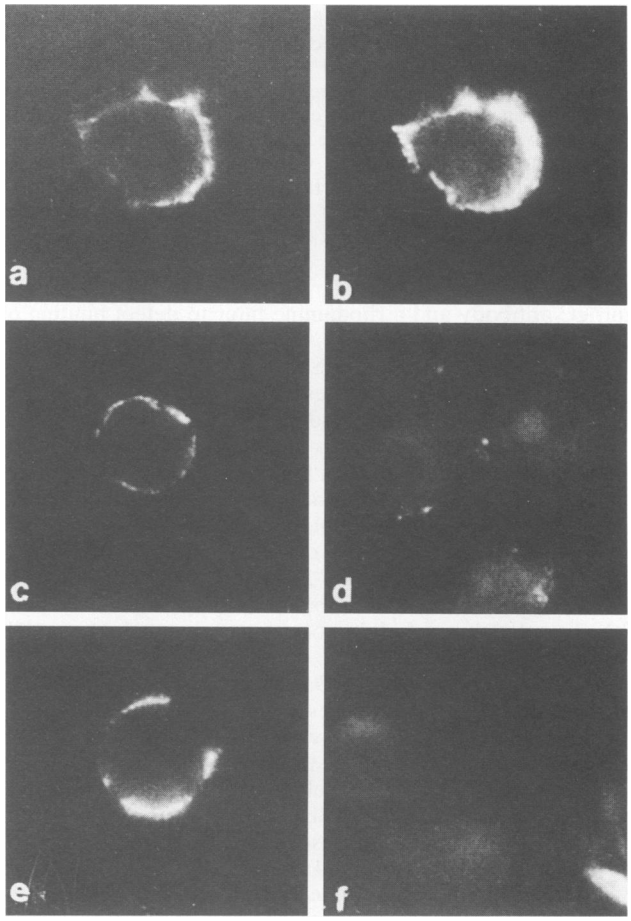

FIG. 7. Double-label immunofluorescence analysis of the gC-2 deletion mutants. Cells expressing each of the three deletion mutants were treated as described in the legend to Fig. 4 and viewed with a fluorescein filter to detect binding of polyclonal anti-gC-2 serum (a, c, and e) or a rhodamine filter to detect binding of C3b (b, d, and f). (a and b) Cell expressing gC-2-(Δ26-73); (c and d) cell expressing gC-2-(Δ219-244); (e and f) cell expressing gC-2-(Δ318-346).

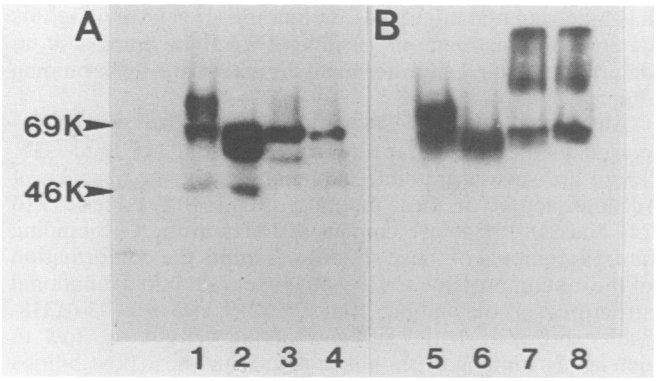

FIG. 8. Western blot analysis of gC-2-(pCD45) and the gC-2 deletion mutants. (A) Cytoplasmic extracts prepared from NIH 3T3 cells transfected with pCD45 and each of the deletion mutants were electrophoresed under denaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gC-1 serum. Lanes: 1, gC-2-(pCD45); 2, gC-2-(Δ26-73); 3, gC-2-(Δ219-244); 4, gC-2-(Δ318-346). The arrows designate the positions of markers of 69,000 (69K) and 46,000 molecular weight. (B) Cytoplasmic extracts of NIH 3T3 cells transfected with pCD45 and the deletion mutants were electrophoresed under nondenaturing conditions, transferred to nitrocellulose, and reacted with polyclonal anti-gC-2 serum. Lanes: 5, gC-2-(pCD45); 6, gC-2-(Δ26-73); 7, gC-2-(Δ318-346); 8, gC-2-(Δ219-244).

only with native gC-2. Blocking studies (10) showed that these two MAbs recognize distinct sites on the glycoprotein (M. Ponce de Leon, C. Seidel-Dugan, G. H. Cohen, and R. J. Eisenberg, unpublished observations).

