Herpes simplex virus entry into cells requires a multipartite fusion apparatus made of glycoprotein D (gD), gB, and heterodimer gH/gL. gD serves as a receptor-binding glycoprotein and trigger of fusion; its ectodomain is organized in an N-terminal domain carrying the receptor-binding sites and a C-terminal domain carrying the profusion domain, required for fusion but not receptor binding. gB and gH/gL execute fusion. To understand how the four glycoproteins cross-talk to each other, we searched for biochemical defined complexes in infected and transfected cells and in virions. Previously, interactions were detected in transfected whole cells by split green fluorescent protein complementation (Atanasiu, D., Whitbeck, J. C., Cairns, T. M., Reilly, B., Cohen, G. H., and Eisenberg, R. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 18718–18723; Avitabile, E., Forghieri, C., and Campadelli-Fiume, G. (2007) J. Virol. 81, 11532–11537); it was not determined whether they led to biochemical complexes. Infected cells harbor a gD-gH complex (Perez-Romero, P., Perez, A., Capul, A., Montgomery, R., and Fuller, A. O. (2005) J. Virol. 79, 4540–4544). We report that gD formed complexes with gB in the absence of gH/gL and with gH/gL in the absence of gB. Complexes with similar composition were formed in infected and transfected cells. They were also present in virions prior to entry and did not increase at virus entry into the cell. A panel of gD mutants enabled the preliminary location of part of the binding site in gD to gB to the amino acids 240–260 portion of gB and gH/gL. gD serves as a receptor-binding glycoprotein and trigger of fusion, and requires a multipartite apparatus made of a glyco-
exposes the fusogenic domain; and (iv) the active form of gD ultimately leads to the activation of gH/gL and gB. It was hypothesized that gB and gH/gL activation occurs through their recruitment to activated gD and that the C-terminal fusion domain carries the actual binding sites for gB and gH/gL. An alternative possibility is that the C-terminal fusion domain simply enables the conformational changes in gD but does not carry the actual binding sites for gB and gH/gL.

Efforts to validate the model prompted the search of interactions among the glycoprotein quartet. So far, it was found that HSV-infected cells harbor a co-immunoprecipitable complex made of HVEM, gD, and gH (28). A number of interactions were detected in transfected whole cells by split green fluorescent protein (or variations thereof) complementation assay (29, 30). The interactions were gD-gH/gL, gD-gB, and gB-gH/gL. The former two were found in cells transfected with two or three glycoproteins and are believed to mirror interactions that take place before gD activation. In contrast, the gB-gH/gL interaction was detected at fusion. Whether such interactions occur in infected cells or are seen only in transfected cells under conditions of high overexpression and whether they lead to biochemically defined complexes have not been investigated so far. In addition, as much as the split green fluorescent protein complementation irreversibly stabilizes weak and transients interactions, it tends to overemphasize interactions. For other herpesviruses, evidence is accumulating of complex formation among the glycoproteins involved in virus entry. Thus, Epstein-Barr virus gH and gL form a complex with the receptor-binding glycoprotein gp42; human cytomegalovirus and murine γ-herpesvirus 68 gH and gL form a complex with gB (31–33).

The objective of this work was to provide biochemical evidence for complex formation among the glycoprotein quartet, to verify whether complexes are present in infected cells and in virions and whether they are formed at virus entry into the cell. We analyzed the composition of the complexes by two approaches, co-immunoprecipitation and a pulldown assay that exploits the ability of One-strep-tagged proteins to be specifically retained by the Strep-Tactin resin. Complexes with undistinguishable composition were detected in infected and transfected cells and in virions prior to entry into the cell. A panel of mutants enabled the preliminary location of part of the gD regions critical to gB- and gH/gL-binding sites at the fusion domain.

EXPERIMENTAL PROCEDURES

Cells and Viruses—The cells were grown in Dulbecco’s modified minimum essential medium containing 5–20% fetal calf serum, HSV-1(F) was described (34). The AgD F-gDB (35), ΔgB-KAT (36), ΔgH SCgH7 (37), and ΔgL (38) HSV mutants were grown and titrated in the respective complementing cells.

Antibodies—R8 polyclonal antibody (pAb) to gD and BD80 monoclonal antibody (mAb) to aa 264–275 epitope of mature gD were generously provided by Dr. G. H. Cohen and Dr. R. Eisenberg; mAbs HD1, HC1, and H233 were a gift of Dr. L. Pereira. pAbs to gH and gL were a gift from Dr. H. Browne (Cambridge, UK) and D. Johnson (Portland). mAb H170 (reactive to aa 1–23 epitope), H1817, and H633 were purchased from Goodwin Institute. mAbs 52S, 53S, 30, and 5E1 were described (39–41). mAb 52S reacts to a conformational-dependent epitope. mAb 53S reacts to a conformational-dependent and gL-dependent epitope. V5 mAb was from Invitrogen. pAb to gM was described (42). A pAb to gH/gL was derived to a soluble form of gH truncated at aa 789/gL produced in insect cells.3

Virus Yield Assay—Vero cells grown in 12-well plates were infected with the indicated viruses at 0.1 PFU/cell for 90 min at 37 °C. The inoculum was removed, and unpenetrated virions were inactivated by means of an acidic wash (40 mM citrate acid, 10 mM KCl, 135 mM NaCl, pH 3). Replicate cultures were frozen at indicated times, i.e. 3, 24, and 48 h after infection. The progeny virus was titrated in Vero cells.

Plasmids—the mammalian expression plasmids encoding gH, gL, and gB in MTS vector, and gD in pcDNA3.1, all under the cytomegalovirus promoter, were described (43). Plasmids encoding HVEM (pBEC) (5), HER2 (human epidermal growth factor receptor 2) (44), gDAPED (herein renamed gD_{E838M}), gD_{CFP}, and gD_{DP} were described (9, 10).

