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The Nectin-1α Transmembrane Domain, But Not The Cytoplasmic Tail, Influences Cell Fusion Induced by HSV-1 Glycoproteins

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Abstract

Nectin-1 is a receptor for herpes simplex virus (HSV), a member of the immunoglobulin superfamily, and a cellular adhesion molecule. To study domains of nectin-1α involved in cell fusion, we measured the ability of nectin-1α/nectin-2α chimeras, nectin-1α/CD4 chimeras, and transmembrane domain and cytoplasmic tail mutants of nectin-1α to promote cell fusion induced by HSV-1 glycoproteins. Our results demonstrate that only chimeras and mutants containing the entire V-like domain and a link to the plasma membrane conferred cell-fusion activity. The transmembrane domain and cytoplasmic tail of nectin-1 were not required for any viral receptor or cell adhesion function tested. Cellular cytoplasmic factors that bind to the nectin-1α cytoplasmic tail, therefore, did not influence virus entry or cell fusion. Interestingly, the efficiency of cell fusion was reduced when membrane spanning domains of nectin-1α and gD were replaced by glycosylphosphatidylinositol tethers, indicating that transmembrane domains may play a modulatory role in the gD/nectin-1α interaction in fusion.

Keywords

glycosylphosphatidylinositol; fusion; syncytium; nectin-1; herpes simplex; transmembrane domain; cytoplasmic tail

INTRODUCTION

Herpes simplex virus (HSV) entry into susceptible cells and virus-induced cell fusion require glycoproteins B (gB), gD, gH, and gL (Cai *et al.* 1988a;Cai *et al.* 1988b;Highlander *et al.* 1988;Huff *et al.* 1988;Johnson and Ligas 1988;Ligas and Johnson 1988;Forrester *et al.* 1992;Hutchinson *et al.* 1992;Roop *et al.* 1993;Balan *et al.* 1994;Davis-Poynter *et al.* 1994;Wilson *et al.* 1994). HSV particles can fuse directly with the plasma membrane in a pH-independent manner (Wittels and Spear 1991). However, recent studies suggest that a cell-type or cell-line dependent internalization of viral particles and a reduction in pH may increase efficiency of virus entry (Nicola *et al.* 2003;Gianni *et al.* 2004;Nicola and Straus 2004). Regardless, HSV glycoprotein-induced cell fusion occurs at neutral pH. Fusion of HSV-infected cells with adjacent uninfected cells can result in the formation of large multi-nucleated cells called syncytia (Spear 1993;Pertel and Spear 1998). Because syncytia survive for a relatively short period of time, syncytium formation may represent a mechanism of virus-induced cell killing. To study the fusion events that occur during virus entry and virus-induced cell fusion, plasmid-based expression systems have been developed. Cells expressing a gD receptor form syncytia when transfected with plasmids expressing gB, gD, gH, and gL

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(Connolly *et al.* 2003;Jones and Geraghty 2004). Also, when mixed together, cells expressing gB, gD, gH, and gL fuse with cells expressing a gD receptor, and the extent of fusion can be measured by reporter gene expression (Turner *et al.* 1998;Pertel *et al.* 2001;Jones and Geraghty 2004;Tiwari *et al.* 2004).

Efficient fusion requires the interaction of viral gD with a cell surface receptor from one of three classes of receptors (Spear *et al.* 2000). HVEM (herpesvirus entry mediator) is a member of the tumor necrosis factor receptor family. HVEM is expressed on lymphocytes and other cells and can mediate the entry of most HSV-1 and HSV-2 isolates (Montgomery *et al.* 1996;Kwon *et al.* 1997). A second class of gD receptor, 3-*O*-sulfotransferase-modified heparan sulfate present on cell surface proteoglycans, mediates the entry of many HSV-1 strains (Shukla *et al.* 1999). The final class of receptors contains the immunoglobulin (Ig) superfamily members nectin-1 and nectin-2. Nectin-1 expression in normally resistant cells allows entry of all viable isolates of HSV-1 and -2 tested thus far (Cocchi *et al.* 1998b;Geraghty *et al.* 1998;Krummenacher *et al.* 2004). Nectin-2 mediates the entry of laboratory isolates of HSV-1 with a mutation in the amino-terminus of gD and some strains of HSV-2 (Warner *et al.* 1998;Lopez *et al.* 2000;Yoon and Spear 2004). Once gD binds its receptor, a region of gD, the "pro-fusion" domain, may interact with the other viral fusion glycoproteins to promote membrane fusion (Cocchi *et al.* 2004;Zago *et al.* 2004).

Nectins are members of a growing family of related Ca^{2+}-independent cell adhesion molecules that localize to E-cadherin-based adherens junctions (Takahashi *et al.* 1999;Miyahara *et al.* 2000;Satoh-Horikawa *et al.* 2000;Reymond *et al.* 2001). To promote cell adhesion, dimers of nectin-1 interact in trans with nectin-1, nectin-3, or nectin-4 dimers on adjacent cells (Lopez *et al.* 1998;Miyahara *et al.* 2000;Satoh-Horikawa *et al.* 2000;Sakisaka *et al.* 2001;Momose *et al.* 2002). Trans interactions occur via binding of respective V-like domains and are required for efficient localization of nectins to areas of cell-cell contact (Miyahara *et al.* 2000;Fabre *et al.* 2002). Mutagenesis, monoclonal antibody binding, and in vitro binding studies have identified the V-like domain of nectin-1 as the gD-binding region (Cocchi *et al.* 1998a;Krummenacher *et al.* 1999;Krummenacher *et al.* 2000;Cocchi *et al.* 2001;Geraghty *et al.* 2001;Martinez and Spear 2002;Struyf *et al.* 2002a). The gD-binding region overlaps with the region involved in the trans interactions necessary for cell adhesion (Fabre et al. 2002;Krummenacher et al. 2002). The V-like domain alone, when engineered to be expressed on the surface of the cell, can mediate virus entry although at a level significantly reduced from wild-type nectin-1 (Cocchi *et al.* 1998a). Upon addition of the V-like domain to CD4, nectin-2, or the poliovirus receptor, these chimeric molecules display wild-type nectin-1 virus-entry activity (Cocchi *et al.* 2001;Geraghty *et al.* 2001). However, a nectin-1/CD4 chimera, called 1/1/1/4/4, containing the entire extracellular domain of nectin-1 (including an intact V-like domain) binds gD but does not allow entry of HSV-1 (Geraghty *et al.* 2001), indicating that the V-like domain is necessary but not sufficient for virus entry. The lack of 1/1/1/4/4 entry activity may be due to the extended distance of the V-like domain from the plasma membrane (Jones and Geraghty 2004). The two C-like extracellular domains of nectin-1 have as of yet not been assigned a specific role in virus entry.

The cytoplasmic tail (CT) of many nectins, including the nectin-1αisoform, binds the PDZ domains of the F-actin-binding protein afadin (Mandai *et al.* 1997;Takahashi *et al.* 1999;Satoh-Horikawa *et al.* 2000) and the cell polarity protein PAR-3 (Takekuni *et al.* 2003). Trans interactions between nectins result in the activation of Rac and Cdc42 small G proteins, independent of afadin or PAR-3, via a mechanism involving activation of c-Src (Kawakatsu *et al.* 2002;Fukuhara *et al.* 2003;Honda *et al.* 2003;Fukuhara *et al.* 2004;Hoshino *et al.* 2004). It has not been reported whether Cdc42/Rac activation occurs upon gD binding but since the gD-binding region overlaps with the region involved in trans interactions (Fabre *et al.* 2002;Krummenacher *et al.* 2002), gD binding could result in small G protein activation.

Manipulation of the actin cytoskeleton or other cytoskeletal elements by afadin or activation of Rac and Cdc42 could impact nectin-dependent processes such as virus entry and cell fusion. Actin remodeling facilitates membrane fusion during cellular processes such as intracellular trafficking (Eitzen 2003). Previous reports found that HSV-1 "fusion from without" and "fusion from within" were inhibited by the addition of the actin filament-disrupting agent cytochalasin D, suggesting a role for actin in syncytium formation (Heeg *et al.* 1986;Walev *et al.* 1991;Yura *et al.* 2000). It has yet to be determined whether association of nectin-1α and cellular cytoplasmic proteins influences the efficiency of virus entry or cell fusion.

Nectin-1α, therefore, has multiple extracellular and intracellular domains that function in virus entry and cell adhesion. To better understand the process of nectin-1α-mediated cell fusion and to compare and contrast the requirements for fusion in virus entry and cell fusion, we analyzed nectin-1α mutants and chimeras to identify the domains important for cell fusion. We found that only nectin-1α mutants and chimeras with an intact V-like domain and a link to the plasma membrane, such as a transmembrane domain (TM) or a glycosylphosphatidylinositol (GPI) link, functioned in cell fusion. The efficiency of cell fusion was reduced when membrane spanning domains of nectin-1α and gD were replaced by a GPI tether, indicating that TMs play a modulatory role in the gD/nectin-1 interaction in fusion. Recently, a nectin-1gpi mutant was demonstrated to mediate virus entry and cell fusion (Gianni *et al.* 2004). Here we confirm and extend those results to quantify virus entry, cell fusion, and trans interactions for a nectin-1gpi mutant and also a nectin-1α mutant lacking a CT. The nectin-1α CT, and therefore interactions with cytoplasmic cellular factors, were not important for virus entry or cell fusion.

RESULTS

Nectin-1 chimeras

The major domains of nectin-1 (3 Ig-like folds in the extracellular domain, the transmembrane domain, and the cytoplasmic tail) have functions assigned to them such as gD binding, trans interactions for cell adhesion, cellular localization, association with the actin cytoskeleton, and activation of small G proteins. Our goal was to determine the importance of each domain/function in cell fusion to better understand the process of nectin-1-mediated cell fusion. To study general domains important for cell fusion involving nectin-1, we utilized nectin-1α/nectin-2α and nectin-1α/CD4 chimeras. These chimeras have been previously characterized for their ability to bind gD and to mediate virus entry (Geraghty *et al.* 2001) allowing us to compare and contrast the receptor requirements for fusion occurring during virus entry and cell-cell fusion.

