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Mutations in the N-Terminal Domains of Nectin-1 and Nectin-2 Reveal Differences in Requirements for Entry of Various Alphaherpesviruses and for Nectin-Nectin Interactions

Frank Struyf,[1,2] Wanda M. Martinez,[1] and Patricia G. Spear[1]*

Department of Microbiology-Immunology, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611,[1] and Laboratory of Clinical and Epidemiological Virology, Department of Microbiology and Immunology, Rega Institute for Medical Research, 3000 Leuven, Belgium[2]

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Nectin-1 and nectin-2 are related molecules that can function with different specificities as entry receptors for mammalian alphaherpesviruses through interaction with viral glycoprotein D (gD). The normal function of members of the nectin family is to mediate cell-cell adhesion through homotypic and heterotypic nectin-nectin interactions in cadherin-based adherens junctions. We examined mutations in three equivalent regions of the N-terminal V-like domains of nectin-1 and nectin-2 to test the effects on entry of various alphaherpesviruses, nectin-nectin interactions, and interactions of the mutant nectins with gD. Mutations in region I previously shown to severely impair herpes simplex virus (HSV) entry activity, but not pseudorabies virus (PRV) or bovine herpesvirus 1 (BHV-1) entry, did not reduce homotypic *trans* interactions for either nectin-1 or nectin-2 or binding of nectin-3 to nectin-1. Mutations in region II, patterned after a reported single-nucleotide polymorphism in nectin-2, enhanced intracellular accumulation of both nectin-1 and nectin-2 and had a deleterious effect on all of the activities under study. Mutations in region III previously shown to reduce homotypic *trans* interactions of nectin-2 impaired the entry of PRV and BHV-1 when introduced into either nectin-1 or nectin-2, but only the nectin-2 mutation reduced HSV entry activity. Binding of nectin-1 to nectin-3 was not affected. Effects of the nectin-1 and nectin-2 mutations on interactions with gD did not necessarily correlate with entry activity of the mutant receptors. We can conclude that structural requirements for HSV entry, PRV and BHV-1 entry, and homotypic and heterotypic *trans* interactions are all different despite the previously reported ability of HSV and HSV gD to inhibit *trans* interactions.

Nectin-1 and nectin-2 are closely related cell surface molecules of the immunoglobulin superfamily and are expressed in a variety of cell types. To date, four members of the nectin family have been described, each with multiple isoforms (10, 23, 31, 32, 34), and all related to the poliovirus receptor CD155 (26). Most members of the nectin family are membrane-bound proteins with an extracellular N-terminal variable region-like (V-like) domain, two extracellular constant region-like (C-like) domains, a transmembrane region, and a cytoplasmic tail; however, a soluble isoform of nectin-1 has also been described (21).

Nectin-1 and nectin-2, but not nectin-3 or nectin-4, can function with different specificities as entry receptors for mammalian alphaherpesviruses (5, 14, 22, 39). After initial binding of the virion via viral glycoprotein B (gB) or gC to heparan sulfate on the cell surface, nectin-1 or nectin-2 can mediate viral entry through interaction with viral gD, followed by fusion of the viral envelope with the cellular plasma membrane. The amino-terminal V-like domain has the critical determinants for binding to gD (4, 17, 20) and for viral entry (3, 4, 12, 25). Nectin-1 and nectin-2 have different specificities for wild-type alphaherpesviruses. While nectin-1 shows activity as a receptor for herpes simplex virus type 1 (HSV-1), HSV-2, pseudorabies virus (PRV), and bovine herpesvirus type 1 (BHV-1) (5, 14), nectin-2 mediates entry of HSV-2 and PRV but not that of HSV-1 or BHV-1 (39). Mutant strains of HSV-1 that have single amino acid substitutions at position 25 or 27 in gD (2, 7) can enter cells via both nectin-1 and nectin-2 (14, 22, 39).