Tagging of gH, gL, and gC—To enable detection or retention to resin, gH, gL, and gC were tagged with heterologous epitopes, as follows. The 5E1 epitope consists of a 27-aa-long sequence recognized by mAb 5E1, initially derived to human herpesvirus 7 (39). The 5E1 epitope was inserted in gH (gH_{E838M}), and the thrombin plus 5E1 and His (polyhistidine) epitopes were inserted in gL (gL_{5E1+His}) upstream of the stop codon. To generate gH_{E838M}, a Strep restriction site was inserted in the cytoplasmic tail of gH, in place of the stop codon, by site-directed mutagenesis with oligonucleotides 5′-GGG TTA TTT TTT TGT AGA GCG ATG CAA AGT GGG GAT CGT TCT GCC CAC C-3′ and 5′-GGG CGG AGA AAC GGC GGA AGG TAT CGG TCT GCC CAC CAC CTT CCA GTC TCC AAC AAA ACG G-3′. The oligonucleotides contained EcoRI restriction site for screening and introduced a single mutation, E838M. Two annealing oligonucleotides, encoding the 5E1 epitope 5′-ACA TGC ATG CAT GCT TGT ACT GAG CTA AGA CCA GGA AGC ACT ACA CCC TCT GGG AAC TCT GCA AGA TAT GGG-3′ and 5′-GGA AGA TCT GGG ATT GTA CCC TTA AAC CTA GGT ATG TAT ACT TCT AGG GTG TGT ATT CCC ATA TAT TCT TCG AGA GTT CCC C-3′ were ligated to Strep/BgIII-digested gH. The oligonucleotides contained the Asp718 restriction site for easiness of screening. The aa sequence of gH_{E838M} cytoplasmic tail was modified to ILKVLRTSVPFVWFRMHRVSRPGSTTTPGSNARYGNNTPRSTTP. To generate gH_{E838M}, indicated below also as gH_{E838M}, we followed a similar strategy; the annealing oligonucleotides encoding the Factor Xa protease cleavage site, followed by V5 and One-strep-tag epitopes, were 5′-GGA GAC GCA TGG TAC TCG AAG GAC GAG GAA GAT CTA TCC TCA ACC CTC TCC TAG GCC TCG ATT CTA CGA GCG CTT GGA GCC ACC CGC AGT TCG AGA AAG G-3′ and 5′-GGT AGT AGA TCT CAT CAT TTT TGT TCG AAG TAC GGG TGG TCC CAC GAT CCA CCT CCC GAT CCA CCT CCG GAA CCT CCA CCT TTC TCG AAC ‘TGC GGG TCG CTC GAA G-3′. They were ligated to the Strep/BgIII-digested gH. The aa sequence of gH_{E838M} cytoplasmic tail became ILKVLRTSVPFVWFRMHRVSRPGSTTTPGSNARYGNNTPRSTTP.

3 T. Gianni and G. Campadelli-Fiume, manuscript in preparation.
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GSGGGSGGGSW/SHPQFE/L. To generate gL_{5E1,His}, we followed essentially a similar strategy and inserted the NheI restriction site by site-directed mutagenesis, in place of the stop codon, by means of oligonucleotides 5'-CCC AAC CCC TCC CGG CGC CTG CTA GCA ATT CAC GGA AAC CGG TCC GGG TCT GGG-3' and 5'-CCC GAA CCC GGA CGG GTT TCC GTG ATAT TCA TGC TAG CAG GCC CCC AGA GTG GGG-3'. The annealing oligonucleotides encoding the trumbin-5E1-His epitopes were 5'-CTA TAG CTA GCC CGT TGT CCG GTG GAA TCG TGG GTAG ATG GTG TGG TAT CGG AGT TAT TCT AGG TGT GTT ATT CCC ATA CGG GTG TGG GAA GCT TCG GTA-3' and 5'-GGA AGT TAT ACT TCT AGG TGT GTT ATT CCC ATA CGG GTG TGG GAA GCT TCG GTA-3'. The aa sequence of the gL C terminus was changed from NheI/BglII-digested gL. The aa sequence of the gL C terminus was changed from NheI/BglII-digested gL. To generate gCV5 was generated as follows. The gC ORF was changed from SRRL to SRRLLAGLPIPNPLLGLDSTRTVHHH. To generate gLV5.His, the annealing oligonucleotides encoding the V5 and His epitopes were 5'-ATG CTC GCT AGC TGG TAA GCC TAT CTC TAA CCC TCT CGG TCT CGA TTC TAC GC-3' and 5'-TGA CGG TGG GCT TGG GAA GCT TCG GTA-3' and were ligated to NheII/BglII-digested gL. The aa sequence of the gL C terminus was changed from SRRL to SRRLLALVPRGSSRPGSTTPSGNSARYG-NNTPRSITPHHHHHH. To generate gLV5.His, the annealing oligonucleotides encoding the V5 and His epitopes were 5'-CTA TAG CTA GCC CGT TGT CCG GTG GAA TCG TGG GTAG ATG GTG TGG TAT CGG AGT TAT TCT AGG TGT GTT ATT CCC ATA CGG GTG TGG GAA GCT TCG GTA-3' and 5'-GGA AGT TAT ACT TCT AGG TGT GTT ATT CCC ATA CGG GTG TGG GAA GCT TCG GTA-3'. The Stul-Notl-digested gc amplimer was ligated into MTS vector. For all plasmids, the ORF was sequenced.