Diagrams of nectin-1α, nectin-2α, CD4, and the general positions where the chimeras were constructed are depicted in Figures 1A and B. Details on the construction of the chimeras and exact amino acid composition of each chimera have been described previously (Geraghty *et al.* 2001). Nectin-1α and nectin-2α are closely related (35% amino acid identity) and have the same general domain organization. The 1/2/2 chimera contains all of the predicted nectin-1α V-like domain replacing the homologous region of nectin-2α. The 1*/2/2 chimera is nearly identical to 1/2/2 except the final 22 amino acids of the V-like domain were derived from nectin-2α. Chimera 1/1*/2 contains the entire nectin-1 V-like domain and most of the first C-like domain joined to nectin-2α at the equivalent amino acid after the fourth conserved Cys. Figure 1B depicts the nectin-1α/CD4 chimeras containing single (1/4/4) or multiple (1/1/4/4 and 1/1/1/4/4) Ig-like domains of nectin-1α fused to a hinge region in CD4 located N-terminal to the two membrane-proximal Ig domains. The nectin-1α region in chimera 1/4/4 consisted of the entire V-like domain except for a 22 amino acid deletion between the second conserved Cys and the predicted end of the V-like domain.

All chimeras were previously shown to be expressed at the cell surface at levels equivalent to wild-type nectin-1α with two exceptions, 1/1/4/4 and 1/1/1/4/4, for which expression was reduced but significantly above background (Geraghty *et al.* 2001). When gD binding was related to cell surface expression, all chimeras bound HSV-1 gD at approximately equivalent levels compared to wild-type nectin-1α (Geraghty *et al.* 2001). The one exception was the 1/4/4 chimera which displayed no detectable gD-binding activity (Geraghty *et al.* 2001).

Cell fusion activity of nectin-1 chimeras

Chinese hamster ovary (CHO) cells (K1 clone) do not express a functional gD receptor and therefore do not support HSV-1-glycoprotein-induced cell fusion, making them ideal for the study of gD receptors in cell fusion. We employed a cell-mixing fusion assay to assess the ability of nectin-1α and chimeras to promote cell fusion induced by HSV-1 glycoproteins. CHO K1 cells were transfected with plasmids expressing gB, gD, gH, gL, and T7 polymerase (1:1:1:1:1 molar ratio). The glycoprotein-transfected cells were mixed (at a 1:1 ratio) with CHO K1 cells transfected with plasmids expressing nectin-1α (or chimera) and pG1NT7β-gal (3:1 molar ratio). The plasmid G1NT7β-gal contains the *lacZ* gene under the control of the T7 promoter (Feng *et al.* 1996). Upon cell fusion, the cell contents mix and T7 polymerase induces the synthesis of β-galactosidase (β-gal). The amount of β-gal produced was measured as an indication of cell fusion. To determine cell surface expression of the nectin-1 chimeras during the fusion assay, the transfected cells were analyzed by CELISA in parallel with each fusion assay. The CELISA technique has been previously described (Geraghty *et al.* 2000;Geraghty *et al.* 2001;Jones and Geraghty 2004). Briefly, transfected cells were incubated with an anti-nectin-1 monoclonal antibody (mAb) CK6, washed, and fixed prior to the addition of secondary antibody and an antibody detection system. CK6 recognizes a linear epitope in the V-like domain of nectin-1 (Krummenacher *et al.* 2000) present in all the chimeras.

The relative results for the nectin-1α/nectin-2α chimeras are shown in Figure 2A. The chimeras with an intact nectin-1α V-like domain, 1/2/2 and 1/1*/2, mediated cell fusion via the HSV-1 (KOS) glycoproteins at levels just below wild-type nectin-1α. The 1*/2/2 chimera, however, mediated cell fusion at a greatly reduced level when compared to nectin-1α (Fig. 2A), despite a higher cell surface expression (Fig. 2B). These results directly paralleled those obtained for gD binding and virus entry (Geraghty *et al.* 2001). A low but significant level of cell fusion was mediated by nectin-2α despite the use of HSV-1(KOS) envelope glycoproteins (Fig. 2B). To determine whether the low fusion activity was caused by low expression of nectin-2α compared to nectin-1α, tagged version of the two receptors were created by fusing the yellow fluorescent protein (YFP) to the C-terminus of the CT of the respective receptors. The fusion results with the YFP-tagged versions of nectin-1α and nectin-2α were identical to those in Figure 2A (data not shown). Nectin-1YFP and nectin-2YFP were expressed at the same level in cells used in the fusion assay based upon western blot analysis using an anti-YFP antibody (data not shown). Therefore, when nectin-1α and nectin-2α were expressed at equivalent levels, nectin-2α was much less efficient at mediating cell fusion with HSV-1(KOS) glycoproteins. Our results are consistent with previous observations for nectin-2α using a similar cell fusion assay (Yoon *et al.* 2003;Zago and Spear 2003), but differed from the negligible activity of nectin-2α for HSV-1(KOS) entry described previously (Warner *et al.* 1998).

Results obtained for the nectin-1α/CD4 chimeras are shown in Figure 3. The 1/4/4, 1/1/4/4, and 1/1/1/4/4 chimeras all mediated HSV-1-induced cell fusion (Fig. 3A). The relative fusion activity (percent of nectin-1α) for 1/4/4 and 1/1/4/4 (Fig. 3A) was higher than the relative cell surface expression (percent of nectin-1α) shown in Figure 3B. The ratio of relative fusion activity to relative surface expression for 1/4/4 and 1/1/4/4 was 147%/71% and 55%/23%, respectively, approximately a value of 2. The ratio for 1/1/1/4/4 was 43%/61%, approximately 0.75. Those values indicate that the 1/4/4 and 1/1/4/4 chimeras demonstrated an enhanced

fusion activity over nectin-1α (which would by definition have a value of 1) if the relationship between cell surface expression of nectin-1α and cell fusion activity was linear for the cell mixing assay. The positive fusion result for the 1/1/1/4/4 chimera was surprising because although 1/1/1/4/4 bound HSV-1(KOS) gD with wild-type nectin-1 efficiency, it did not mediate HSV-1(KOS) entry (Geraghty *et al.* 2001). Therefore, the virus entry and cell fusion results with the 1/1/1/4/4 chimera suggest a difference in the receptor requirements for the two processes. The 1Δ/4/4 chimera failed to mediate cell fusion despite being expressed at high levels at the cell surface (Fig. 3A and B). This result was consistent with the inability of 1Δ/4/4 to bind HSV-1 gD and an inability to mediate virus entry (Geraghty *et al.* 2001).

Taken together, the results with the nectin-1α chimeras indicate that like virus entry, efficient cell fusion requires an intact V-like domain. The domains of nectin-1α other than the V-like domain, the two C-like extracellular domains and the TM and CT, are not required for efficient cell fusion.

Nectin-1 mutants promote trans interactions and virus entry

Because the specific nectin-1α TM and CT were not necessary for efficient cell fusion, we next sought to determine whether any TM or CT was required for cell fusion. The nectin-1cyt^{-} mutant was created to examine the importance of the nectin-1α CT to cell fusion (Fig. 1C). The entire CT in nectin-1cyt^{-} was deleted except for the four Arginine anchor just after the TM. To examine the importance of the nectin-1α TM and CT for cell fusion, we constructed a nectin-1α molecule lacking those regions but still tethered to the outer leaflet of the plasma membrane via a GPI anchor. This mutant, nectin-1gpi, was created using polymerase chain reaction (PCR) to replace the TM and CT of nectin-1α with the 29 aminoacid-GPI-addition sequence from decay-accelerating factor (DAF) (Zhou *et al.* 1997) (Fig. 1C). To further examine whether a link to the plasma membrane was required for cell fusion, we constructed a nectin-1α mutant lacking the entire TM and CT domains, nectin-1tmcyt^{-} (Fig. 1C).

Nectin-1gpi expression was detected at the surface of transfected CHO K1 cells by CELISA using an anti-nectin-1 mAb and the expression was comparable to wild-type nectin-1α (Fig. 4A). We treated CHO K1 cells expressing nectin-1gpi or nectin-1α with phosphatidylinositol phospholipase C (PIPLC), which cleaves GPI-anchored proteins from the surface of cells, to verify that nectin-1gpi was attached to the plasma membrane by a GPI anchor. As depicted in Figure 4A, PIPLC treatment reduced cell-surface expression of nectin-1gpi approximately 95% while the cell-surface expression of nectin-1α was not reduced.

Since nectin-1α is a cell-surface adhesion molecule, we investigated whether the nectin-1gpi mutant was capable of undergoing the trans interactions associated with cell adhesion. Trans interactions between molecules on adjacent cells cause protein accumulation at areas of cell-cell contact (Takahashi *et al.* 1999;Struyf *et al.* 2002b;Yoon and Spear 2002;Yoon *et al.* 2003). Indirect immunofluorescence analysis of B78H1 cell lines expressing nectin-1gpi or nectin-1α using an anti-nectin-1 mAb demonstrated increased staining at areas of cell-cell contact (Fig. 4B). B78H1 cells do not express endogenous nectin-1 or any other gD receptor (Miller *et al.* 2001). Another way to demonstrate trans interactions is to measure the ability to bind a secreted form of a nectin. Nectin-1 and nectin-3 undergo a heterotypic trans interaction important for cell adhesion (Satoh-Horikawa *et al.* 2000;Fabre *et al.* 2002). For the nectin binding experiments, we used a secreted version of nectin-3 with its transmembrane domain and cytoplasmic tail replaced by the constant region of human IgG (Fabre *et al.* 2002). Cells expressing nectin-1α bound nectin-3:Fc more efficiently than a nectin-1:Fc (data not shown) (Fabre *et al.* 2002), so nectin-3:Fc was used here. When analyzed by CELISA for ability to bind nectin-3:Fc, nectin-1cyt^{-} and nectin-1gpi bound nectin-3:Fc approximately as well as nectin-1 (Fig. 5A). Taken together, these studies indicate that the nectin-1α TM and CT, and therefore an association with cytoplasmic factors such as afadin, are not required for trans

interactions. Both the B78H1 and CHO K1 cells were positive for afadin expression by indirect immunofluorescence (data not shown).