Nectins and associated cytoplasmic proteins are components of cadherin-based adherens junctions in both epithelial and nonepithelial cells (37, 38). Some membrane-bound isoforms of the nectins contain in their cytoplasmic tail a carboxy-terminal PDZ-binding domain through which they can bind afadin, which itself binds to F-actin and α-catenin (28, 30). In the presence or absence of cadherin, nectin-1 and nectin-2 can engage in homotypic *cis* and *trans* interactions (28). Both molecules also show heterotypic *trans* interactions with other members of the nectin family. Nectin-1 and nectin-2 can bind to nectin-3 (31, 34), and nectin-1 binds to nectin-4 (32).

It appears that, for both nectin-1 and nectin-2, the V-like domain is important for viral entry (3–5, 12, 20, 24, 25) and nectin-nectin interactions (11, 18, 28, 32) and that regions critical for both activities may overlap. Also, soluble forms of viral gD were able to block cell adhesion mediated by nectin-1 homotypic *trans* interactions (18, 33) and to partially inhibit the binding of soluble forms of nectin-3 and nectin-4 to nectin-1 (11).

By use of hybrid molecules constructed from members of the nectin-CD155 family that differ in functional activities, it has been shown that regions critical to HSV entry are located in

* Corresponding author. Mailing address: Northwestern University, The Feinberg School of Medicine, Department of Microbiology-Immunology, Room Searle 6-447, Mail Code S213, 320 E. Superior St., Chicago, IL 60611. Phone: (312) 503-8230. Fax: (312) 503-1339. E-mail: p-spear@northwestern.edu.

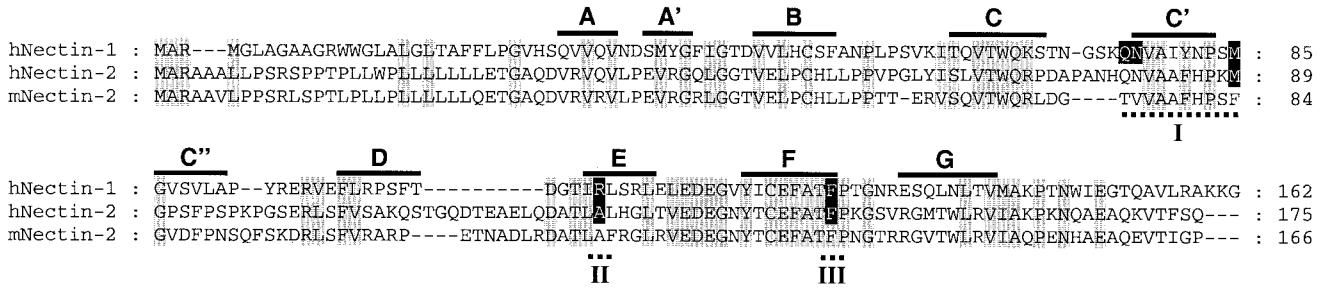

FIG. 1. Alignment of amino acid sequences in the N-terminal V-like domains of human nectin-1 (hNectin-1), human nectin-2 (hNectin-2), and mouse nectin-2 (mNectin-2). The proposed location of beta-strands (labeled A to G), based on a model of the poliovirus receptor (CD155) (15), is indicated above the sequences. Grey shading represents conserved amino acids in all three sequences. Mutated amino acids are indicated by black boxes. Dotted lines indicate the positions of the region I, II, and III mutants.