Genetic Engineering of HSV1(BAC)-gDst—The linear map of the gD constructs employed in this study is shown in Fig. 6. To generate gD_{218–240,260} the starting plasmid contained an Asp718 restriction site at aa 260 (46). An additional Asp718 site was inserted at aa 240 by site-directed mutagenesis by means of oligonucleotides 5'-CAG TTT GAA GAT CGC GGT ACC GAA GCT TCC CAA GGC CCC ATA CAC GAG CAC CCC CC-3' and 5'-GGG TGC TGT TGG GGT TGG GAA GCT TGA GGT GGA GGT ATT CAT TTT TCG AAC TGC GGG TGG CTC CAC GAT CCA CCT CCG GAT CCA CCT CCG-3'. The DNA encoding the 18-aa-long Ser-Gly linker 5'-PHO-GTA CCC AGT AGT GCC GGT GCC TCT GGA TCC TGG TCG TCG TCG GAG GAA CC-3' was ligated into Asp718-digested gD. Orientation was screened by colony PCR and confirmed by sequence. To generate gD_{218–240} we followed essentially a similar strategy. Two Asp718 restriction sites were introduced in gD plasmid at aa 218 and 240 by simultaneous double site-directed mutagenesis. The Asp718 site in aa 218 was inserted by means of oligonucleotides 5'-TGA CGG TGG TAC TGC TGC TCC CCC TCA GAG CAT GGT GAC GTC GGT GGT GAA GCT TCG GTA-3' and 5'-GGG TGC TGG TGG TGG TGG GAA GCT TGA GGT GGA GGT ATT CAT TTT TCG AAC TGC GGG TGG CTC CAC GAT CCA CCT CCG GAT CCA CCT CCG-3'; the Asp718 site at aa 240 was inserted by means of the oligonucleotides described above for gD_{240–260}. The DNA encoding the 18-aa-long Ser-Gly linker was generated as above and ligated with Asp718-digested gD. Orientation was screened by colony PCR and confirmed by sequence. The starting plasmid for gD_{218–240} was generated by Dr. L. Menotti in the course of an independent study. Briefly, the gD ORF, engineered in a vector designed to enable homologous recombination of mutant gD into HSV-BAC, was mutated by insertion of Ndel restriction sites at aa 60 and 218. Two synthetic annealing oligonucleotides encoding a 18-aa-long Ser-Gly linker 5'-PHO-TAG TTT GAA GCC AGG TTC TGG GGG GGT ATT CAT TTT TCG AAC TGC GGG TGG CTC CAC GAT CCA CCT CCG GAT CCA CCT CCG-3' and 5'-PHO-TACC CAC TCC CGC TAC CGC CTC CGC TCG GAG GAA CC-3' were ligated into Asp718-digested gD. Orientation was confirmed by colony PCR and confirmed by sequence.
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fragment containing the partially deleted ORF was subcloned in pcDNA containing gD ORF. Orientation was screened by colony PCR and confirmed by sequence. To generate gDDel-gD, Asp718 restriction sites were engineered at aa 240 and 310 of wt gD, respectively, by site-directed mutagenesis by means of oligonucleotides 5'-CAG CTT GAA GAT CGC GGT ACC GAA GCT TCC CAA GGC CCC ATA CAC GAG CAC CC 3' and 5'-CAT CCC CCG CGG GTA CTA CCG AAC AAG ATG GGC CTG 3'. A CD8 ampler obtained with oligonucleotides 5'-GGT GAC TGG TAG TTC GGC CCT GAG GAA CTC CAT CGG CGG GTA GAT AGG TAC CGC GGA GTC CAG CCC CCT GTG CGT 3' and digested with Asp718 was then ligated into Asp718-digested gD. To generate gDDel-gDHis, two EcoRI restriction sites were introduced in pcDNA-gD plasmid by simultaneous double site-directed mutagenesis with prior elimination of the EcoRI site present in the vector. The EcoRI site at aa 6 was introduced by means of oligonucleotides 5'-CAT GTA TGC CTG GCG GTA CCG CAT ATT TG-3' and 5'-GGC CTC CGA ATT CGC GTA CCG CAT ATT TG-3'; the EcoRI site at aa 59 was introduced by means of oligonucleotides 5'-CAG GGT TTA CTA CGC GAA CAT ATT TG-3' and 5'-CGG CCA GAT GTA GAT GGA CTT AAT TGC-3'. The EcoRI-digested gD was relegated, thus collapsing the EcoRI fragment. For all of the plasmids, the ORF was sequenced.

Co-immunoprecipitation and Pulldown Experiments—Co-immunoprecipitation was carried out from infected or transfected cells. Infected 293 T cells received 5 PFU/cell of one of the following viruses, HSV-1(F), ΔgD, ΔgB, ΔgH, or ΔgL HSV, as titrated in Vero or their respective complementing cells. To enable detection of gH and gL, the cells were transfected with gH5E1 and gL5E1.His (5 and 3 μg of DNA/flask, respectively) 6 h prior to infection with HSV-1(F), ΔgD, and ΔgB viruses. Infected cells were harvested 18 h after infection. U251, human embryonic lung, and human foreskin fibroblast cells were infected with HSV-1(F) or ΔgD virus (5 PFU/cell). We used 3-fold higher amount of cells than in the experiments with infected 293T cells. To enable detection of gH and gL, the cells were transfected with gH5E1 and gL5E1.His (15 and 9 μg of DNA/T75 flask, respectively) 6 h prior to infection with HSV-1(F), ΔgD, and ΔgB viruses. Infected cells were harvested 18 h after infection. Transfected 293T cells in T25 flasks received the appropriate plasmid mixture together with Arrest-in (Celbio, Milano), in the amount of 1.5 μg of DNA for each plasmid (1X amount). When indicated, pBEC was co-transfected at 3 μg/T25 flask (2X amount). Transfection mixtures in which one or more plasmids were omitted contained the HER2-encoding plasmid in place of the omitted plasmid, such that the total amount of DNA transfected in each flask did not vary. The cells were harvested 18 h after infection or transfection, prior to the appearance of syncytia, without freezing. For co-immunoprecipitation, in a typical experiment cells from two replicate T25 flasks (or one T75 flask) were solubilized in 200 (or 300) μl of EA1 buffer (50 mM HEPES, 250 mM NaCl, 0.1% Igepal) containing the protease inhibitors Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride and Nα-p-tosyl-L-phenylalanine chloromethyl ketone (final concentration, 0.3 mM each), as described (28), at 4 °C for 20 min. The lysates were centrifuged at 14,000 rpm for 30 min. The supernatants were cleared by incubation for 1 h at 4 °C with a preimmune serum, followed by absorption to protein A-coupled Sepharose (10 mg) (Sigma-Fluka, Milano). The cleared unbound fraction was then incubated with pAb R8 to gD (2 μl/200 μl) overnight at 4 °C, and thereafter with 10 μg of protein A-coupled Sepharose for 1 h at 4 °C. The beads were washed three times with EA1 buffer containing the protease inhibitors and once with 50 mM Tris-HCl, pH 7.5, and 15 mM NaCl.