We next characterized the virus receptor properties of nectin-1cyt$^-$ and nectin-1gpi. The gD-binding activity of nectin-1cyt$^-$ and nectin-1gpi was analyzed by CELISA using a secreted form of gD, gD:Fc. The extracellular domain of HSV-1(KOS) gD was fused to the constant region of rabbit IgG to create gD:Fc (Geraghty *et al.* 2000). As shown in Figure 5B, cells expressing nectin-1cyt$^-$ and nectin-1gpi bound soluble HSV-1(KOS) gD:Fc with wild-type nectin-1α efficiency. The slight increase in gD-binding efficiency, as well as nectin-3-binding efficiency, may be explained by an increased surface expression of nectin-1cyt$^-$ and nectin-1gpi as determined by CELISA analysis using the anti-nectin-1 mAb, Prr1-PE (Fig. 5C). We further examined the ability of nectin-1cyt$^-$ and nectin-1gpi to mediate virus entry. CHO K1 cells transiently expressing nectin-1α, nectin-1cyt$^-$, nectin-1gpi, or control cells were inoculated with an HSV-1(KOS) isolate capable of expressing β-gal upon virus entry, HSV-1 (KOS)tk12 (Warner *et al.* 1998). Six hours later, the cells were lysed and β-gal activity was measured as an indication of virus entry. The nectin-1cyt$^-$ mutant consistently displayed entry-mediating activity slightly higher than wild-type nectin-1α, most likely due to cell-surface expression that was slightly higher than wild-type nectin-1α (Fig. 6A). The nectin-1gpi mutant also mediated virus entry at a higher level than wild-type nectin-1α while also being expressed at a higher level than wild-type nectin-1α (Fig. 6B). When corrected for cell-surface expression, both nectin-1cyt$^-$ and nectin-1gpi displayed wild-type nectin-1α activity in mediating HSV-1 entry. Identical results were obtained when cells were inoculated for a two hour period, citrate-treated to inactivate bound virus, and incubated for four further hours in cell culture media (data not shown). The lack of a nectin-1α TM and CT, and corresponding lack of signaling through the nectin-1α CT, did not influence the ability of nectin-1α to mediate virus entry. The absence of a link to the plasma membrane altogether, as occurred with the nectin-1tmcyt$^-$ mutant, resulted in undetectable virus entry (data not shown).

Cell fusion mediated by nectin-1 transmembrane domain and cytoplasmic tail mutants

To determine whether the nectin-1α TM and CT were important for cell fusion, we mixed CHO K1 cells expressing nectin-1cyt$^-$, nectin-1gpi, or nectin-1tmcyt$^-$ with cells expressing the four HSV-1 fusion glycoproteins. Both the nectin-1cyt$^-$ and nectin-1gpi mutants were expressed at the cell surface at higher levels than wild-type nectin-1α and both mutants mediated cell fusion at correspondingly elevated levels (Fig. 7A and C). The lack of a TM and CT did not diminish the fusion activity of nectin-1gpi, indicating that the absence of an interaction with cellular cytoplasmic factors did not affect cell fusion in the cell-mixing assay. Because GPI-linked proteins do not span the lipid bilayer but are tethered to the outer leaflet, our results with the nectin-1gpi mutant indicate that a membrane spanning domain was not required for efficient cell fusion with the wild-type HSV-1 fusion glycoproteins. Again, similarly to virus entry, a link to the plasma membrane was required for cell fusion because cells expressing the nectin-1tmcyt$^-$ mutant were unable to fuse with cells expressing the HSV-1 fusion glycoproteins above background levels (Fig. 7A). We were unable to detect nectin-1tmcyt$^-$ expression by CELISA probably because the mutant is secreted and not stably expressed on the cell surface. The number of CHO K1 cells expressing nectin-1tmcyt$^-$ in the fusion assay was similar to the number of CHO K1 cells expressing nectin-1α when examined by indirect immunofluorescence (data not shown).

Previously, we have shown that a GPI-linked version of gD, gDgpi, retained nearly wild-type gD function in the cell mixing fusion assay (Jones and Geraghty 2004). To our knowledge, gDgpi is the only reported fusion glycoprotein that still retains function when the TM and CT are replaced by a GPI tether. We were thus presented with a unique opportunity to examine cell fusion when both gD and nectin-1α lacked a TM and CT but were linked to the plasma

membrane via a GPI tether. Cell fusion using gDgpi occurred with nearly wild-type gD efficiency when envelope glycoprotein-expressing cells were mixed with cells expressing nectin-1α and nectin-1cyt$^-$ (Fig. 7B). A reduction in cell fusion occurred when cells expressing nectin-1gpi were mixed with cells expressing the fusion glycoproteins including gDgpi (Fig. 7B). This reduction is best documented by comparing the ratio of the mean fusion activity observed when gDgpi was used versus the mean fusion activity observed when gD was used. There was approximately equivalent expression of gD and gDgpi in the fusion experiments (Fig. 7D). The ratio of gDgpi-mediated to gD-mediated fusion for nectin-1α and nectin-1cyt$^-$ was 0.90 and 0.95, respectively, whereas the ratio of gD-mediated fusion to gDgpi-mediated fusion for nectin-1gpi was 0.50. The slight reduction in wild-type nectin-1α-dependent fusion with gDgpi, when compared to gD, agreed with our previous findings (Jones and Geraghty 2004). Our results in the cell mixing assay indicate that the gD/nectin-1 interactions required to promote cell fusion were less efficient when both membrane spanning domains were replaced by GPI links.

Syncytium formation mediated by nectin-1 transmembrane and cytoplasmic domain mutants

The cell-mixing fusion assay described above most likely measures fusion of two or a few cells. Another assay to measure cell fusion, the syncytium formation assay, involves fusion of many cells and may, more so than the cell-mixing assay, require an extensive rearrangement of actin or other cytoskeletal elements. To examine syncytium formation mediated by the HSV-1 envelope glycoproteins, we used B78H1 cells. B78H1 cells readily form syncytia when engineered to express a gD receptor such as nectin-1α (Connolly *et al.* 2003;Jones and Geraghty 2004). We created B78H1 cell lines expressing nectin-1α (CJ4E), nectin-1cyt$^-$ (cyt$^-$A3-2), or nectin-1gpi (n1gpiA) to examine the importance of the nectin-1α TM and CT in syncytium formation. The cell-surface expression of nectin-1cyt$^-$ on the cyt$^-$A3-2 cells and nectin-1gpi on the n1gpiA cells was approximately equivalent to wild-type nectin-1α when examined by flow cytometry using an anti-nectin-1 mAb (Fig. 8A and B).

To generate syncytia, the B78H1-based cell lines were transfected with plasmids expressing gB, gH, gL and either gD, gDgpi, or a control plasmid. Replicate transfections of B78H1, B78H1 CJ4E, B78H1 cyt$^-$A3-2, and B78H1 n1gpiA were fixed at 24, 48, or 72 hour times after transfection and giemsa stained. Representative syncytia are depicted in Figure 9A for the B78H1 CJ4E cells, in Figure 9B for the B78H1 cyt$^-$A3-2 cells, and in Figure 9C for B78H1 n1gpiA cells. No syncytia were observed in any transfections using the parental B78H1 cells (data not shown). The absence of the CT of nectin-1α did not influence the efficiency of syncytium formation but the absence of a TM reduced the size of the syncytia formed over time. The size of syncytia decreased incrementally with the number of GPI anchors in the assay. Syncytia formed when gDgpi, gB, gH, and gL were expressed in B78H1 CJ4E cells were approximately 25-50% smaller over time than those formed by expression of gD, gB, gH, and gL (Fig. 9A), consistent with previous results (Jones and Geraghty 2004). Syncytia induced in nectin-1gpi cells by expression of gD and the other fusion glycoproteins were approximately 25-50% smaller than syncytia formed in cells expressing nectin-1α (Fig. 9A and C). However, when fusion was induced in nectin-1gpi-expressing cells using gDgpi, the syncytia were reduced by approximately 50-75% when compared to the wild-type situation (Fig. 9A and C). These results are in agreement with our results using the cell-mixing assay (Fig. 7A and C) in that two GPI anchors present in the fusion reaction yielded the greatest reduction in cell fusion. Syncytia formed in the cells expressing nectin-1cyt$^-$ reached the same approximate size as those observed in wild-type nectin-1α-expressing cells (Fig. 9A and B) indicating that the nectin-1α CT, and the corresponding link to cellular cytoplasmic factors, were not important for cell fusion. However, a membrane spanning domain for both gD and nectin-1 were important for efficient enlargement of syncytia over time.

DISCUSSION

Based upon the results presented here, two properties of nectin-1α are critical for HSV-1 glycoprotein-induced cell fusion, an intact V-like domain and an attachment to the plasma membrane. The specific TM and CT of nectin-1α were not required for cell fusion because all chimeras that functioned in cell fusion contained either a nectin-2α or CD4 TM and CT. Further, a TM and CT were not required for nectin-1α-mediated cell fusion because the nectin-1cyt^{-} and nectin-1gpi mutants were competent for cell fusion. Our results are in agreement with a recently published report describing receptor activity for a nectin-1 mutant containing a GPI link (Gianni *et al.* 2004). We further extend the previous results to quantify virus entry, cell fusion, and trans interactions compared to wild-type nectin-1α. Virus entry and trans interactions occurred with wild-type nectin-1α efficiency for both the CT deletion and GPI-linked mutants. However, cell fusion, particularly the size of syncytia formed over time, was reduced in cells expressing nectin-1gpi. This reduction was most evident when a GPI-linked version of gD was used, demonstrating an importance for membrane spanning domains in gD/nectin-1α contributions to HSV-induced cell fusion.

It was not surprising that the CT of nectin-1α was not absolutely required for cell fusion to occur. The other gD receptors, HVEM and 3-*O*-sulfated heparan sulfate, mediate cell fusion and virus entry. However, nectin-1α has multiple activities linked to its cytoplasmic tail that could influence the efficiency of cell fusion, especially through remodeling of the actin cytoskeleton. The nectin-1cyt^{-} mutant, missing the nectin-1α CT and unlikely to interact with cytoplasmic factors, had approximately wild-type fusion activity in the cell-mixing, syncytium formation, and virus entry assays. Therefore, the absence of an interaction with actin through afadin or possible actin remodeling through Rac and Cdc42 activation did not influence HSV-1 glycoprotein-induced cell fusion. It is formally possible that the cells used in this report, mouse B78H1 and hamster CHO K1, could not support human nectin-1-based Rac and Cdc42 activation. We think this is unlikely because the activation has been demonstrated in a variety of cell lines and also in mouse L cells expressing human nectin-1 (Kawakatsu *et al.* 2002;Fukuhara *et al.* 2003;Honda *et al.* 2003;Fukuhara *et al.* 2004;Fukuyama *et al.* 2004;Hoshino *et al.* 2004;Kawakatsu *et al.* 2004). Previous reports describing an inhibition of HSV-1 syncytium formation by the actin filament-disrupting drug, cytochalasin D, suggest a role for actin in fusion (Heeg *et al.* 1986;Walev *et al.* 1991;Yura *et al.* 2000). The potential pleiotropic effects of cytochalasin D on cellular physiology and virus replication, however, make it difficult to attribute the observed reduction in syncytium formation to a nectin-1-dependent influence on the actin cytoskeleton. Similar results have been seen with HIV fusion in that cytochalasin B and D inhibit fusion (Frey *et al.* 1995;Iyengar *et al.* 1998;Gallo *et al.* 2001;Pontow *et al.* 2004), but cytoplasmic tail mutants of chemokine co-receptors, predicted to reduce or abolish signaling, mediate fusion like wild-type co-receptor (Lu *et al.* 1997;Amara *et al.* 2003). Thus, rearrangement of the actin cytoskeleton is most likely important for fusion, but the genesis of the signal for rearrangement does not appear to result from signaling through the CT of viral receptors.