Cytoplasmic extracts containing gC-2-(pCD45), gC-2-(Δ26-73), gC-2-(Δ219-244), or gC-2-(Δ318-346) were separated by SDS-PAGE under nondenaturing conditions, transferred to nitrocellulose, and probed with polyclonal antibody (Fig. 9, lanes 1), MP-1 (lanes 2), or MP-5 (lanes 3). The wild-type protein gC-2-(pCD45) reacted with each of the antibodies (Fig. 9A, lanes 1 to 3). gC-2-(Δ26-73) also reacted with each of the antibodies (Fig. 9B, lanes 1 to 3), suggesting that removal of residues 26 to 73 did not disrupt the protein conformation. In contrast, removal of residues 219 to 244 or 318 to 346 had a dramatic effect on MAb binding; gC-2-(Δ218-244) (Fig. 9C) and gC-2-(Δ318-346) (Fig. 9D) did not react with either MP-1 (lane 2) or MP-5 (lane 3). In addition, other MAbs against gC-2 which react only with native gC-2 and recognize sites distinct from those recognized by MP-1 or MP-5 also did not react with these mutant proteins (data not shown). Thus, it appears that removal of residues 26 to 73 had no effect on the overall conformation or extent of processing of the protein, whereas deletion of residues 219 to 244 or 318 to 346 had a dramatic effect on the folding of the protein and on the extent of processing of the oligosaccharides.

DISCUSSION

gC from HSV acts as a receptor for the C3b fragment of the third component of complement (11, 13, 37). Although the biological significance of this activity is not known, some evidence suggests that it may play a role in modulating the effects of the complement cascade, thus protecting the virus or virally infected cells from complement-mediated damage (11, 15, 37; S. Harris, unpublished observations). In previous studies, receptor activity could be demonstrated on HSV-1- but not HSV-2-infected cells (4, 13, 14). However, purified gC-2 binds C3b to the same extent as gC-1 (11, 37). In this study, we used a transient transfection assay to address this problem and to begin to characterize the structural sites on gC involved in C3b binding.

Transient expression of gC-1 and gC-2 in transfected cells. Previous studies of gC-1 expression utilized cell lines stably transfected with the gC-1-coding region under the control of either its own promoter (2) or a gD-1 promoter (46). In both cases, infection of the cell line with HSV was necessary to produce abundant amounts of gC-1. Since we were interested in studying gC-1 and gC-2 in uninfected cells, we chose to use a transient transfection assay with the genes for gC-1 or gC-2 placed under the control of a strong eucaryotic promoter. Although each glycoprotein was expressed, we found that gC-2-(pCD45) was approximately 20-fold more abundant in the transfected cells than gC-1-(pCD14). Cell lines carrying the gC-1 gene under the control of an inducible promoter lose their ability to express gC-1 after multiple passages (H. Friedman, unpublished observations), suggesting that gC-1 expression is toxic to cells. Perhaps gC-2 expression is not as detrimental.

The expressed proteins gC-1-(pCD14) and gC-2-(pCD45) were similar in size and antigenic properties to gC-1 and gC-2 produced during viral infection (11). Both proteins were transported to the membrane of the transfected cell. gC-1-(pCD14) contained only complex N-linked oligosaccharides characteristic of the product form of the protein seen in infected cells. The absence of a precursor form suggests that the protein was rapidly and efficiently processed in these cells. A similar phenomenon was observed with gD-2 expressed in transfected cells (30). In contrast, the 68K polypeptide of gC-2-(pCD45) did contain some high-mannose oligosaccharides characteristic of a precursor form. It therefore appears that either the efficiency or the rate of processing of gC-2 in transfected cells is reduced compared with that of gC-1 or gD-1. The significance of this is not known.

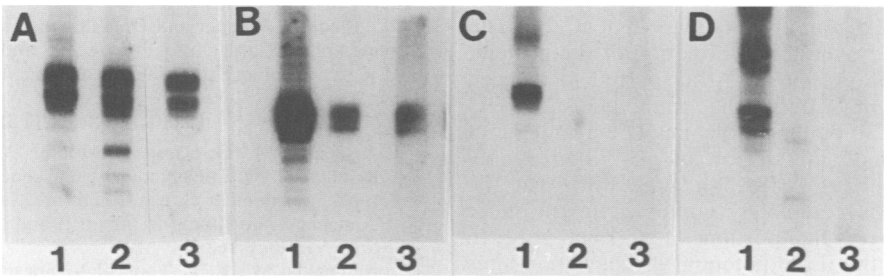

FIG. 9. Antigenic analysis of gC-2-(pCD45) (A), gC-2-(Δ26-73) (B), gC-2-(Δ219-244) (C), and gC-2-(Δ318-346) (D). Cytoplasmic extracts were electrophoresed in a combless gel under nondenaturing conditions and transferred to nitrocellulose, and strips were reacted with polyclonal anti-gC-2 serum (lanes 1), MAb MP-1 (lanes 2), or MAb MP-5 (lanes 3).