gH1 pulldown experiments were carried out from transfected 293T cells (2 T25 flasks/sample), and gD pulldown experiments were carried out from HSV1(BAC)-gDΔvirions alone or absorbed to cells. For pulldown experiments from cells, two replicate T25 flasks (or virions in the indicated amounts) were solubilized with 200 μl of EA1plus buffer (50 mM HEPES, 250 mM NaCl, 0.5% Igepal, pH 8) containing 0.3 mM each Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride and Nα-p-tosyl-L-phenylalanine chloromethyl ketone at 4 °C for 20 min. The lysates were centrifuged at 14,000 rpm for 60 min. The supernatants were cleared by preabsorption for 1 h at 4 °C to protein A-coupled Sepharose (10 mg) (Sigma-Fluka). The unbound fraction was then incubated with Strep-Tactin Sepharose (IBA, GmbH, Gottingen, Germany) (30 μl/200 μl of lysate) for 1 h at 4 °C and washed five times with the resin washing buffer (100 mM Tris-HCl, pH 8, 150 mM NaCl, and 1 mM EDTA), according to the manufacturer.

For both co-immunoprecipitation and pulldown experiments, the material deriving from two flasks was solubilized with 120 μl of sample buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 50 mM Tris-HCl, pH 7, and 2.5% sucrose), boiled, and loaded in two different gels: one for Western blot (WB) detection of gD and gB and one for detection of gH, gL, and gC.

Western Blot—Proteins separated by 8.5% PAGE were transferred to Hybond ECL nitrocellulose membrane from GE Healthcare (Milano) and developed by Amersham Biosciences ECL Advance Western blotting detection kit (GE Healthcare).

Cell Surface Expression—Cell enzyme-linked immunosorbent assay (CELSA) was performed as described (48, 49). Briefly, 293T cells in 48 wells were transfected with plasmids encoding wt or mutant gD (125 or 375 ng/well, corresponding to 1× or 3× amounts, as specified). Sixteen h later they were reacted with mAb H170, or BD80 and fixed with paraformaldehyde, followed by anti-mouse peroxidase. To measure gH cell surface expression, 293 T cells were transfected with mixture of plasmids encoding wt gH/wt gL, gH5E1/gL5E1.His or gH5E1/gl5E1.His. Sixteen h later they were reacted with mAb 53S, fixed with paraformaldehyde, followed by anti-mouse peroxidase. All of the samples were run in triplicate.

Cell-Cell Fusion Assay—The luciferase-based cell-cell fusion assay was performed as detailed (49, 50) by means of a luciferase assay system from Promega (Florence, Italy) in 293T cells. The total amount of transfected plasmid DNA was made equal by the addition of human epidermal growth factor receptor 2 plasmid DNA. All of the samples were run in triplicate.

Infectivity Complementation—The assay was performed as detailed elsewhere (46). Briefly, 293T cells in T75 flasks were
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transfected with the appropriate gD plasmid. Four h later, the cells were infected with a gD\(^{-/-}\) stock of FgD\(_B\) (3 PFU/cell). Unpenetrated virions were removed by two phosphate-buffered saline rinses and inactivated by means of 40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3, for 1 min. The monolayers were then rinsed twice with phosphate-buffered saline and overlaid with medium containing 1% fetal calf serum. The cells were incubated overnight at 37 °C. The extracellular progeny virions were ultracentrifuged, analyzed by WB, and titrated in the gD-complementing R6 cells.

RESULTS

Tagging of HSV-1 Glycoproteins—Biochemical evidence of complex formation among the HSV glycoprotein quartet by means of co-immunoprecipitation or pulldown experiments relied on WB-positive antibodies, able to detect the complexed glycoproteins. The most limiting factor throughout this study was the detection of gH and gL, which greatly affected the sensitivity of the assays. To improve their detection, gH and gL were tagged with the following epitopes: 5E1 (51) plus or minus polyhistidine, V5-polyhistidine, and One-strep-tag. All of the epitopes were engineered just upstream of the stop codon. gC was similarly tagged with the V5 epitope. As shown in Fig. 1, the tagged glycoproteins, named gH\(_{5E1}\), gL\(_{5E1}\), gH\(_{V5}\), and gL\(_{V5}\), maintained immunofluorescence reactivity to conformation-dependent mAbs 52S, 53S, and HC1, reactive to gH/gL, and gC, respectively. They were transported to the cell surface, as measured in the CELISA test (Fig. 1B). When cotransfected with gD and gB, the tagged gH and gL maintained the ability to induce cell-cell fusion, even though efficiency was reduced, as compared with the respective untagged version (Fig. 1C).

The Glycoprotein Quartet Forms Complexes Co-immunoprecipitated with gD in Cells Infected with wt or Deletion HSV Mutants—The aim of the this series of experiments was to detect the complexes formed by the glycoprotein quartet in infected cells by gD co-immunoprecipitation. Prior to infection, 293T cells were transfected with gH\(_{5E1}\) and gL\(_{5E1}\) to facilitate their detection and with HVEM to potentially increase complexes dependent on receptor-bound gD. Six h later, they were infected with HSV-1(F) or HSV deletion mutants in the glycoprotein genes and lysed 18 h after infection. gD was immunoprecipitated with pAb R8; the proteins, separated by denaturing PAGE, were identified by WB. The results in Fig. 2 show that, from wt HSV-infected cells, gD co-immunoprecipitated not only gH (Fig. 2C) but also gB (Fig. 2B) and gL (Fig. 2C). The co-immunoprecipitation of gB, gH, and gL was specific by two criteria. First, gB, gH, and gL failed to be detected when cells were infected with ΔgD-HSV, instead of wt virus. Second, gC, which is present in the same subcellular compartments as the other glycoproteins but is not expected to form any complex with them, was indeed absent from the co-immunoprecipitate (Fig. 2D); the only gC-positive lane in Fig. 2D is that of an aliquot of the lysate, not subjected to immunoprecipitation.