Our analysis of cell fusion activity of the chimeric and mutant receptors revealed similarities for receptor requirements between virus entry and cell fusion. An intact V-like domain attached to the surface of the cell was required for both virus entry and cell fusion. The 1*/2/2 chimera was deficient in mediating cell fusion consistent with a defect in virus entry (Geraghty *et al.* 2001). The 1*/2/2 chimera differs from the 1/2/2 chimera, which displayed near wild-type nectin-1α receptor activity (Geraghty *et al.* 2001) (Fig. 2), by 10 amino acid substitutions and the absence of an N-linked glycosylation site near the end of the V-like domain (Geraghty *et al.* 2001). Both 1*/2/2 and 1/2/2 bind gD equivalently to nectin-1α (Geraghty *et al.* 2001), emphasizing the idea that mutations outside of the gD binding domain can impact receptor function. These findings further indicate that cell fusion and virus entry require gD-receptor

events that are not entirely conveyed by binding of a secreted form of gD. In determining the importance of the TM and CT to nectin-1-dependent fusion, the cell mixing fusion assay appeared to more closely delineate fusion during virus entry than did the syncytium formation assay. The nectin-1cyt^{-} and nectin-1gpi mutants mediated entry of HSV-1(KOS) with wild-type nectin-1α efficiency. Both mutants also mediated cell fusion in the cell mixing fusion assay with approximately nectin-1α efficiency when wild-type gD was expressed. The nectin-1gpi mutant, however, displayed reduced ability to support syncytium formation when either gD or gDgpi was used. Syncytium formation requires the fusion of many cells and may amplify subtle differences in fusion especially over time.

Receptor requirements also differed for virus entry and cell fusion in the experiments with the nectin-1/CD4 chimeras. Previous results demonstrated that 1/1/1/4/4 was unable to mediate HSV-1 entry despite binding a secreted form of HSV-1 gD efficiently (Geraghty *et al.* 2001). The V-like domain of the 1/1/1/4/4 chimera was postulated to extend too far from the plasma membrane to mediate HSV-1 entry, even though the chimera mediated pseudorabies virus entry efficiently (Geraghty *et al.* 2001). In contrast to virus entry, the 1/1/1/4/4 chimera mediated HSV-1 glycoprotein-induced cell fusion efficiently. Therefore, if there are distance requirements for the V-like domain in relation to the plasma membrane during virus entry, those distance requirements are not important for cell fusion. Unlike the results obtained previously for virus entry (Geraghty *et al.* 2001), the ratio of relative cell fusion activity to relative cell surface expression was higher for 1/4/4 and 1/1/4/4 than nectin-1α, suggesting the chimeras have increased cell fusion activity. The chimeras may be more efficient at mediating cell fusion due to the addition of CD4 sequences or the removal of nectin-1α sequences. However, because nectin-1cyt^{-} displays fusion activity comparable to nectin-1α, it is unlikely that cytoplasmic factors binding the nectin-1α CT negatively influence fusion. Elements in the two C-like extracellular domains of nectin-1 could influence cell fusion or alternatively, the extracellular domain, TM, or CT of CD4 may have a positive influence on fusion via a mechanism not yet clear. Our interpretation of the chimera results is based upon the assumption of a linear relationship between nectin-1α cell surface expression and fusion activity. If no such relationship exists, fusion activity for nectin-1α and the 1/4/4 and 1/1/4/4 proteins may be equivalent if there is excess nectin-1α expressed on the cell surface not involved in fusion. However, higher surface expression of nectin-1cyt^{-} and nectin-1gpi yields higher fusion activity (Fig. 7A and C). Also, greater cell surface expression of nectin-1α yields larger and more numerous syncytia when the four fusogenic glycoproteins are expressed (unpublished results). The mAbs used to detect cell surface expression of nectin-1α and the chimeras may also affect the determination of fusion efficiency. CK6 recognizes a linear epitope in the V-like domain (Krummenacher *et al.* 2000) and would be expected to equivalently recognize nectin-1α and chimeras containing an intact V-like domain. Similar results were obtained with the mAb CK8 which also recognizes a linear epitope in the V-like domain, although the epitope at least partially overlaps that of CK6 (Krummenacher *et al.* 2000).

Another difference between cell fusion and virus entry was observed in experiments using nectin-2α. Nectin-2α mediates entry of HSV-1 isolates with mutations in the amino terminus of gD and not HSV-1(KOS) (Warner *et al.* 1998;Lopez *et al.* 2000). Despite the inability of nectin-2α to mediate HSV-1(KOS) entry, reproducibly low but significant levels of cell fusion occurred when cells expressing HSV-1(KOS) glycoproteins were mixed with cells expressing nectin-2α, in agreement with previously published results (Yoon *et al.* 2003;Zago and Spear 2003). The cell fusion assay could simply be more sensitive in detecting HSV-1(KOS) gD and nectin-2α interactions and resulting fusion. Alternatively, the requirements for interactions between gD and receptor for cell fusion could be less stringent compared to requirements for virus entry, such that nectin-2α bound HSV-1(KOS) gD efficiently enough to mediate cell fusion but not virus entry.

The fusion perpetrated by viral fusion proteins is characterized by many common steps such as binding receptor, bringing membranes into close proximity, and forming and enlarging fusion pores. These steps have primarily been characterized during fusion with the class I fusion proteins. The mechanism of fusion conducted by HSV glycoproteins is highly likely to involve equivalent steps to those taken by the class I fusion proteins. However, whereas class I fusion proteins are often single proteins, HSV requires four distinct proteins to conduct the steps to fusion. Thus, the functionality present in class I fusion proteins may be spread among all four HSV fusion proteins such that one or more proteins is involved in each step towards fusion. It has been proposed that gD serves largely a receptor binding role in fusion because, unlike class I fusion proteins, gD does not require a membrane spanning domain to function in fusion (Cocchi *et al.* 2004;Jones and Geraghty 2004). The results presented here indicate that the TM of gD and the TM of nectin-1α influence fusion beyond gD/receptor binding and beyond simply attaching the respective protein to the membrane. The binding of gD to nectin-1 occurs equivalently regardless of how the proteins are attached to the membrane. It is likely that a post-receptor-binding event is affected when both gD and nectin-1α are GPI linked. A conformational change is thought to occur after gD binds receptor, enabling the gD pro-fusion domain to interact with gB and/or gH-gL to form a “tri-partite complex” (receptor/gD/gBgH-gL) that mediates fusion (Cocchi *et al.* 2004;Zago *et al.* 2004). When both gD and nectin-1 are GPI linked, the gD conformational change may occur less efficiently, thereby reducing the level of fusion. Alternatively, the conformational change may be unaffected but the GPI anchors could cause improper localization of gDgpi/nectin-1gpi in the membrane, thereby hindering interactions necessary for complex formation and reducing fusion efficiency. The localization of gDgpi/nectin-1gpi may differ from gD/nectin-1 either because GPI-anchored proteins can be sequestered in lipid rafts or because of the enhanced membrane mobility of GPI anchored proteins. Regardless, if tri-partite complex formation is necessary for HSV fusion, understanding how gD/receptor interacts with gB/gH-gL will be key to designing strategies to block fusion.

MATERIALS AND METHODS

Cell lines and antibodies

CHO-K1 cells were provided by P. Spear (Northwestern Univ.) and were grown in F12 media supplemented with 7% fetal bovine serum and pen/strep. B78H1 cells (provided by P. Spear, Northwestern Univ.) were grown in DMEM supplemented with 7% fetal bovine serum and pen/strep.

B78H1 cells constitutively expressing nectin-1 (CJ4E), nectin-1cyt$^-$ (cyt$^-$A3-2), and nectin-1gpi (n1gpiA) were created by transfecting B78H1 cells with 1.5ug of the corresponding expression plasmid, selecting the cells with 500μg/ml G418, and sorting via flow cytometry for a population of cells expressing high levels of nectin-1 or a mutant. The cells were sorted three to four times until greater than 95% of the cells expressed the appropriate protein.

The antibodies against nectin-1 (all bind the V-like domain) were the mouse monoclonals CK5, CK6, and CK41 (provided by G. Cohen and R. Eisenberg, Univ. of Pennsylvania) (Krummenacher *et al.* 2000), and also Prr1-PE (Immunotech, Beckman Coulter). CK5 and CK6 recognize linear epitopes while CK41 and Prr1-PE (also known as R1.302) recognize conformation dependent epitopes (Cocchi *et al.* 1998a;Krummenacher *et al.* 2000). HSV-1 gD antibody was a polyclonal rabbit anti-HSV-1 gD serum, R7 (G. Cohen and R. Eisenberg, Univ. of Pennsylvania). The biotin- and FITC-conjugated secondary antibodies were α-mouse biotin, α-rabbit biotin, α-mouse FITC, and α-rabbit FITC (Sigma).

Construction of expression vectors

Plasmids expressing HSV-1 gB (pPEP98), HSV-1 gD (pPEP99), HSV-1 gH (pPEP100), and HSV-1 gL (pPEP101) were provided by P. Pertel (Bayer Pharmaceuticals, West Haven, Connecticut) and previously described (Pertel *et al.* 2001). Other previously described plasmids include the nectin-1α expression plasmid pCJ4 (Geraghty *et al.* 2000), CD4 expression plasmid pBG53 (Geraghty *et al.* 2001), T7 RNA polymerase plasmid pT7pol (provided by P. Pertel, Northwestern Univ.) (Pertel *et al.* 2001), the plasmid expressing β-gal under the control of the T7 promoter, pG1NT7β-gal (provided by E. Berger, National Institutes of Health) (Nussbaum *et al.* 1994), and the gDgpi expression plasmid pgDgpi (Jones and Geraghty 2004). The plasmid expressing gD:Fc was pBG64 (Geraghty *et al.* 2001) and the plasmid expressing the nectin-3:Fc protein was pCFR3.a2 (provided by M. Lopez, Institut De Cancerologie Et D'Immunmunologie Marseille, France) (Fabre *et al.* 2002).