loops bounding the predicted C′ beta strand in the V domain of both nectin-1 and nectin-2 (3, 24, 25) and that regions critical to heterotypic *trans* interactions are located within a region including the C-C′-C″-D beta strands in the V domain of nectin-1 (11) (Fig. 1). For both HSV entry and heterotypic *trans* interactions, numerous amino acid substitutions within the V domain but outside of these critical regions are tolerated. Mutations in one particular region of the V domains of both nectin-1 and nectin-2 have been shown to significantly reduce HSV entry activity (24, 25) but have not been tested for effects on *trans* interactions between nectins. A mutation in a different highly conserved region of the V-like domain of mouse nectin-2 has been shown to prevent homotypic *trans* interactions (28), but no information on viral entry activity was presented. In this study, we aimed to clarify the requirements for human nectin-1 and nectin-2 engagement in homotypic and heterotypic *trans* interactions and mediation of the entry of various alphaherpesviruses. We constructed nectin-1 and nectin-2 mutants with single amino acid changes in the V-like domain of the molecules on the basis of information from previous studies of mutations that were shown to have deleterious effects on HSV entry or homotypic *trans* interactions and on a nectin-2 V domain polymorphism reported in a public database. Viral entry properties, gD binding, nectin-3 binding, cellular localization indicative of homotypic *trans* interactions, and colocalization with gD were examined by expressing the receptor molecules in Chinese hamster ovary (CHO) cells, which normally lack alphaherpesvirus entry receptors. The results show that the structural requirements of the nectin activities examined partially overlap but differ.

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MATERIALS AND METHODS

Viruses and cells. Reporter viruses used in this study included the previously described HSV-1(KOS)tk12 and HSV-1(KOS)Rid1/tk12 strains (below named HSV-1 and HSV-1/Rid1, respectively), in which the viral thymidine kinase gene was replaced with the *Escherichia coli lacZ* gene (39). HSV-1/Rid1 has an amino acid substitution at position 27 in gD (Q27P) that allows it to use nectin-2, as well as nectin-1, for entry (7, 39). Entry activity of HSV-2 was tested by using HSV-2(333)gJ$^-$ (designated HSV-2), a replication-competent reporter virus in which part of the nonessential gJ-encoding gene was replaced with a cytomegalovirus-*lacZ* cassette (25). Other β-galactosidase reporter viruses used were gH-negative PRV(Kaplan) (1), designated here PRV and provided by T. Mettenleiter (Federal Research Center for Virus Diseases of Animals, Insel Riems, Germany), and BHV-1(Cooper)TK-bgal+v4a (27), designated here BHV-1 and provided by L. Bello (University of Pennsylvania, Philadelphia).

CHO cells, provided by J. Esko (University of California, San Diego), were cultured in F12 medium with 10% fetal bovine serum, penicillin, and streptomycin.

Creation of mutant plasmids. Nectin-2 mutant plasmids were constructed from pMW20 (39), containing the coding region of nectin-2 in pcDNA3.1 (Invitrogen), by site-directed mutagenesis with the QuickChange kit (Stratagene). The same method was used to construct nectin-1 mutants from pBG38 (13), which contains the coding region of nectin-1 in pcDNA3.1. The plasmids and mutations are listed in Table 1.

Genotyping. Exon 2 of the nectin-2-encoding gene was amplified by PCR with primers 5′-CGA CTA CTT CAC TCT CTG TCC-3′ and 5′-AAG CCA AGA GAG AAA CTG ACC-3′ from genomic DNA obtained from a population of 200 healthy Caucasian individuals as described in reference 36. The 508-bp PCR products were incubated with *Bgl*I. This enzyme recognizes a restriction site that is present in the wild-type allele but absent in the A126P form of the gene, resulting in 317- and 191-bp fragments for the wild-type allele and an undigested 508-bp fragment for the A126P mutant.

Entry assays. CHO cells were grown to subconfluency in six-well tissue culture dishes and transfected with 1.5 μg of plasmid DNA and 5 μl of Lipofectamine (Gibco BRL Life Technologies). The cells were transferred to 96-well flat-

TABLE 1. Nectin-1 and nectin-2 mutations analyzed in this study

Region[a]	Nectin-1 mutant (plasmid)	Nectin-2 mutant (plasmid)	Comment; reference(s)
I	QN76-77AA;M85F[b] (pWM114)	M89F (pWM101)	Defective for HSV entry but not for PRV entry; 24, 25
II	R110P (pFS21)	A126P (pFS17)	Nectin-2 A126P mutation in dbSNP database[c]
III	F129L (pFS23)	F145L (pWM112)	F136L mutation in mouse nectin-2 reduced homophilic trans-interactions; 28

