Complexes between Herpes Simplex Virus Glycoproteins gD, gB, and gH Detected in Cells by Complementation of Split Enhanced Green Fluorescent Protein[▽]

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The interactions between herpes simplex virus gD and its nectin1 receptor or between gD, gB, and gH were analyzed by complementation of the N and C portions of split enhanced green fluorescent protein (EGFP) fused to the glycoproteins. The gD$_N$-Nect$_C$ complex was readily detected; the gD$_N$-gC$_C$ complex was undetectable, highlighting the specificity of the assay. Split EGFP complementation was detected between proteins designated gD$_N$+gH$_C$, gD$_N$+gB$_C$, and gH$_N$+gB$_C$+wtgD (gB was deleted of endocytosis motifs), both in cells transfected with two-tree glycoproteins and in syncytia. The in situ assay provides evidence that gD interacts with gH and gB independently of each other and supports a model whereby gH and gB in complex exert their activities to gD.

The entry of herpes simples virus (HSV) into cells requires a multipartite fusion system made of a quartet of glycoproteins (3, 24, 28). The receptor-binding glycoprotein gD interacts with three alternative receptors, nectin1, herpesvirus entry mediator, and modified heparan sulfate (5, 11, 22, 27). gD also encodes a profusion domain at the ectodomain C terminus, which is required to trigger fusion (4). In the unliganded gD, the ectodomain C terminus folds around the N terminus. At receptor binding, the C terminus is displaced, gD adopts an open conformation, and fusion is triggered (8, 20). Three glycoproteins conserved across the *Herpesviridae* family, gB and gH · gL, execute fusion (2, 7, 25). The identity of the executor—whether it is gB, gH · gL, or the three glycoproteins together—remains unclear. Thus, gB exhibits a trimeric structure, properties typical of class I and II viral fusion proteins, and a candidate fusion loop (16, 17). On the other hand, gH exhibits elements typical of class I fusion glycoproteins, including two heptad repeats able to form a coiled coil and a candidate fusion peptide, besides having additional hydrophobic regions (9, 10, 12–15). Hemifusion (the fusion of the outer layers of the virion and cell membranes) requires gD and gH · gL; complete fusion (the mixing of both outer and inner lipid layers) additionally requires gB (29). Fusion between perinuclear virions and the outer nuclear membranes, which culminates in capsid release into the cytoplasm, requires gD plus either gB or gH · gL, implying that, under particular conditions, either gB or gH · gL suffice for fusion execution (6).

A key question in HSV entry/fusion centers on how gD signals the encounter with its receptor to the downstream glycoproteins and thus triggers fusion. The working model investigated in this laboratory envisions that the receptor-bound gD

forms complexes with the downstream glycoproteins or with a subset of them (3). Indeed, by coimmunoprecipitation, gD was shown to be in a complex with gH (23).

The aim of this work was to investigate, by means of a protein complementation assay (CA) (19, 21), in intact cells, the molecular interactions that take place between gD and nectin1 and between the four glycoproteins. In the CA, proteins like enhanced green fluorescent protein (EGFP) are split into two portions that, if brought to an 8- to 10-Å proximity of each other, refold together and emit fluorescence (19, 21). In the current adaptation to membrane proteins, the EGFP N terminus (N; amino acids [aa] 1 to 157) was fused to the endodomain of HSV type 1 gD or gH, and the C terminus (C) was fused to the endodomain of gB, gH, gC, or nectin1. The glycoproteins under examination interact, or are likely to interact through their ectodomains; the EGFP portions were located in the endodomains. The assumption was made that any specific interaction occurring between the ectodomains would result in refolding and fluorescence emission of the endodomain-located EGFP fragments. Fluorescence intensity from the complemented EGFP was reported to be about 10% of that produced by the unsplit protein, essentially because only a subset of the fragments have a likelihood of associating with each other (see reference 19). This drawback is balanced by the fact that complementation is driven by specific interactions that occur between the proteins under investigation, to which the EGFP portions are fused. Moreover, the refolded EGFP adopts an irreversible conformation that contributes to stabilizing the complex and thus enables the detection of transient and weak complexes (18, 19).