The nectin-1cyt^{-} plasmid was created by first amplifying the nectin-1α expression plasmid, pBG38, (Geraghty *et al.* 1998) with the primers CD3prim (5′CACTGCTTACTGGCTTATCG) and Prr1cyt^{-} (5′GCTCTAGACCGGCGCCGACGCAGGGCGACCAC). The PCR product was digested with *HindIII* and *BamHI* and ligated into pcDNA3 digested with *HindIII* and *BamHI*. The nectin-1 gpi mutant was created by amplifying the extracellular domain of the glycoprotein, amplifying the gpi-addition sequence of DAF, combining the two purified PCR products, and conducting PCR using the most 5′ and 3′ primers to yield the final full-length product. The nectin-1 gpi expression plasmid was constructed by amplifying pCJ4 with the primers CD3prim and Nec1gpc (5′CGTGGGCACCGGCCCGGCGC). The plasmid pDAF-12 (provided by J. White, Univ. of Virginia) (Kemble *et al.* 1993) was amplified with the primers Necgpi (5′GCGCCGGGCCGGTGCCCACGCCAAATAAAGGAAGTGGAACC) and DAFC (5′CCAACCGAAGGAAAGATG). The two PCR products were gel purified, combined, and amplified with the primers CD3prim and DAFC. The final product was digested with restriction enzymes *BstEII* and *BglII*. This product was ligated into pCJ4 digested with *BstEII* and *BglII*. The plasmid expressing nectin-1tmcyt^{-}, pBG41, was constructed by amplifying pBG38 with the primers Prr113 (5′ CGGGATCCGAATTCTGTGATATTGACCTCCACC) and BG1-2a (5′ GCTCTAGAATGGCTCGGATGGGGCTTGCG), digestion of the resulting product with *XbaI* and *BamHI*, and ligation into pcDNA3.1mychisA (Invitrogen) digested with *XbaI* and *BamHI*. To ensure appropriate construction, all newly created expression plasmids were verified by determining the DNA sequence (Davis Sequencing, Davis, CA).

Transfections

In each well of a 6-well plate, approximately 80% confluent CHO-K1 or B78H1-derived cells were incubated with 1.5μg of plasmid DNA and 5 μl of LipofectAMINE (GibcoBRL), according to the manufacturer's instructions. The cells were incubated with the transfection reagents for 6-8 hours and the transfection media was replaced with F12 or DMEM media/ 20% fetal bovine serum.

CELISA

The CELISA technique has been previously described (Geraghty *et al.* 2000;Geraghty *et al.* 2001;Jones and Geraghty 2004). The anti-nectin-1 mouse monoclonal CK6 was used at 1:500 dilution of ascites fluid; Prr1-PE at 1:10 dilution, and anti-gD R7 at 1:2000. The CELISA experiments using PIPLC were performed as described (Jones and Geraghty 2004). The primary anti-nectin-1 mAb was added at dilution described above.

To produce the gD:Fc and nectin-3:Fc protein, CHO-K1 cells were transfected with pBG64 and pCFR3.a2 respectively. Cells expressing the hybrid proteins secreted gD:Fc or nectin-3:Fc

into the culture medium. The cells were incubated in Opti-MEM containing 4% Ultra-low Ig fetal bovine serum (Invitrogen), and 48 hrs later the culture supernatant was collected. The culture supernatant was clarified by low speed centrifugation prior to use. The supernatant was used neat and after a 30 minute incubation with nectin-1- or nectin-1 mutant-expressing cells, CELISA analysis was conducted as described above.

Flow cytometry

To remove the cells from the tissue culture dishes, the cells were rocked in a 37°C incubator in a solution of PBS/4 mM EDTA. The cells were washed in FACS buffer (PBS/2% heat-inactivated fetal bovine serum) and then incubated for 10 minutes on ice in 100 μl of primary antibody diluted in FACS buffer. The cells were again washed in FACS buffer and then incubated for 10 minutes on ice in 100 μl of FITC-conjugated secondary antibody diluted in FACS buffer. The cells were washed in FACS buffer and stored on ice in a solution of FACS buffer/1.25 μg/ml propidium iodide prior to flow cytometry analysis. The antibodies used to detect nectin-1 expression were CK6, at a 1:100 dilution of ascites fluid, and FITC-conjugated anti-mouse, at a 1:100 dilution.

Virus entry assay.

CHO-K1 cells were transfected as described above and replated in 96-well plates. The next day the cells were exposed to various dilutions of HSV-1 in PBS containing calcium and magnesium. After 6 hrs, the cells were lysed and β-gal activity determined, as previously described (Montgomery 96), as a measure of viral entry. The β-gal-reporter virus used has been described previously, HSV-1(KOS)tk12 (Warner *et al.* 1998).

Cell mixing fusion assay

The assay conditions used were as previously described (Jones and Geraghty 2004). CHO-K1 effector cells were transfected with the plasmids expressing the HSV-1 fusion glycoproteins (gB, gD, gH and gL) and T7 RNA polymerase. Where appropriate, the gDgpi expression plasmid was substituted for the gD plasmid. Target CHO-K1 cells were transfected with the plasmids expressing nectin-1 (pCJ4), nectin-1gpi (pNectin-1gpi), nectin-1 deleted for the cytoplasmic tail (pNectin-1cyt^{-}), or CD4 expression plasmid (pBG53) and β-gal under control of the T7 promoter (pG1NT7β-gal). Twenty-four hours later, effector and target cells were mixed in a 1:1 ratio and co-cultivated for 18 hr. β-gal activity was quantitated using the substrate CPRG (0.7 mg/ml in PBS with 0.5% NP40) and spectrometry.

Syncytium formation assay

B78H1 CJ4E, B78H1 n1gpiA, B78H1 cyt^{-}A3-2, and B78H1 cells were transfected with plasmids expressing gB (pPEP98), gD (pPEP99), gH (pPEP100), and gL (pPEP101), or transfected with a control plasmid or pgDgpi substituted for pPEP99. At 24, 48, and 72 hours, the cells were fixed with methanol and stained with giemsa. The cells were examined on an Axiovert S100 inverted microscope at the same magnification and photographs taken using Axiovision 3 software (Zeiss) at the same exposure. Each independent experiment was blinded, 12-15 different syncytia imaged, and images depicted were deemed representative of syncytia for a particular cell line.

Indirect Immunofluorescence

B78H1 cells expressing nectin-1 (CJ4E), nectin-1gpi (n1gpiA), or control B78H1 cells were plated onto cover slips. The next day, the cells were fixed in −20°C methanol, blocked in PBS/10% normal goat serum, incubated in primary anti-nectin-1 antibody, CK41 (1:300 dilution of ascites fluid). The cells were then incubated with goat anti-mouse FITC-conjugated secondary

antibody (1:100 dilution), mounted in mounting media (40mM Tris pH 8, 75% glycerol, and 1mg/ml p-phenylenediamine). Pictures were taken on a Zeiss Axioscope.

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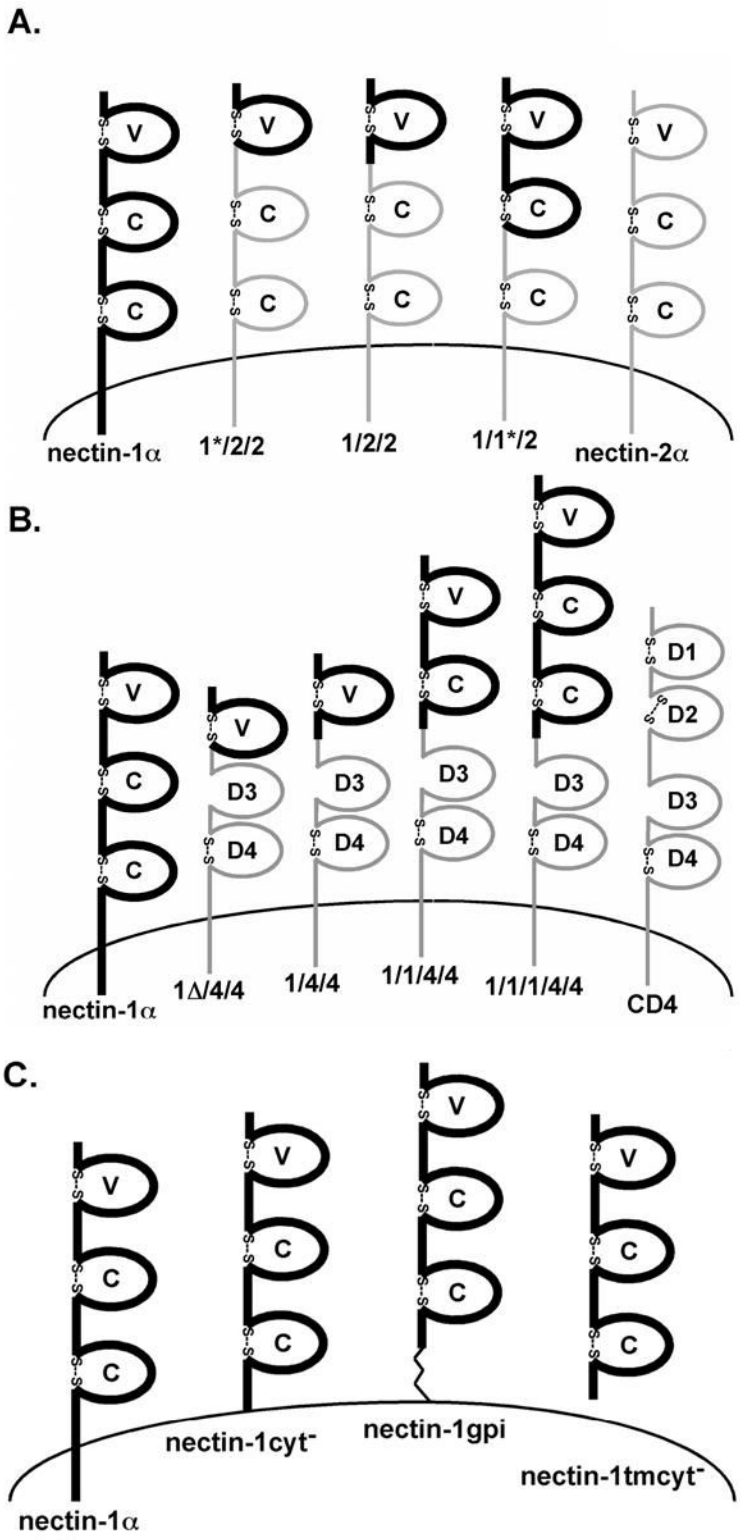

Figure 1.