[a] Equivalent regions of nectin-1 and nectin-2, as indicated on the alignment in Fig. 1.
[b] Mutants QN76-77AA;M85F, N77A;M85F, and N77A;M85A had the same phenotype with respect to viral entry. The former mutant was used in this study.
[c] dbSNP accession number rs371523.

bottom microtiter plates 24 h after transfection. Thirty-six hours after transfection, the cells were washed once with phosphate-buffered saline (PBS) plus 0.1% glucose and 1% heat-inactivated calf serum. After addition of serial dilutions of virus prepared in PBS plus 0.1% glucose and 1% heat-inactivated calf serum, cells were incubated for 6 h at 37°C. Cells were washed three times with PBS containing 0.1 g of $MgCl_2$ per liter and 0.1 g of $CaCl_2$ per ml, incubated with PBS containing 3 mg of *o*-nitrophenyl-β-D-galactopyranoside per liter and 0.5% NP-40 (Calbiochem), and read at 410 nm in a SpectraMax 250 enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices) at various time intervals.

CELISA. The cell ELISA (CELISA) used for detection of receptor expression on the cell surface and binding of soluble gD was described in detail elsewhere (13). Briefly, CHO cells transfected as described above were subjected to a blocking step with PBS–3% bovine serum albumin (PBS-BSA) for 1 h at room temperature; this was followed by incubation with primary antibodies in 50 μl of PBS-BSA for 30 min at room temperature at the following dilutions: anti-nectin-1 monoclonal antibodies (MAbs) CK6, CK8, CK31, and CK41 (17, 18) at a 1:500 dilution and polyclonal rabbit anti-nectin-2 R146 serum (39) at a 1:500 dilution (R146 serum was preadsorbed against CHO cells at room temperature for 15 min before use). For detection of soluble gD binding, the cells were incubated with culture supernatants containing HSV-1 gD:Fc, HSV-1/Rid1 gD:Fc, or PRV gD:Fc at a concentration of 0.5 μg of gD:Fc per ml. Production of the gD:Fc hybrid proteins was done as described previously (12, 13). After washing, the cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature, incubated with biotinylated secondary antibodies against mouse immunoglobulin G (IgG) or rabbit IgG (Sigma) at a 1:500 dilution, and subsequently incubated with AMDEX streptavidin-conjugated horseradish peroxidase (Amersham) at a 1:20,000 dilution in PBS-BSA with 0.1% Tween 20. After incubation with 3,3′,5,5′-tetramethylbenzidine substrate (BioFX), the plates were read at 380 nm in a Victor ELISA reader (Perkin-Elmer).

Immunofluorescence assay. CHO cells were either singly transfected with plasmids encoding wild-type or variant forms of nectin-1 or nectin-2 or cotransfected with plasmids encoding one of the receptor variants and pCJ1 or pCJ3 (13), containing the coding region of HSV-1/Rid1 gD and HSV-1 gD, respectively, in pcDNA3.1 (Invitrogen). After transfection, cells underwent several rounds of selection with G418 (Invitrogen). Transfected cells were grown on coverslips in six-well tissue culture plates, washed once with PBS, fixed with methanol at −20°C for 5 min, and washed again with PBS. Nonspecific binding was blocked by incubation with 1% BSA in PBS for 5 min at 37°C, after which cells were washed with PBS for 5 min at room temperature. Incubation with primary antibodies occurred at 37°C for 25 min at the following concentrations: chicken polyclonal anti-HveC (nectin-1) serum (13) at 1:500, R146 rabbit polyclonal anti-HveB (nectin-2) serum (39) at 1:100, R7 rabbit anti-gD serum (16) at 1:500, anti-gD MAb DL6 (6) at 1:1,000. Cells were washed with PBS for 15 min at room temperature and incubated with the appropriate secondary antibody (Alexa Fluor 488 goat anti-chicken IgG, 488 goat anti-rabbit IgG, 568 goat anti-rabbit IgG, or 568 goat anti-mouse IgG, all from Molecular Probes, Eugene, Oreg.) at 37°C for 25 min at a 1:200 dilution. After incubation with a secondary antibody, cells were washed with PBS for 15 min at room temperature, mounted on microscope slides with Gelvatol (Air Products and Chemicals, Inc., Allentown, Pa.) containing DABCO (Sigma Chemical Co.) at 1 mg/ml, and examined on an LSM510 confocal microscope (Zeiss).