Plasmid construction. The mammalian expression plasmids for gH in the MTS vector, and gD and nectin1 in pcDNA3.1 (5, 31) were site-directed mutagenized 0 to 10 aa upstream of the stop codon, in order to generate restriction sites for the insertion of N or C amplimers. The sites were SphI for gH and BglII for gD or nectin1. Where necessary, the BglII site of pcDNA3.1 was preliminarily eliminated by digestion, filling in

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by T4-DNA polymerase and religation. N and C sequences were PCR amplified from pCMS-EGFP (Clontech) with the primer pairs (or variations thereof) 5′CCCAGATCTC CATG GTGAGC AAGGGCGAGG AGCTGT plus 5′GGGAAG CTTC TACTTGTCGG CCATGATATA GACGTTG or 5′C CCGCTAGCT CAGAAGAACG GCATCAAGGT GAACT plus 5′GGGAGATCTT ACTTGTACAG CTCGTCCATG CCGAGA, respectively. N amplimer was ligated with BglII-HindIII-digested gD plasmid or SphI-BglII-digested gH plasmid, generating gD_N and gH_N. The C-EGFP amplimer was ligated with digested nectin1 (BglII-XhoI) and gH (SphI-BglII) plasmids, generating $Nect_C$ and gH_C. The gC gene sequence was PCR amplified from DNA of HSV type 1 (F) with primers 5′AGATCTAGGC CTATGGCCCC GGGGCG GGTG GGCCTTGCCG TGGTCCTGTG GAGCCTG and 5′GAAGATGCGG CCGCTTAGCT AGCCGCCGAT GAC GCTGCCG CGACTGTGAT GTGCG. The StuI-NheI-digested gC amplimer and the NheI-BglII-digested C amplimer were ligated with StuI-BglII-digested MTS vector. The gB-encoding plasmid in pcDNA3.1 was deleted of the endodomain sequences that carry endocytosis motifs, from aa 867 to the stop codon ($gB_{\Delta 867}$), in order to maximize gB expression (1). The gB_C chimera was generated by mixing $gB_{\Delta 867}$ and C amplimers, generated with primer pairs 5′GGCTGGATCC TCCCCGTAGT CCCGCCATGC plus CCTTGATGCC GTT CTTCTGA GATCTCTTCT TCTTGGCCTT GTGTTC and 5′GAACACAAGG CCAAGAAGAA GAGATCTCAG AAG AACGGCA TCAAGG plus 5′GGGAAGCTTT TACTTG TACA GCTCGTCCAT GCCGAGA, followed by ligation with BamHI-HindIII-digested pcDNA3.1. Arrestin-transfected 293T or COS cells, mounted without fixation with Fluoromount, were observed with a Leica TCS-SL confocal microscope, set at 100% excitation at 488 nm with emission between 490 to 540 nm. Images were collected with a 63× 1.62 Leica oil immersion objective; confocal slices were 1.7 to 2.3 μm thick. For each experimental series—see Figure 2A to T and U to Z and Fig. 3A to F, G to I, J to L, and M to P—images were collected on the same day, under the same settings, applying 1,024- by 1,024-pixel resolution and an 8-bit intensity scale. Specifically, the first sample to be analyzed was the negative one, containing gCc; for subsequent observations of the samples belonging to the same series, the settings were then kept unmodified. Figure 1 shows the electrophoretic mobility of the N-EGFP or C-EGFP fusion proteins generated in this study, made in transfected COS or 293T cells, detected by Western blotting with monoclonal antibodies H170, H1817, H633, and CK6 to gD, gB, gC, and nectin1, respectively, and polyclonal antibodies to gH. As expected, all the fusion proteins exhibited a slower electrophoretic mobility relative to their respective wild-type versions.