Nectin-1α/nectin-2α chimeras and nectin-1α/CD4 chimeras. (A) Regions of nectin-2α (thin gray lines) were replaced with the homologous nectin-1α regions (thick black lines). The names

indicate origin of first, second and third Ig-like domains. (B) Nectin-1α sequence indicated by thick black lines and CD4 sequence indicated by the thin gray lines. (C) Nectin-1α transmembrane and cytoplasmic domain mutants. Nectin-1cyt^{-}, cytoplasmic tail deleted after arginine 382. Nectin-1gpi, transmembrane domain and cytoplasmic tail removed after threonine 354 and GPI addition sequence from DAF added. Nectin-1tmcyt^{-}, transmembrane domain and cytoplasmic tail deleted after phenylalanine 333. Amino acid numbering starts with the initiator methionine residue for the nectin-1α sequence (GenBank accession number **AF060231**). Characteristic disulfide bonds predicted for many Ig domains indicated by "S—S". V= variable-like domain, C= constant-like domain, D= Ig-like domain. Drawing not to scale. (A) and (B) reprinted from (Geraghty *et al.* 2001) with permission from Elsevier.

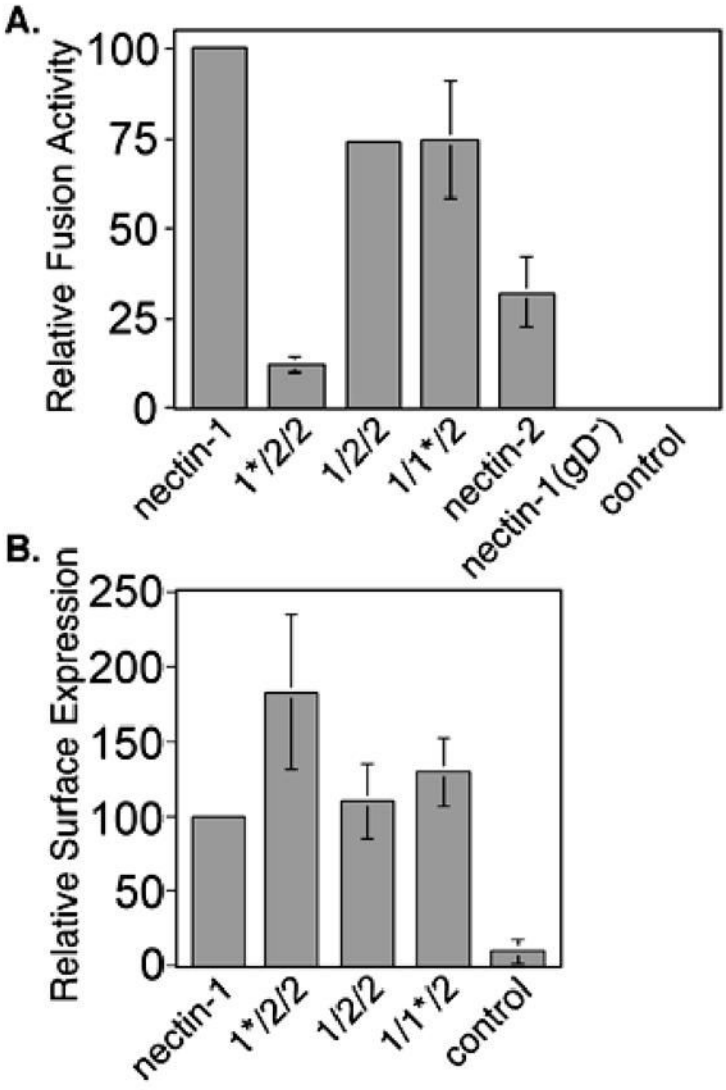

Figure 2.

Fusion activity of nectin-1α/nectin-2α chimeras in a cell-mixing fusion assay. (A) Fusion assay. CHO-K1 cells expressing gB, gD, gH, gL, and T7 polymerase, or control plasmid pCAGGS substituted for gD plasmid (gD^{-}), or all control plasmid (control) were mixed with CHO-K1 cells expressing nectin-1α or chimera and pG1NT7β-gal. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). (B). Cell-surface expression of chimeras in the fusion assay. CELISA analysis of receptor expressing cells from (A) using the nectin-1 mAb CK6. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). The absence of error bars for relative values given is due to standard deviations too small to generate a visible error bar. The fusion/CELISA experiments were performed three times and the mean values plus standard deviations for the combined relative results are depicted.

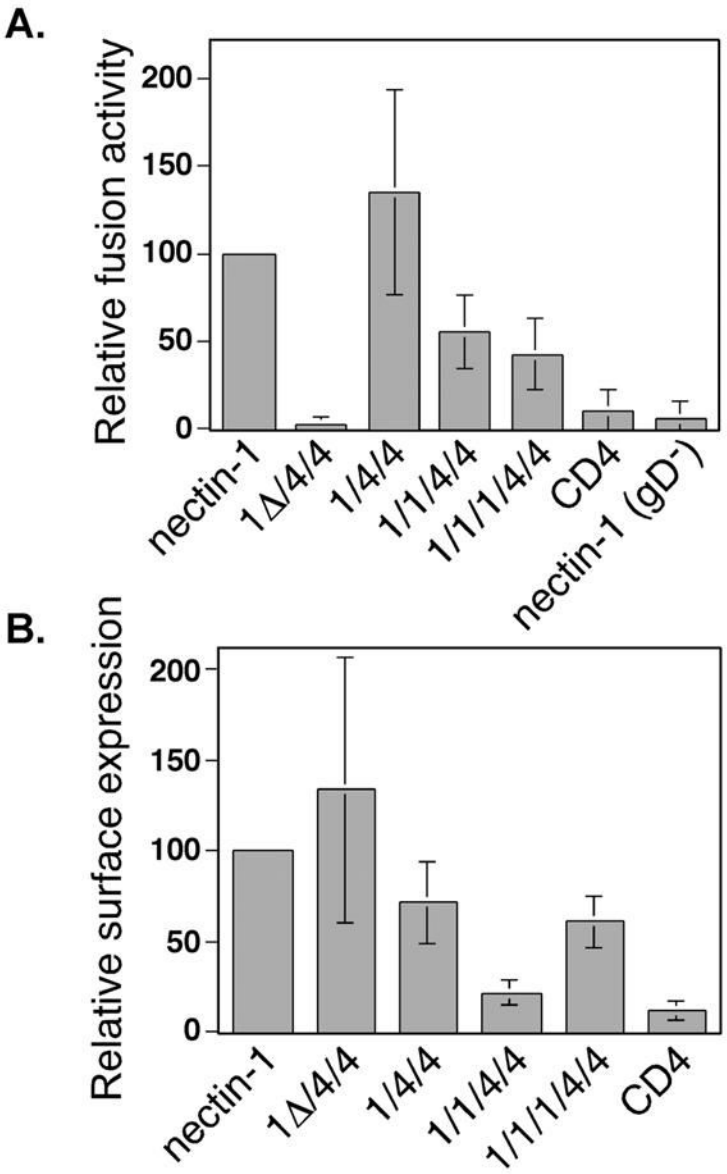

Figure 3.

Fusion activity of nectin-1α/CD4 chimeras in a cell-mixing fusion assay. (A) Fusion assay. CHO-K1 cells expressing gB, gD, gH, gL, and T7 polymerase, or control plasmid pCAGGS instead of gD plasmid (gD^{-}), or all control plasmid pCAGGS (control) were mixed with CHO-K1 cells expressing nectin-1α or chimera and pG1NT7β-gal. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). (B). Cell-surface expression of chimeras in the fusion assay. CELISA analysis of cells from (A) using the nectin-1 mAb CK6. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). The 1Δ/4/4 chimera, however, was not efficiently recognized by CK6 so another anti-nectin-1 V-like domain mAb, CK5, was used and the resulting values were expressed as a percentage of the results obtained for nectin-1-expressing cells with CK5. The fusion/CELISA experiments were performed three times and the mean values plus standard deviations for the combined relative results are depicted.

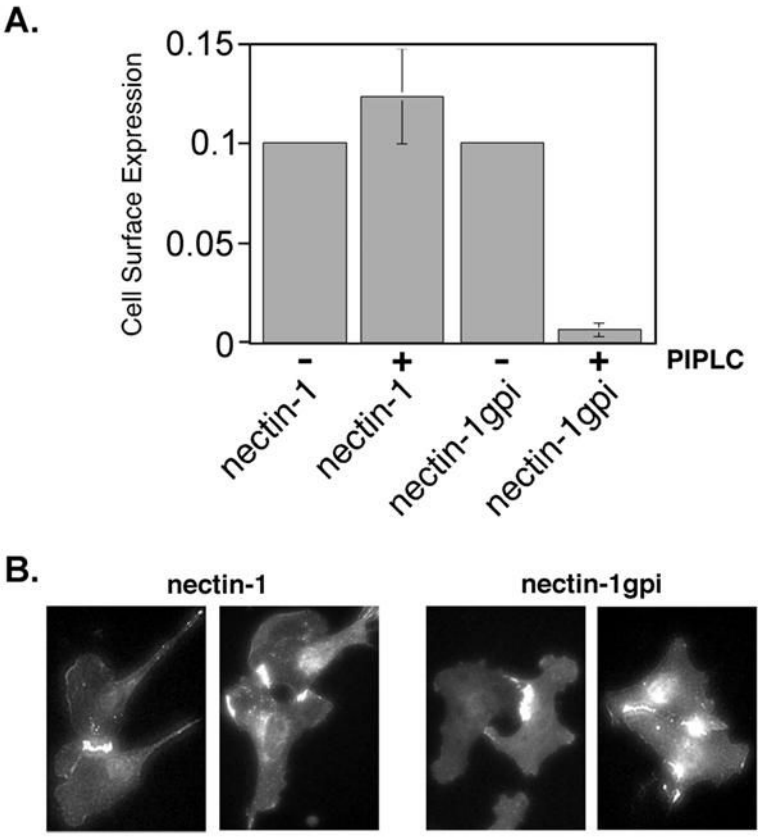

Figure 4.