Nectin-3:Fc binding. Plasmid pCFR3, containing the ectodomain of human nectin-3 fused to the Fc portion of human IgG (32), was provided by Marc Lopez (INSERM, Marseille, France). PEAK cells (EDGE BioSystems, Inc.) were grown to 50% confluency in Dulbecco's minimal essential medium (Invitrogen) containing 10% heat-inactivated serum, penicillin, and streptomycin; transfected with pCFR3 or COS Fc Link vector control (GlaxoSmithKline); and maintained in serum-free medium (OptiMEM; Invitrogen) for harvest of the culture supernatants after 24 h. CHO cells transfected with mutant forms of nectin-1 as described in the paragraph on entry assays underwent multiple rounds of selection with G418 (Invitrogen). Approximately 10^6 cells were washed once with Versene (Dow Chemical Company), detached with trypsin-EDTA (Life Technologies), and washed once with F12 medium containing 10% heat-inactivated calf serum and once with fluorescence-activated cell sorter (FACS) buffer (PBS with 1% BSA, 10 mM HEPES, and 0.01% sodium azide). Cells were incubated for 40 min at 4°C with 250 μl of FACS buffer containing a 1:5 dilution of the supernatant of PEAK cells transfected with either pCFR3 or the vector control and anti-nectin-1 MAb CK31 (17) at 1:500. After being washed once with FACS buffer, cells were incubated with a 1:100 dilution of the secondary antibodies, fluorescein isothiocyanate-labeled goat anti-human IgG (Jackson Immunoresearch Laboratories), and allophycocyanin-labeled goat anti-mouse IgG (CalTag) for 40 min at 4°C and analyzed on a FACScalibur flow cytometer (Becton Dickinson).

Amino acid sequence accession numbers and nomenclature. The protein sequences used as references for the numbering of amino acids in this report have GenBank accession numbers AAC23798 (human nectin-1α) and AAC23797 (human nectin-2α). We refer to these as wild-type sequences. Mutations were named in accordance with published recommendations (8, 9).

RESULTS

Mutant forms of nectin-1 and nectin-2 with amino acid substitutions in the N-terminal V-like domain. Figure 1 shows an alignment of the amino acid sequences in the N-terminal V-like domains of human nectin-1 and nectin-2 with that of mouse nectin-2. Conserved regions are shaded in grey. The black shading indicates the positions of amino acids that were substituted in mutant forms of the human nectins examined in this study. These mutations are in equivalent positions of nectin-1 and nectin-2 in regions I, II, and III. These region designations are used for convenience only, to facilitate reference to mutations in equivalent positions of nectin-1 and nectin-2, and are not meant to indicate structural or functional domains.

Previous studies had demonstrated that a single amino acid substitution in region I of nectin-2 (M89F) and the equivalent substitution in nectin-1 (M85F), along with additional substitutions (QN76-77AA or N77A), could severely reduce the HSV entry activities of these receptors with no effect on PRV entry (24, 25). Plasmids expressing these mutant forms, as indicated in Table 1, were used in this study to assess the effects of these mutations on *trans* interactions important for cell adhesion. Plasmids expressing mutant nectins with amino acid substitutions in region II (R110P in nectin-1 and A126P in nectin-2) were constructed on the basis of a single-nucleotide polymorphism in the nectin-2 gene that was reported in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) and that would result in the A126P mutation. It is not clear whether this polymorphism is found in human populations, but we did not detect it in genomic DNA samples from 200 healthy Caucasians (36) by examination of PCR products for loss of a diagnostic restriction endonuclease site (data not shown; see Materials and Methods). Finally, plasmids expressing mutant nectins with amino acid substitutions in region III (F129L in nectin-1 and F145L in nectin-2) were constructed on the basis of the report that the same substitution at the equivalent position in mouse nectin-2 reduced homophilic *trans* interactions (28). Plasmids expressing the human nectin mutant forms altered in regions II and III were used to assess both viral entry activities and nectin-nectin interactions.