gD-nectin1 complex detection. Inasmuch as EGFP-CA has been applied mainly to analysis of soluble mammalian or bacterial proteins (19), we first validated its application to membrane proteins—in particular, to HSV glycoproteins—by analysis of gD and its nectin1 receptor. Figure 2C and M documents complex formation between gD_N and $Nect_C$ as fluorescence emission from EGFP-CA in transfected 293T or COS cells. Cells were observed 36 h after arrestin-mediated transfection. Results with the two cell lines were essentially similar, although the level of expression and number of fluorescent cells was higher with 293T cells. In agreement with
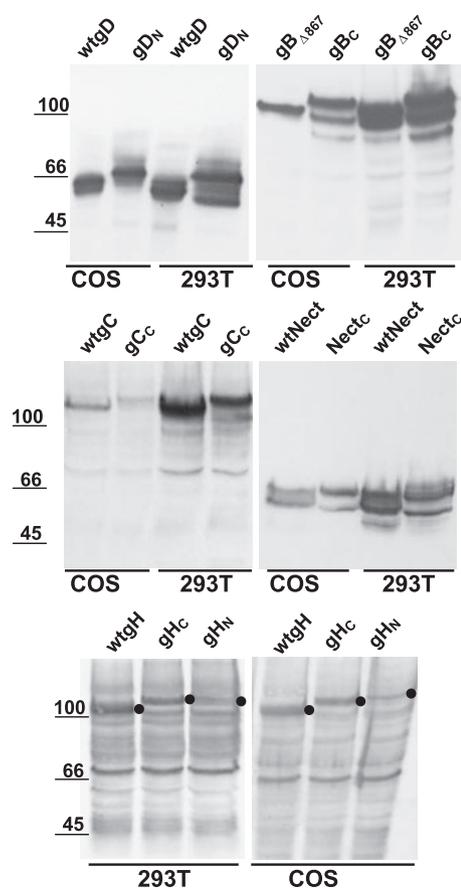

FIG. 1. Electrophoretic mobility of proteins fused to N- or C-EGFP portions, or their wild-type alleles, expressed in COS or 293T cells. Numbers on the left indicate the migration positions of molecular mass markers (in kilodaltons).

previous reports, the overall fluorescence emitted by complementation of split EFGP fragments was lower than that from the unsplit protein (19).

The specificity controls that validated the assay were as follows. First, neither gD_N nor $Nect_C$ emitted fluorescence when transfected singly (Fig, 2A, B, K, and L) or in combination with the wild-type alleles of nectin1 or gD (not shown), ruling out autofluorescence. Second, fluorescence was reconstituted only when the EGFP chimeric proteins were in a specific complex and not simply present in the same subcellular compartment. For this control, we selected gC, which is involved in virus attachment but not virus entry and is present in the same subcellular compartments as gD. The coexpression of gD_N and gC_C resulted in no or background fluorescence (Fig. 2D and N), ruling out the possibility that proteins that exhibit no specific interaction, but that are abundantly present in the same cellular compartment, give rise to EGFP complementation. We took advantage of the lack of EGFP complementation by gC_C-containing samples and, in all experiments, used the gC_C-containing sample to adjust the confocal microscope settings. The settings were then kept constant throughout the observation period of a same series of samples. Third, we ascertained by immunofluorescence assay (IFA) that all proteins were expressed, even those expressed singly (gD_N, $Nect_C$) (Fig. 2E, F,
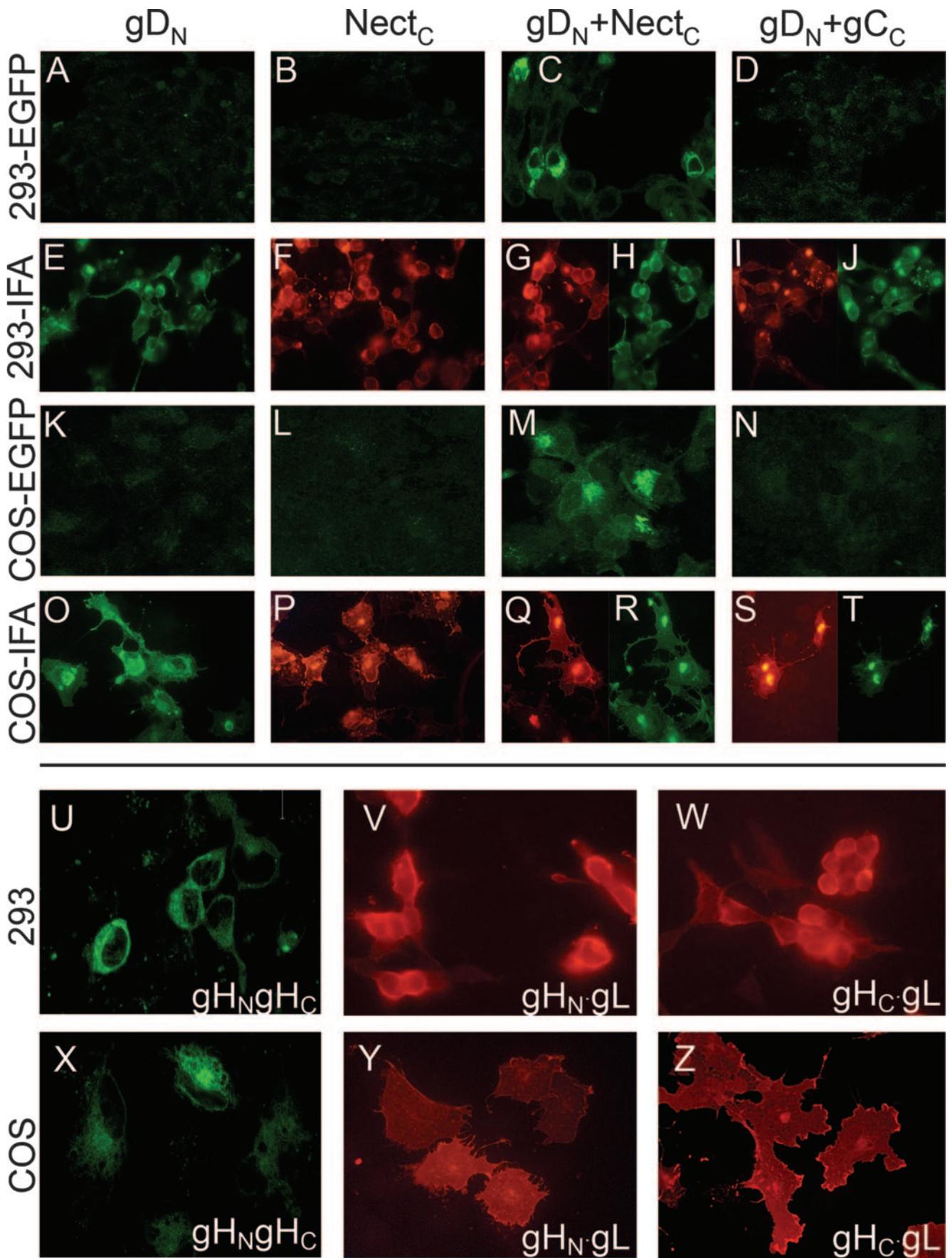
O, and P) or in the gD_N-gC_C combination that did not yield EGFP fluorescence (Fig. 2I, J, S, and T). Importantly, the EGFP-glycoprotein chimeras were not hampered in plasma membrane localization (Fig. 2). We conclude that EGFP-CA fulfills the criteria for detection of specific interactions between membrane-bound proteins, particularly HSV-1 gD and its receptor.