Cell surface expression and PIPLC sensitivity of GPI-linked nectin-1. (A) CELISA analysis. CHO-K1 cells expressing nectin-1 or nectin-1gpi were treated with PIPLC or mock treated, incubated with mAb CK6, followed by an antibody detection system. The assays were performed in triplicate and repeated two times with similar results. The mean values plus standard deviations for a representative experiment are depicted. The absence of error bars for mean values given is due to standard deviations too small to generate visible error bars. (B) Indirect immunofluorescence of cells expressing nectin-1α or nectin-1gpi. B78H1 cells expressing nectin-1α, B78H1 CJ4E, or nectin-1gpi, B78H1 n1gpiA, were fixed with ice-cold methanol and stained with anti-nectin-1 mAb CK41 followed by an FITC-conjugated secondary antibody.

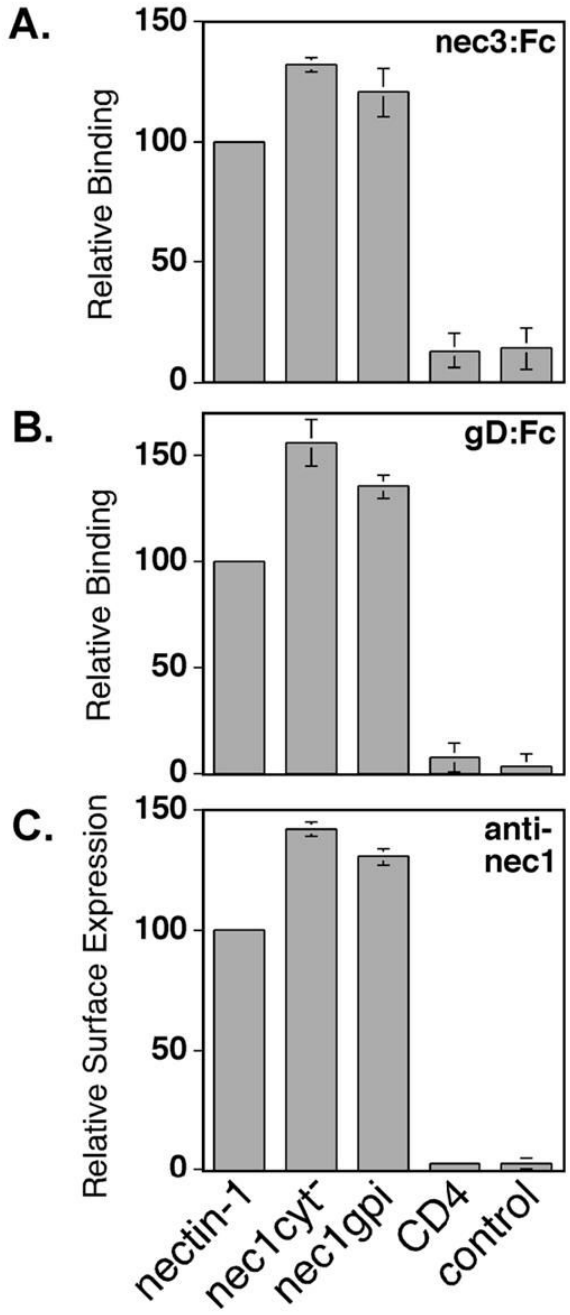

Figure 5.

Binding of nectin-3:Fc, HSV-1(KOS) gD:Fc, or an anti-nectin-1 mAb to CHO-K1 cells expressing nectin-1α and nectin-1cyt^{-}, or nectin-1gpi. CHO-K1 cells were transfected with plasmids expressing the receptors indicated, replated in 96 well plates and incubated in triplicate with nectin-3:Fc (A), gD:Fc (B), or Prr1-PE anti-nectin-1 mAb (C). The cells were then washed, fixed, incubated with biotinylated secondary antibodies, and an avidin-HRP detection system. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). The values graphed are the means and standard deviations of the relative results from three independent experiments.

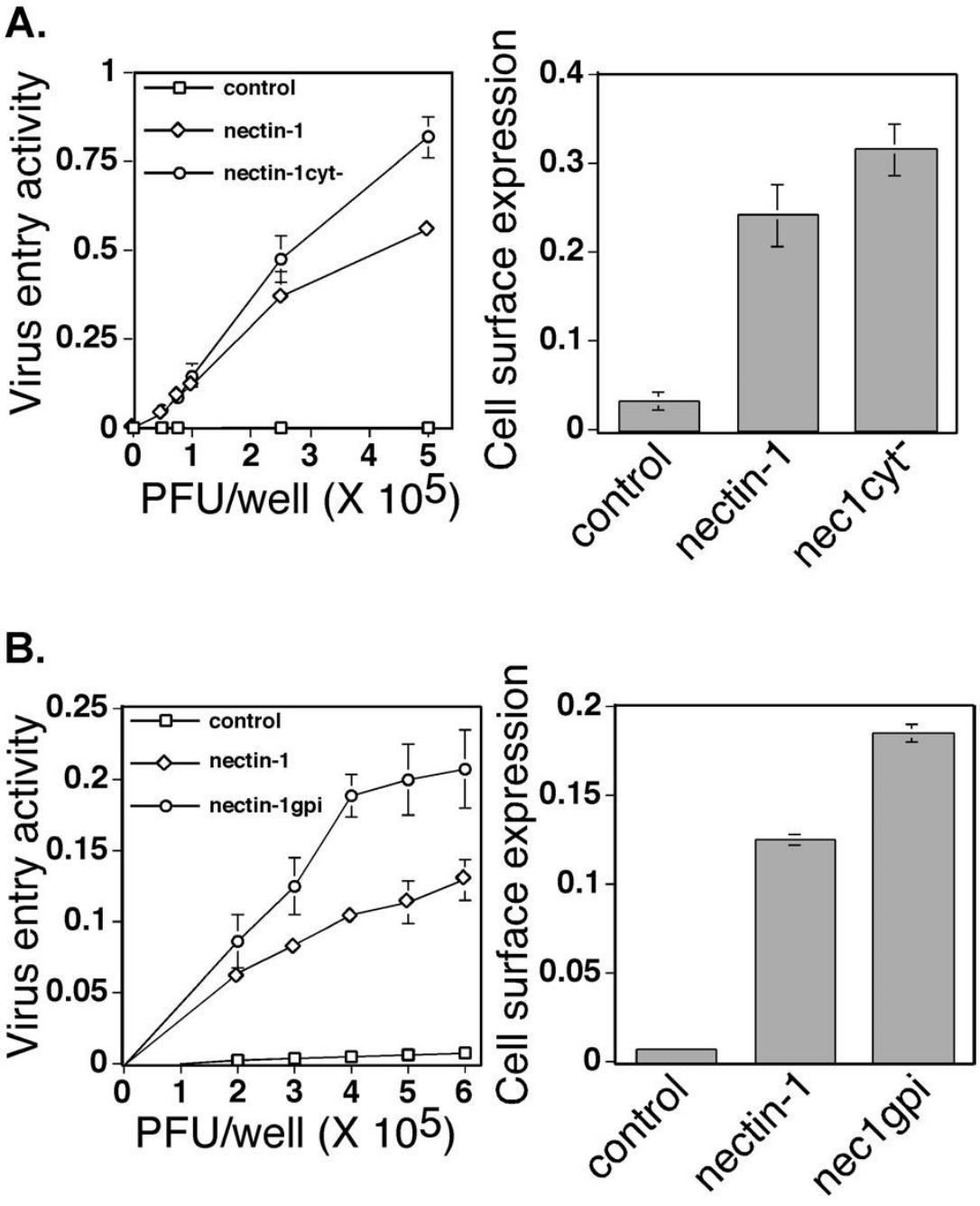

Figure 6.

HSV-1 entry activity and surface expression of the nectin-1α mutants nectin-1cyt⁻ (A) and nectin-1gpi (B). CHO-K1 cells were transfected with plasmids expressing the proteins indicated or with a control plasmid and then replated in 96 well plates. The next day the cells were inoculated with an HSV-1 recombinant expressing β-galactosidase. Six hrs after inoculation, cells were lysed and β-galactosidase activity determined as a measure of virus entry. The assays were performed in triplicate and repeated three times with similar results. The mean values plus standard deviations for representative experiments are depicted. The graphs on the right represent CELISA analysis with anti-nectin-1α mAb CK6 to detect cell surface expression of nectin-1 or mutant on transfected cells used in the entry assay. The assays

were performed in triplicate and repeated three times with similar results. The mean values plus standard deviations for representative experiments are depicted.