To determine whether the mutant nectins were expressed on cell surfaces at levels equivalent to those of the wild-type forms, CHO cells were transfected with plasmids expressing the mutant and wild-type nectins and then incubated, prior to fixation, with a panel of MAbs specific for nectin-1 or a polyclonal antiserum specific for nectin-2 in a CELISA. MAb CK31 is directed against an epitope on the second Ig-like domain of nectin-1, a domain that is unaltered in all of the mutant forms, whereas the other anti-nectin-1 MAbs bind to the V domain (17, 18). As shown in Fig. 2A, the binding of CK31 to all three mutant forms was equal to or greater than that to wild-type nectin-1, indicating that all of the mutant forms were expressed on the cell surface and that their extracellular domains were

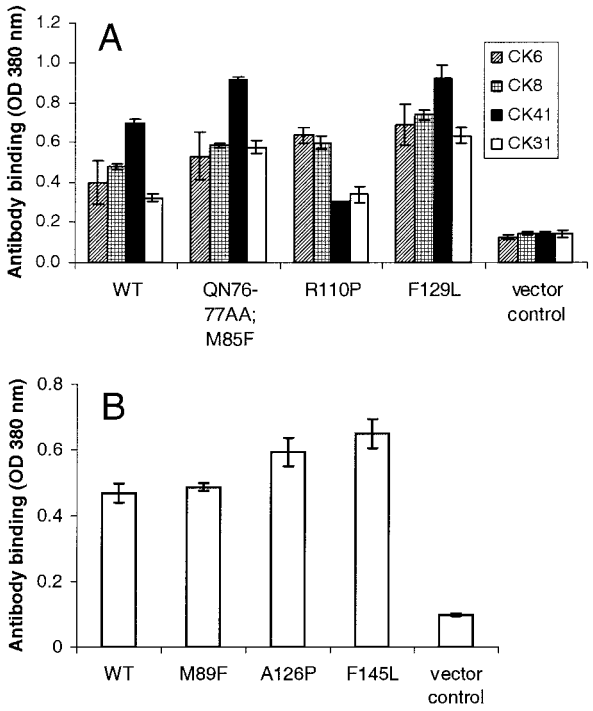

FIG. 2. Binding of antibodies to intact CHO cells expressing wild-type (WT) or mutant forms of nectin-1 (A) or nectin-2 (B). CHO cells were transfected with plasmids expressing the various forms of nectin-1 or nectin-2, replated in 96-well plates, and then incubated with anti-nectin-1 MAbs CK6, CK8, CK31, and CK41 (A) or with rabbit anti-nectin-2 R146 antiserum (B). Following washes to remove unbound antibodies, the cells were fixed and incubated with secondary antibodies and the horseradish peroxidase detection system described for the CELISA in Materials and Methods. Results of the nectin-1 and nectin-2 CELISAs are expressed as optical density (OD) at 380 nm and are means of triplicate measurements, with error bars indicating one standard deviation. The results shown are representative of three independent experiments.

accessible for binding. We reproducibly found that binding of CK31 to the F129L and QN76-77AA;M85F mutant forms was greater than that to wild-type nectin-1, suggesting that they were expressed on cell surfaces at levels higher than wild-type nectin-1 or were more accessible to the MAb. The levels of binding of the V domain-specific MAbs (CK6, CK8, and CK41), relative to that of CK31, were comparable for F129L, QN76-77AA;M85F, and wild-type nectin-1. In contrast, for R110P, binding of CK6 and CK8 was elevated and binding of CK41 was reduced relative to CK31 binding. CK6 and CK8 recognize linear epitopes in both native and denatured proteins, whereas CK41 binds to a conformational epitope that overlaps the gD-binding domain (17). These results reveal an altered conformation in the V domain for nectin-1 R110P.