To determine the requirements for complex formation, the cells were infected with ΔgB, ΔgH, or ΔgL. HSVs, gD was immunoprecipitated with pAb R8, and the co-immunoprecipitated proteins were identified as above. Fig. 2 shows that gD co-immunoprecipitated gB from cells infected with ΔgH or ΔgL viruses (Fig. 2B), i.e. in the absence of gH and gL. Conversely, gD co-immunoprecipitated gH and gL from cells infected with ΔgB virus (Fig. 2C), i.e. in the absence of gB.

In the experiments reported in Fig. 2 (A–D), HVEM was hyperexpressed by transfection. To assess whether HVEM hyperexpression was necessary or at least augmented detection of the glycoprotein complexes, 293T cells were infected with wt HSV-1(F) or ΔgD-HSV, with or without prior transfection of HVEM. All of the cells were transfected with gH\(_{5E1}\) and gL\(_{5E1}\); gD was co-immunoprecipitated with pAb R8, and the co-immunoprecipitated proteins were detected by WB, as above. The results in Fig. 2 (E and F) show that HVEM hyperexpression did not modify the ability of gD to co-immunoprecipitate gB, gH, and gL and affected the quantities
of co-immunoprecipitated glycoproteins to a very minor extent.

Complex Formation among the HSV Glycoprotein Quartet Occurs in Cell Lines Relevant to HSV Infection—HSV exhibits a broad tropism in vivo and in cultured cells. In humans, it generally infects the skin and the central nervous system; under certain conditions, including immunosuppression, it infects a variety of organs, testifying for the wide tropism. Here, we asked whether complexes were detected in human cells relevant to HSV infection. We analyzed neural glioblastoma U251 cells and human fibroblasts. Glioblastoma is the target of HSV oncolytic virotherapy (52). Fig. 3 shows that gD co-immunoprecipitated gB and gH/gL in all cell lines.

The Glycoprotein Quartet Forms gD Co-immunoprecipitated Complexes in Transfected Cells—Next, we verified whether the complexes formed in infected cells were formed also in transfected cells. 293T cells were co-transfected with plasmid mixtures encoding the glycoprotein quartet plus HVEM (to increase the amounts of receptor-bound gD) and gCV5, as negative control. Where indicated, the transfection mixtures lacked gD, gB, or gH/gL. gD was immunoprecipitated with pAb R8; co-immunoprecipitated proteins were identified by WB. The results in Fig. 4 show that gB (Fig. 4B), and gH/gL (Fig. 4C) were co-immunoprecipitated from lysates of cells transfected with the quartet. From cells transfected with mixtures lacking gB, gD could still co-immunoprecipitate gH/gL (Fig. 4C). From cells transfected with mixtures lacking gH/gL, gD could still co-immunoprecipitate gB (Fig. 4B). The specificity of co-immunoprecipitation was assessed by the absence of gC from the co-immunoprecipitated proteins (Fig. 4D). The results indicate that complexes formed in transfected cells closely mirror those formed in the infected cells and imply that no viral protein other than those that are recruited to the complexes is required for complex formation. Because the complexes formed in transfected cells did not differ from those in infected cells, most of the subsequent experiments were carried out in transfected cells.
Genetic Engineering of HSV-1 Carrying One-strep-tagged gD (HSV1(BAC)-gDst) — The HSV glycoprotein complexes identified in this study and elsewhere (29, 30) were detected in infected or transfected cells. As mentioned above, a constant major limit in our assays was detection of gH and gL. To investigate glycoprotein complexes in virions and to augment complex capture, we engineered an HSV carrying One-strep-tagged form of gD (gDst). One-strep-tagged proteins, herein named strep-tagged proteins, specifically absorb to Strep-Tactin resin (IBA GmbH, Goettingen, Germany) and retain any protein complexed to them. The virus was engineered through the BAC technology, as detailed under “Experimental Procedures,” and named HSV1(BAC)-gDst. The strep-tag was well tolerated by gD. In a virus yield experiment HSV1(BAC)-gDst replicated to very similar extent as wt HSV1(F) (Fig. 5A). gD, as well as gB, gH/gL, and gC, maintained reactivity to conformation-dependent antibodies (Fig. 5B).

Glycoprotein Complexes Are Present in Virions — HSV1(BAC)-gDst was employed to ask whether glycoproteins already interact with each other in virions independent of entry into the cells and whether complexes formed at virus entry into the cell. Partially purified extracellular HSV1(BAC)-gDst virions were lysed, and gDst was absorbed

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**FIGURE 4.** gD co-immunoprecipitates gB and gH/gL, and not gC, from transfected cells. 293T cells were co-transfected with plasmid mixtures encoding gD (D), gB (B), gH5E1/gL5E1.His (HL), gC5 (C), plus HVEM or with mixtures lacking the indicated glycoproteins (—). The cells were harvested 18 h after transfection. All other details are as in the legend to Fig. 2. Lys, lysate of transfected cells prior to immunoprecipitation.