C3b binding to uninfected cells expressing gC-2. An important result of the present study was the observation that gC-2 expressed on the surface of transfected cells can act as a C3b receptor. Under the same assay conditions, HSV-2-infected cells did not exhibit this property, even though they reacted with polyclonal anti-gC-2 antibody. It is possible that the lack of C3b receptor activity on infected cells is due to low surface expression of gC-2. Earlier studies have shown that less gC-2 is expressed on infected cell membranes than gC-1 (26); this pattern of cellular distribution is likely to be influenced by the presence of other HSV-2 gene products in the infected cell. In transfected cells, any restraint on surface expression of gC-2 by viral gene products would be absent, so more gC-2 may be located on the surface of transfected cells than infected cells. It will be important to quantitate the amount of gC-2 expressed on infected versus transfected cell membranes to address this issue. Alternatively, a protein or proteins associated with the membrane of HSV-2-infected cells may prevent the binding of C3b to gC-2. Such a protein could directly block the C3b-binding site on gC-2, or it might interact with gC-2 in such a way as to alter the conformation of gC-2, thus preventing C3b binding. Likely candidates are one or several of the structural glycoproteins expressed in the membrane of an HSV-2-infected cell. Cotransfection experiments with cloned genes of each of the HSV-2 glycoproteins would be useful in addressing this problem. Another way to address the issue would be to infect cell lines which express gC-2 with a gC-2-negative mutant and measure the effect on C3b binding. It is also conceivable that a nonstructural protein associated with the infected cell membrane is responsible for preventing receptor activity. It would be of interest to determine whether HSV-2 virions express C3b receptor activity.

C3b binding by gC-2 deletion mutants. We are interested in defining regions of gC involved in C3b binding. Initial attempts to identify sites on gC which bind C3b employed anti-gC-1 MAbs to block receptor activity and gC-1 MAb-resistant mutants with single amino acid changes in each of the different antigenic sites of gC-1 (23, 36). The results indicated that the antigenic structure of gC-1 does not define one particular region of gC which binds C3b (14). As an alternative approach, we are using site-directed mutagenesis to localize domains on gC important for C3b receptor activity. In this initial study, we constructed three in-frame deletion mutants of gC-2.

In one mutant, pCD51, amino acids 26 to 73 of the predicted gC-2 sequence were deleted, including three potential N-linked glycosylation sites. Since the first 25 amino acids probably constitute the signal peptide of gC-2 (8, 54), the processed protein should start at residue 74. The expressed protein gC-2-(Δ26-73) had an electrophoretic mobility appropriate for its predicted size, was processed to the same degree as gC-2-(pCD45), and reacted with the gC-2 MAbs. Thus, removal of amino acids 26 to 73 of gC-2 had no apparent effect on the overall conformation of the protein. Most importantly, the protein was transported to the cell surface and bound C3b, showing that amino acids 26 to 73 are not part of a C3b-binding domain. The result is of particular interest since this region of gC-2 shares only limited homology with gC-1 (8, 54), suggesting that the C3b-binding domain is completely contained in the more homologous carboxy-terminal portions of gC-1 and gC-2. In addition, deletion of the three potential glycosylation sites included in residues 26 to 73 had no effect on C3b-binding activity. This is of interest because it had been previously shown that removal of N-linked oligosaccharides from gC-2 has a detrimental effect on C3b binding (11). Extending this deletion downstream of residue 73 will be important in determining the amino-terminal limits of the C3b-binding domain.

Although gC-2-(Δ219-244) and gC-2-(Δ318-346) were transported to the cell surface, the proteins did not bind C3b. There are several possible explanations for the absence of receptor activity in these mutant proteins: (i) residues 219 to 244 and 318 to 346 are contact residues in the C3b-binding site; (ii) removal of these residues disrupts the conformation of the mutant proteins so they no longer fold into a functional structure; (iii) the amount of gC-2-(Δ219-244) or gC-2-(Δ318-346) expressed on the surfaces of the cells is too low to detect C3b binding. It should be noted that these possibilities are not mutually exclusive. Quantitation of the amount of each protein found on the membrane will be necessary to rule out the third possibility. Our evidence suggests that the second possibility is an important consideration. The expressed proteins shared several properties suggesting that they had lost the native conformation of gC-2 and were no longer folding properly. The proteins migrated as high-molecular-weight aggregates in nondenaturing gels, they no longer reacted with anti-gC-2 MAbs, and their oligosaccharides appeared not to be processed beyond the precursor stage. HSV-1 gD exhibited similar characteristics when cysteine residues were removed by site-directed mutagenesis (7, 56). Although no cysteine residues were removed in these two gC-2 deletion mutants, the spacing of the cysteines was altered. We suggest that alterations in the spacing of critical cysteine residues can have a profound affect on the conformation of a molecule. Thus, although actual C3b contact residues could very well be missing in the two mutants, the effect of each deletion on conformation prevents a conclusion from being made. It is of interest that amino acids 318 to 346 are located in a region of gC-2 which has structural features reminiscent of the human C3b receptor, CR1 (31), including four cysteine residues whose positions are similar to cysteines in CR1 which are highly conserved among the C3/C4-binding proteins.

We conclude that amino acids 26 to 73 are not involved in C3b binding but that residues 219 to 244 and 318 to 344 are necessary for C3b receptor activity. To further understand the role of these regions in binding C3b, smaller deletions or insertions in the areas of interest which do not alter the conformation of the protein will be invaluable.

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