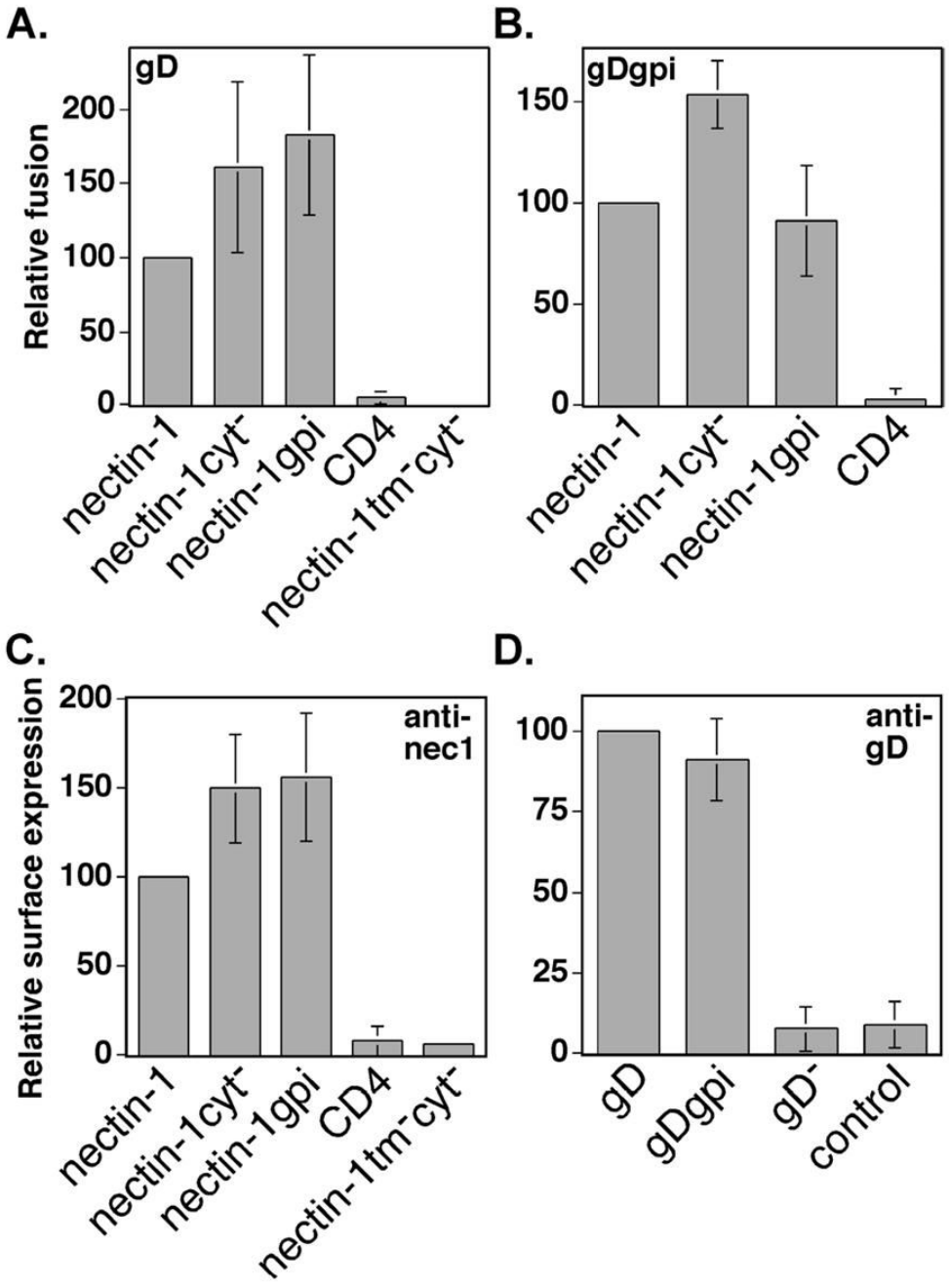

Figure 7.

Fusion activity of nectin-1α mutants using gD or gDgpi. (A) Fusion assay using wild-type gD. CHO-K1 cells expressing gB, gD, gH, gL, and T7 polymerase were mixed with CHO-K1 cells transiently expressing nectin-1α or mutant and pG1NT7β-gal. Within each experiment, all values were made relative to the value obtained for the positive control (nectin-1). (B) Fusion assay using gDgpi. CHO-K1 cells expressing gB, gDgpi, gH, gL, and T7 polymerase were mixed with CHO-K1 cells expressing nectin-1α or mutant and pG1NT7β-gal. Within each experiment, all values were expressed as a percentage of the value obtained for the positive control (nectin-1). (C) Cell-surface expression of nectin-1α mutants in the fusion assay. CELISA analysis of cells from (A) and (B) using the nectin-1 mAb Prr1-PE (anti-nec1). Within

each experiment, all values were expressed as a percentage of the value obtained for the positive control, nectin-1. (D) Cell-surface expression of gD and gDgpi in the fusion assay. CELISA analysis of cells from (A) and (B) using the rabbit polyclonal anti-gD serum R7 (anti-gD). Cells transfected with plasmids expressing gB, gD, gH, gL, and T7 (labeled gD), or gB, gDgpi, gH, gL, and T7 (labeled gDgpi), or gB, gH, gL, control plasmid pCAGGS, and T7 (labeled gD^{-}) or control plasmid pCAGGS (labeled control). Within each experiment, all values were expressed as a percentage of the value obtained for the positive control, gD. The fusion/CELISA experiments were performed at least three times and the mean values plus standard deviations for the combined relative results are depicted.

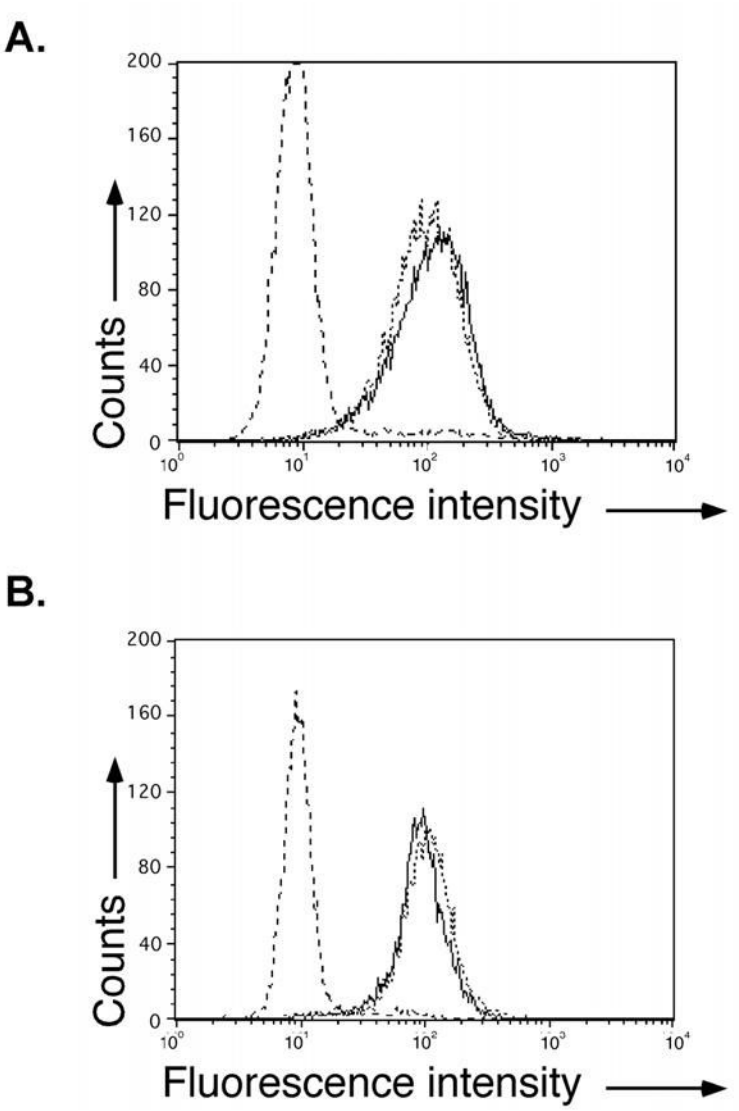

Figure 8.

Cell surface expression of nectin-1α and nectin-1α mutant cells lines using flow cytometry analysis. (A) B78H1 (dashed line), B78H1 CJ4E (solid dark line), or B78H1 cyt$^-$A3-2 cells (fine dashed line). (B) B78H1 (dashed line), B78H1 CJ4E (solid dark line), or B78H1n1gpiA cells (fine dashed line). Cell-surface nectin-1 was detected with anti-nectin-1 mAb CK6 and addition of goat anti-mouse FITC conjugated antibody. The experiment was repeated three times and results from a representative experiment are shown.

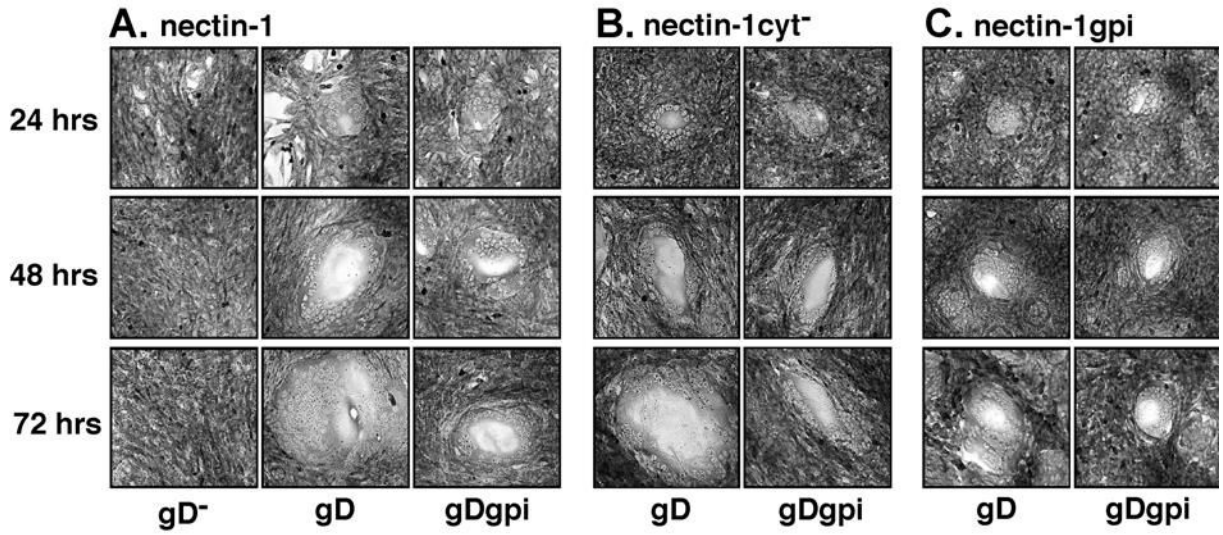

Figure 9.

Syncytium formation using wild-type envelope glycoproteins and gpi-linked mutants. Cells were transfected with plasmids expressing gB, gD, gH, and gL (labeled gD), or gB, gDgpi, gH, and gL (labeled gDgpi), or gB, gH, gL, and control plasmid pCAGGS (labeled gD⁻). At 24, 48, or 72 hours after transfection, the cells were fixed with methanol and stained with giemsa. (A) Cells expressing nectin-1α, B78H1 CJ4E (B) Cells expressing the cytoplasmic tail deletion mutant, nectin-1cyt⁻, B78H1 cyt⁻A3-2 (C) Cells expressing the GPI linked mutant of nectin-1, nectin-1gpi, B78H1 n1gpiA. Images were obtained under identical conditions. Transfections with control plasmid alone, pCAGGS, appeared identical to the results with gB, gH, gL and were not included. The results for gB, gH, gL transfections using cells in (B) and (C) were identical to those depicted in (A) and were not included.