**FIGURE 5.** Characterization of HSV1(BAC)-gDst, and analysis of glycoprotein complexes in virions and during virus entry. A, kinetics of HSV1(BAC)-gDst replication. Vero cells were infected with HSV1(BAC)-gDst or HSV-1(F), 0.1 PFU/cell. Replicated samples of infected cells were frozen at the indicated times after infection, and progeny virus titrated in Vero cells. No major difference was observed in the yield of HSV1(BAC)-gDst relative to wt HSV-1. B, replicate samples of HSV1(BAC)-gDst-infected 293T cells (0.1 PFU/cell) characterized for immunofluorescence reactivity. Infected cells were incubate with the indicated mAbs, reactive to conformation-dependent epitopes of the protein indicated in parentheses. C, detection of glycoprotein complexes in HSV1(BAC)-gDst virions. Partially purified extracellular HSV1(BAC)-gDst virions were lysed; a small aliquot of the lysate (corresponding to 0.2 × 10^8 PFU) was loaded in lane 1. The remaining lysate was absorbed onto strept-Tactin resin; gDst and complexed glycoproteins were detected by WB to gD (mAb H170), gB (mAb H1817), gH (pAb to gH), and gL (pAb to gL). The gD-gB lane (lane 2) was loaded with material, retained by the step-Tactin resin, derived from 2.8 × 10^8 PFU virions. The gH/gL lane (lane 3) was loaded with material, retained by the step-Tactin resin, derived from 1.4 × 10^8 PFU. The upper blot containing lanes 1–3 was stripped and reacted again with pAb to gM (lower panel); it can be seen that only the lane containing the virion lysate showed reactivity, indicating that gM was not present in the Strep-Tactin-retained fraction. D, detection of glycoprotein complexes at virus entry. Partially purified extracellular HSV1(BAC)-gDst virions were absorbed to 293T cells in T25 flask (4 × 10^8 PFU/T25 flask, ~140 PFU/cell) at 4 °C for 2 h. The unabsorbed virus was removed and titrated and accounted for 75% of the input virus. The cells were rinsed twice with ice-cold medium, overlaid with warm medium, immediately shifted to 37 °C for 0, 15, and 30 min (lanes 4 – 6, respectively) and lysed. The lysate was absorbed to Strep-Tactin resin. The lane of each time point contained material derived from one T25 flask. gDst and complexed gB were detected by WB. The bottom panel shows a lighter exposure of the gD, to highlight the decrease in the cell-associated virion gDst during the 30-min time interval.
to Strep-Tactin resin; complexed glycoproteins were analyzed by WB. The results in Fig. 5C show the WB reactivity of virion lysate (lane 1) and furthermore that small amounts of gB, gH, and gL were complexed to gDwt in resting HSV(BAC)-gDwt virions (lanes 2 and 3). The amounts of pulled down gB and gH/gL was small, and to visualize them we added virus to 293T cells for 2 h at 4 °C (∼140 PFU/cell), unabsorbed virus was rinsed, and the cells were shifted to 37 °C for 0, 15, or 30 min and lysed. The lysate was absorbed to the Strep-Tactin resin. Retained proteins were identified by WB.

Some were pre-existing complexes and to analyze the destiny of the pre-existing complexes and to ask whether new complexes were formed during virus infection, HSV(BAC)-gDwt virions were allowed to attach to 293T cells for 2 h at 4 °C (∼140 PFU/cell), unabsorbed virus was rinsed, and the cells were shifted to 37 °C for 0, 15, or 30 min and lysed. The lysate was absorbed to the Strep-Tactin resin. Retained proteins were identified by WB. Fig. 5D shows that in the 30-min interval, the amount of virion gDwt associated to cells decreased; gB decreased in parallel. We did not detect any gH/gL, likely because of limits of detection. The results indicate that virion gDwt and the complexed gB, tended to decrease rather than increase during virus fusion to 293T cells.

Characterization of gD Mutants Carrying Substitutions, Deletions, or Mutations across the Ectodomain—The objective of the next series of experiments was to preliminarily define the gD regions involved in complex assembly with gB and gH/gL. We employed a panel of gD mutants carrying substitutions, deletions, or mutations across the entire gD ectodomain (linear maps shown in Fig. 6A). Some were previously described, and some were generated for the purpose of this study. As mentioned in the Introduction, gD ectodomain can be schematically subdivided into two regions; the N-terminal region up to aa 240/260 includes the receptor-binding sites; the downstream region, spanning from aa 240/260 to 310 carries the profusion domain. In gD260–310 (previously named gDΔPFPD1) (10) and in gD240–310, the indicated sequences were substituted with the CD8 sequences corresponding to the pretransmembrane region. CD8 was initially chosen as donator of heterologous sequence because it is a transmembrane protein, totally unrelated to HSV attachment sites; the downstream region, spanning from aa 240/260 to 310 carries the profusion domain. In gDΔPFPD1, wt gD, gDΔH, and gDΔL mutants, herein and elsewhere referred as gDpp and gDTP, respectively, carry the indicated substitutions in the profusion domain. Both are partially impaired in infection (9). In gD218–260, the 218–240-aa segment, carrying α-helix 3, was substituted with an 18-mer Ser-Gly linker. gDΔ61–218 was generated in this study following the discovery that, in a very peculiar form of gD split into two fragments, one of which carried the Kringle domain from urokinase plasminogen activator, the 61–218-aa region was dispensable (54). Studies from our laboratory confirm that this sequence can be substituted with heterologous sequences.4 gDΔ6–60 was designed during the course of the study; of note, it is known that the gD N terminus can be deleted at least up to aa 38 (55) and leave a functional gD that cannot interact with HVEM any longer.

The panel of gD mutants was preliminarily characterized with respect to ability to be transported to the cell surface and to mediate cell-cell fusion and virus infection. Cell surface expression was measured in transfected 293T cells and expressed as a percentage relative to wt gD-transfected cells. Fig. 6B shows that the gD260–310, gD240–260, and gDTP mutants were expressed at the cell surface in amounts ranging from 50 to 80% of wt gD; the remaining mutants were severely impaired. Increasing the amount of transfected plasmid DNA by 3-fold did not result in substantially increased cell surface expression (data not shown). We next measured the ability of the mutants to induce cell-cell fusion when co-transfected with gB, gH, and gL. The results in Fig. 6C show that all of the mutants were severely hampered, irrespective of their cell surface expression. Lastly, the ability of gD mutants to promote virus infection was measured in a complementation assay, whereby the gD deletion virus FgDB, which does not encode gD, is grown in cells transiently expressing transgenic wt or mutant-gD. The complemented progeny virions were titrated in R6 cells that express wt gD and allow plaque formation once the virus has entered the R6 cells by means of the complementing gD (Fig. 6D). We further ascertained that the complemented virions carried in their envelope the mutants gD, and, for comparison, gB. Aliquots of virions were pelleted by ultracentrifugation and analyzed for the presence of gD and of gB by WB. As shown in Fig. 6E, all forms of mutant gD were incorporated at substantial amounts, except gDΔ61–218. Cumulatively the results indicated that the gD mutants were severely hampered in infectivity complementation, even though, with one exception, they were incorporated in virion envelopes.