Complexes between HSV glycoproteins. The second series of experiments was performed with 293T and COS cells transfected with three membrane proteins in combinations that included gD_N+gB_C, gD_N+$gB_{\Delta867}$, gD_N+gH_C+wtgL, gD_N+ wtgH+wtgL, and gD_N+gC_C. We used a form of gB deleted for endocytosis motifs, to maximize its expression and localization in exocytic and plasma membranes (1). Transfection mixtures were made equal in DNA amounts (900 ng/well, 300 ng/plasmid) by the addition of a plasmid encoding epidermal growth factor receptor 1 deleted of signaling sequences (26). This control ensured that exocytic membranes were loaded with comparable amounts of proteins. In both cell types observed 36 h after transfection, the gD_N+gH_C+wtgL combination resulted in a readily detectable fluorescence (Fig. 3B and E). The gD_N+gB_C combination gave rise to a somewhat weaker fluorescence (Fig. 3A and D) that nonetheless was much higher than the background fluorescence emitted by the gD_N+gC_C combination (Fig. 3C and F). Even though the subcellular localization cannot be clearly defined, EGFP appeared to localize to a perinuclear position, consistent with a Golgi compartment localization, to a cytoplasmic reticular compartment, consistent with endoplasmic reticulum, and to nuclear membranes. By IFA, all proteins resulted to be expressed, even those that did not yield EGFP fluorescence (not shown). We infer that gD can recruit gH to a complex. gD can also recruit gB to a complex. The gD-gH combination results in a stronger EGFP fluorescence than the gD-gB combination, possibly reflecting a stronger interaction, a more stable or longer half-life complex, a higher number of complexes at steady state, or peculiar behaviors of the fusion proteins.