Ability of gD Mutants to Form Complexes with gH/gL—In the following two series of experiments, the panel of mutants was employed to define the gD regions critical for the interactions with gH/gL and with gB. To detect gD-gH/gL complexes, we optimized a pulldown assay based on gHr. 293T cells were transfected with gHr and gLHis (both described in Fig. 1), wt, or mutant gD, plus or minus gB. The cleared lysates were adsorbed to Strep-Tactin resin. The retained proteins were analyzed by WB. Fig. 7A shows that gDwt pulled down wt gD, gD220–260, gDTP, and gDΔ61–218, gDΔ6–60. It is worth noting that gD240–310 and gD260–310 accumulate as three bands (Fig. 8) with apparent molecular masses of ≥56, 50, and 38 kDa. The ≥56-kDa species (marked with diamonds in Figs. 7 and 8) represent the mature forms of the glycoprotein. The 50-kDa species (marked with circles) was observed in all forms of gD and represents the precursor form. The 38-kDa species (marked with stars) likely represents an
FIGURE 6. Characterization of a panel of gD mutants. A, linear map of the gD mutants. gD<sub>TP</sub> and gD<sub>PP</sub> carry the gD<sub>P291L-P292A</sub> or gD<sub>T304-P305L</sub> substitutions (9). In gD<sub>H9018-260–310</sub> (previously named gD<sub>H9004-260–310</sub>) the indicated sequences were substituted with the CD8 sequences corresponding to the pretransmembrane region. gD<sub>H9018-240–310</sub> carries a 18-aa-long Ser-Gly linker in place of the endogenous 240–260-aa sequence. In gD<sub>H9018-218–240</sub>, the 218–240-aa segment, carrying α-helix3, was substituted with a 18-aa-long Ser-Gly linker. In gD<sub>H9018-61–218</sub> the deleted sequences were replaced with a 18-mer Ser-Gly linker. gD<sub>Δ6-60</sub> carries the indicated deletion. B, gD cell surface expression measured in transfected 293T cells by CELISA with mAb H170 (or mAb BD80 for gD<sub>H9004-6–60</sub>). The extent of expression is relative to cells expressing wt gD (100%). C, cell-cell fusion in 293T cells transfected with wt or mutant gD, pluss gB, gH, and gL. All other details as in legend to Fig. 1C. D, infectivity complementation of gD mutants. 293T cells in T<sub>75</sub> flasks were transfected with one of the gD mutants and 4 h later were infected with ΔgD-HSV (FgD<sub>Δ</sub>). In this assay, progeny virus harvested at 18 h after infection is complemented by the transiently expressed transgenic gD; it was titrated in R6 cells (which express wt gD and allow plaque formation); titer was expressed as PFU/ml. It can be seen that all of the gD mutants were dramatically hampered in infection, relative to wt gD. In all histograms, each column represents the average of triplicate samples. The bars denote ± S.E. E, extracellular virions from the experiment shown in D ultracentrifuged and analyzed by WB for the presence of gD and for comparison of gB. It can be seen that, except gD<sub>Δ61–218</sub>, all mutant forms of gD were incorporated in virions.

Complexes Formed by Herpes Simplex Virus gD, gB, gH, and gL
N-terminal degradation product. Its reactivity to mAb H170, used for WB, which recognizes an epitope located at aa 1–23, indicates that this is an N-terminal peptide. Interestingly, with respect to gD<sub>240–310</sub> and gD<sub>260–310</sub>, gH<sub>st</sub> pulled down the 38-kDa N-terminal species and only small amounts of the higher molecular mass species (Fig. 7A). A striking difference was observed also between gD<sub>pp</sub> and gD<sub>TP</sub>, which carry substitutions at Pro<sup>291</sup>–Pro<sup>292</sup> or Thr<sup>304</sup>–Pro<sup>305</sup>, respectively. gD<sub>pp</sub> but not gD<sub>TP</sub> failed to be pulled down by gH<sub>st</sub>. The pattern of gD pulled down by gH<sub>st</sub> in cells transfected with gD, gH, and gL, in the absence of gB (Fig. 7D) was essentially similar to that in Fig. 7A. We interpret the results with gD<sub>240–310</sub> and gD<sub>260–310</sub> to mean that gD carries a composite contact surface to gH/gL. Part is made by N-terminal region and enables gH<sub>st</sub> to pull down the N-terminal 38-kDa degradation product. The other involves segments downstream of aa 240, is lost in gD<sub>240–310</sub> and gD<sub>260–310</sub> because of the substitutions, and accounts for inability of gH<sub>st</sub> to efficiently pull down the mature forms of these two chimeras. The Pro<sup>291</sup>–Pro<sup>292</sup> doublet mutated in gD<sub>pp</sub> is critical to the latter interaction site.

**Ability of gD Mutants to Form Complexes with gB**—In this latter series of experiments 293T cells were transfected with wt or mutant gD plus gB. gD was immunoprecipitated with pAb R8. The results in Fig. 8 show that gD<sub>240–260</sub> and to a lesser extent gD<sub>260–310</sub> and gD<sub>TP</sub> co-immunoprecipitate gB in reduced amounts. The results suggest that a gD region critical for interaction with gB lies between aa 240 and 260 and likely extends downstream; critical residues are Thr<sup>304</sup>–Pro<sup>305</sup>.