Cells transfected with the quartet of gD, gB, gH, and gL form syncytia (30). A series of experiments was designed to verify whether the glycoprotein-EGFP chimeras were still functional in cell-cell fusion, and whether complexes were detectable under conditions that lead to cell-cell fusion. 293T or COS cells were cotransfected with combinations of five plasmids (1.25 µg/well, 250 ng/plasmid) encoding gD, $gB_{\Delta867}$, gH, gL, and gC or their EGFP chimeras. The transfected combinations included gD_N+gB_C+wtgH+wtgL+wtgC, gD_N+ gH_C+wtgL+$gB_{\Delta867}$+wtgC, and gD_N+$gB_{\Delta867}$+wtgH+wtgL+ gC_C. Cells were observed 24 h (293T) or 40 h (COS) after transfection. The results in Fig. 3G to L show that syncytia were formed for any combination, indicating that the EGPF-glycoprotein chimeras were not hampered in fusion activity. The strongest fluorescence was observed with the combination that included gD_N+gH_C (panels H and K). A somewhat
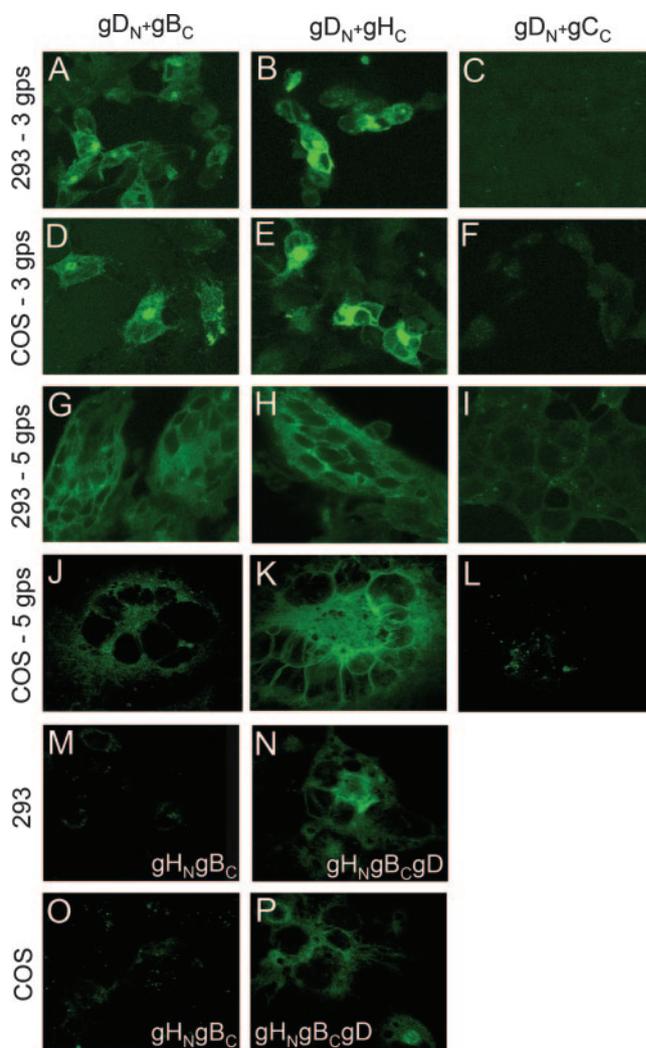

FIG. 3. EGFP CA between HSV glycoproteins. COS or 239T cells were transfected with the indicated plasmids, gD_N+gB_C (A, D, G, and J), gD_N+gH_C (B, E, H, and K), gD_N+gC_C (C, F, I, and L), gH_N+gBc (M and O), and gH_N+gB_c+gD (N and P). Cells transfected with three glycoproteins (gps) received, in addition, plasmids encoding wtgL or epidermal growth factor receptor, as appropriate. Cells transfected with five glycoproteins received gD, $gB_{\Delta867}$, gH, gL, and gC as the wild type or EGFP chimeras, as indicated.

weaker fluorescence was observed with the combination that included gD_N+gB_C (panels G and J), particularly in COS cells. No fluorescence above background level was observed with the combinations that included gD_N+gC_C (panels I and L). The stronger fluorescence in panels B relative to H and in panels E relative to K reflects (i) higher amounts of transfected DNA for each plasmid, (ii) a longer time interval after transfection (panels B versus H), (iii) the lack of dilution of complemented

FIG. 2. EGFP CA between gD_N and $Nect_C$ or between gH_N+gHc, and lack of complementation between gD_N and gC_C. COS or 239T cells were transfected with the indicated plasmids, gD_N (A, E, K, and O), $Nect_C$ (B, F, L, and P), gD_N+$Nect_C$ (C, G, H, M, Q, and R), gD_N+gC_C (D, I, J, N, S, and T), and gH_N+gH_c (U to Z). (A to D, K to N, U, and X) CA. (E to J, O to T, V, W, Y, and Z) IFA. Green, gD; red, all others. Antibodies used were R8 to gD, R1.302 to nectin1, H633 to gC, and 53S to gH (reacts to a gL-dependent epitope).

EGFP molecules consequent to fusion of transfected fluorescent cells with adjacent untransfected nonfluorescent cells, or (iv) possibly a longer half-life of the complexes when cell-cell fusion does not ensue.

We next tested whether gH and gB interact with each other and whether the interaction was dependent on the presence of gD. Cells were transfected with $gH_N+wtgL+gB_C$ in the absence or presence of wtgD. Interaction between gH_N-gB_C was readily documented in the presence (Fig. 3N and P) but not in the absence (Fig. 3M and O) of gD.

While gB is known to be a trimer (17), the oligomeric state of gH is unknown. Here, we addressed the question of whether the split EGFP-CA was suitable to define the oligomeric state of gH. Cells were transfected with gH_N+gH_C+wtgL. As shown in Fig. 2U to Z, the CA readily documented that the gH · gL heterodimer (detected by its reactivity to MAb 53S) formed oligomeric structures in transfected cells.

Concluding remarks. We validated the adaptation of the EGFP-CA to membrane proteins by first applying it to the gD-nectin1 interaction. The fluorescence emitted from the gD_N-$Nect_C$ combination was readily detectable, whereas that from the gD_N-gC_C combination was detected at only background levels, testifying to the assay specificity. For every series of observations, the gC_C-containing sample was therefore used to adjust the confocal microscope settings. Samples exhibiting readily detectable fluorescence under these conditions were considered positive.

We detected a complex made of gD and gH, in agreement with coimmunoprecipitation data (23). The complex formed even in the absence of gB. In addition, we detected a complex made of gD and gB that formed even in the absence of gH · gL. We further documented the interaction between gH and gB; its gD dependence suggests that the interaction is triggered by gD. A notable property of the EGFP-CA as applied here was that complex formation between HSV glycoproteins was detected in intact cells, i.e., in the intracellular compartment and microenvironment and under the very conditions in which the interactions do occur. Importantly, the EGFP chimeric glycoproteins were not hampered in cell-cell fusion activity. Hence, the detected interactions were a faithful mirror of the interactions that take place under conditions that lead to cell-cell fusion. Inasmuch as EGFP reconstitution from split portions is an irreversible reaction, the assay does not allow us to infer whether the complexes between the HSV glycoproteins were stable or transient.

By taking advantage of the fact that CHO but not Vero cells lack the lipid ganglioside GM1, hemifusion, i.e., the mixing of the outer lipid leaflets of the virion envelope and cell membrane or cell-cell membranes, was differentiated from fusion, i.e., complete lipid mixing and content mixing (29). In that assay, gD and gH · gL are sufficient to induce hemifusion. Complete fusion additionally requires gB (29). Those findings support the view that HSV fusion occurs through steps, i.e., the juxtaposition of membranes and the triggering of fusion, hemifusion, and complete fusion. They do not shed light on the sequential order and mechanism of gB recruitment. Current data agree with those from the hemifusion study and, moreover, argue that hemifusion is carried out by the gD-gH · gL complex. Of note, the fact that three independent assays—coimmunoprecipitation, hemifusion and split EGFP CA—concordantly showed the interaction between gD and gH strongly substantiates the current approach.

Cumulatively, the current assay provides in situ evidence for the following. (i) gD recruits gH · gL and gB to complexes. (ii) gH and gB can be recruited to gD independently of one another. Thus, gD carries binding sites for both gH · gL and gB. The independent recruitment of these glycoproteins to gD is consistent with and substantiated by the observation that, at the outer nuclear membrane, virions deleted for gB but carrying gD+gH, or deleted for gH but carrying gD+gB are capable of fusion (6). (iii) Once gH · gL and gB are recruited to gD, they possibly interact with each other. (iv) gH · gL and gB are not necessarily recruited in a sequential order or one to the other. Current data support a model of HSV entry-fusion whereby gH and gB exert their activity through complex formation with gD, or following activation mediated by complex formation with gD.

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