**DISCUSSION**

An extensive literature has documented that the entry of HSV-1 into cells involves the fusion of the virion envelope with a cellular membrane and that a quartet of glycoproteins, gD, gB, gH, and gL, are both necessary and sufficient for fusion (1, 3, 4). The discovery that gD serves as the trigger of fusion (9) implied that it cross-talks with at least one of the downstream glycoproteins gB and gH/gL and boosted a search on the interactions that take place among the glycoprotein quartet. Interactions were identified by co-immunoprecipitation between gD and gH/gL (28) and by split green fluorescent protein complementation assay between gD and gB and between gD and gH/gL (29, 30). In the latter assay weak and transient interactions are rendered irreversible, thus enhancing their detection. The studies presented here were designed to (i) provide biochemical evidence of the complexes formed by the four glycoproteins; (ii) to verify whether complexes form not only in transfected cells but also in infected cells and in virions; and (iii) to preliminarily identify the gD regions critical for complex forma-
Complexes Formed by Herpes Simplex Virus gD, gB, gH, and gL

Transfect gD, gB

IP-gD

A WB: gD

B WB: gB

FIGURE 8. Ability of gD mutants to co-immunoprecipitate gB. 293T cells were co-transfected with plasmid mixtures encoding wt or mutant forms of gD, plus gB, and HVEM. The lane marked No_gD indicates a transfection mixture lacking gD; it served as a control that the proteins under examination were specifically co-immunoprecipitated by gD. The cells were harvested 18 h after transfection. All other details are as in the legend to Figs. 2 and 7. Essentially, gD was immunoprecipitated with pAb R8 (IP-gD) (panel A); the co-immunoprecipitated proteins were identified by WB (panel B). In A it can be seen that different forms of gD migrate with different electrophoretic mobility and apparent molecular mass. The symbols are as explained in legend to Fig. 7. An arrowhead points to heavy IgG band.

from lysates of cells infected with mutant viruses lacking gB or from lysates of cells transfected with gD and gH/gL, showed that gD forms a complex with gH/gL in the absence of gB. Similar experiments with cells infected with gH or gL deletion mutants, or with cells transfected with gD and gB, highlighted that gD forms a complex with gB in the absence of gH/gL. The same types of complexes were detected in physiologically relevant cells, including neural cells and human fibroblasts. The key conclusions are 2-fold. First, complexes are formed in infected cells; they do not differ in composition from those formed in transfected cells. This finding, which legitimates the use of transfected cells in studies of complex formation, implies that formation of the complexes requires no viral protein other than the afore-mentioned quartet. Moreover, the interactions detected previously (29, 30) are authentic and do not reflect artifacts caused by transient hyperexpression or irreversible stabilization of transient interactions. Second, gD can form more than one type of complex and carries distinct and independent binding sites for gB and for gH/gL. This property argues somehow against a stepwise recruitment of glycoproteins to gD, as predicted by hemifusion model (25).

gD Regions Critical for Enabling the Binding of gB and gH/gL—One aim of the current experiments was to identify the gD regions critical for complex formation with gB and with gH/gL. gD ectodomain can be divided into two functionally and topologically distinct regions. The N-terminal region up to aa 240/260 carries the binding sites for HVEM (aa 1–32) and for nectin1 (aa ∼30–240); it includes the Ig-folded core (aa 56–184). The C-terminal region carries the profusion activity, required for triggering of fusion but not for receptor binding. Here, we employed a panel of gD mutants carrying substitutions or deletions across the entire ectodomain. The key results were that (i) none of the mutants was completely hampered in ability to recruit gB or gH/gL, indicating that the binding sites in gD for gB and for gH/gL are each made of multiple contacts, or anyway by a composite contact surface. Consequently, alteration of any one residue critical to the contact surface does not result in ablation of the overall binding, but in impaired interaction. (ii) The gD regions critical for recruitment of gH/gL and of gB were differently affected in different gD mutants; hence they are distinct one from the other. (iii) The gD region critical to gH/gL recruitment maps partly at the gD N terminus and partly at the gD C terminus; critical residues are Pro291-Pro292. (iv) The gD region critical to gB recruitment includes the aa 240–260 segment and extends downstream; the critical residues are Thr164-Pro305. Altogether, the current experiments provide the first evidence that the C terminus of gD ectodomain harbors regions critical for complex formation with gB and gH/gL.

Glycoprotein Complexes Are Present in Resting Virions, Albeit in Small Amounts—A key question is whether the glycoprotein complexes are present in resting virions or whether they form at the time of virus entry into the cells and of virion envelope fusion with cell membranes. To address this question we engineered a HSV carrying a strep-tagged form of gD, named HSV1(BAC)-gD_st. gD_st was found to be
complexed with gB and with gH/gL in resting virions; the amount of complexed gB and gH/gL was indeed low. When HSV1(BAC)-gD<sub>st</sub> virions were allowed to enter cells for a 30-min time interval, we did not obtain evidence of de novo gD-gB complex formation; rather, the amount of gD-gB complex decreased. This finding is in agreement with an earlier report showing that gE from input virions first associate with and then disappear from cell surfaces (56).

The Significance of the Glycoprotein Complexes and Interactions Identified in These and Preceding Studies—A key question raised by the results presented in this report and elsewhere (29, 30) relates to the significance of the diverse complexes involving members of the glycoprotein quartet. There are two fundamental possibilities. Foremost, current evidence indicates that resting virions harbor small amounts of preformed complexes; these complexes did not increase during virus entry; rather, they tended to decrease. In principle, the process of HSV-mediated fusion must entail activation of gB and/or gH/gL from fusion-inactive to fusion-active conformations. A possibility compatible with the current results is that conformational changes in gD may be signaled from gD to the precomplexed gB or gH/gL and in this way induce conformational changes to the fusion executors. A nonexclusive possibility is that complexes made by the glycoproteins, or one of the complexes, either is disassembled or is triggered to become fusogenic, once the fusion executors encounter cellular proteins (e.g., receptors), without any appreciable biochemical modification to the glycoproteins themselves.

Second, the possibility exists that members of the glycoprotein quartet, like most HSV proteins analyzed to date, perform multiple functions. In support of this view is the observation that gB is targeted to the multivesicular body compartment and plays a role in HSV envelopment and exit (57), gD and gB traffic to inner nuclear membrane to enable primary envelopment (58), and gD encodes an anti-apoptotic function (47). Conceivably, the formation of these complexes requires the involvement (e.g. direct binding, post-translational modifications, etc) of cellular proteins that enable the execution of these functions. A corollary of this notion is that a fusogenic complex, if it exists, is but one of the complexes formed by members of the quartet. The resolution of function of the various complexes will ultimately require cell-free assembly as well as identification and characterization of the cellular partners with which the glycoproteins interact.

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