Figure 2B shows that all of the nectin-2 mutant forms bound the rabbit antibodies at levels equal to or higher than that of wild-type nectin-2. Thus, none of the nectin-1 or nectin-2 mutant proteins was significantly impaired in expression or transport to the cell surface although the nectin-1 mutant form R110P appeared to assume an altered conformation. The equivalent mutant nectin-2 could also be similarly altered without a large effect on binding of the polyclonal antibodies used.

Localization of nectin-1 and nectin-2 mutant forms to cell-cell contact sites. CHO cells transfected with nectin-1 or nectin-2 were fixed with methanol and incubated with chicken polyclonal antibodies against human nectin-1 (13) or rabbit R146 antiserum against nectin-2 (39) and then with fluorescently labeled secondary antibodies. Expression of the wild-type forms of nectin-1 and nectin-2 was readily detectable, with little evidence of intracellular accumulation, except in cells well separated from other cells. There was clear localization of cell surface nectin-1 or nectin-2 to regions where adjacent transfected cells were in direct contact (Fig. 3), confirming previous results (28, 38). This localization to cell-cell contact regions correlates with the ability of the nectins to mediate cell-cell adhesion (38) and was observed only when the touching cells were both transfected, indicating that the nectin-nectin interactions are homotypic and not with endogenous forms of nectin.

Region I nectin-1 mutant QN76-77AA;M85F and nectin-2 mutant M89F, both defective for HSV entry, were indistinguishable from their wild-type counterparts in cellular localization (Fig. 3). Region II nectin-1 mutant R110P and nectin-2 mutant A126P accumulated at intracellular sites but not at cell-cell contact sites (Fig. 3), even though significant amounts were clearly present on the cell surface (Fig. 2 and 3). Region III nectin-2 mutant F145L did not accumulate intracellularly but also did not localize to cell-cell contact sites (Fig. 3), as could be expected since the homologous mutation in mouse nectin-2 (F136L) significantly reduced homotypic *trans* interactions (28). The nectin-1 counterpart F129L also did not accumulate intracellularly but did localize to cell-cell contact sites, although a significant fraction was distributed over the rest of the cell surface, in contrast to the results obtained with wild-type nectin-1. This indicates that nectin-1 mutant F129L retained some capacity to engage in homotypic *trans* interactions. For both the nectin-1 and nectin-2 mutants, three different patterns of cellular distribution were observed: similar to the wild type for the region I mutants or indicative of impaired transport from intracellular compartments for the region II mutants and impaired homotypic interactions for both the region II and III mutants, particularly for the nectin-2 region III mutant.

Heterotypic interactions between nectin-1 mutants and nectin-3:Fc. Nectin-3 can bind to nectin-1 or nectin-2 on adjacent cells (34), and this heterotypic *trans* interaction involves the N-terminal variable region-like domains of each (11, 32). The nectin-1–nectin-3 interaction, but not the nectin-1–nectin-1 or nectin-2–nectin-3 interaction, is of high enough affinity to be detected by flow cytometry for quantitation of the binding of one receptor in soluble form to cells expressing the other in membrane-bound form (32). In order to examine heterotypic *trans* interactions between the nectin-1 mutants and nectin-3, we expressed the nectin-1 mutants in CHO cells and quantitated the binding of nectin-3:Fc, a soluble form of the ectodomain of human nectin-3 fused to the Fc portion of human IgG (32). To control for differences in expression of the nectin-1 mutants in transfected cells, we gated only on cells that were positive for binding to antibody CK31 (17), a MAb specific for an epitope on the second Ig(C2)-like domain of human nectin-1 (Fig. 4A). Consistent with results shown in Fig. 2, fewer cells transfected with the R110P mutant scored positive for

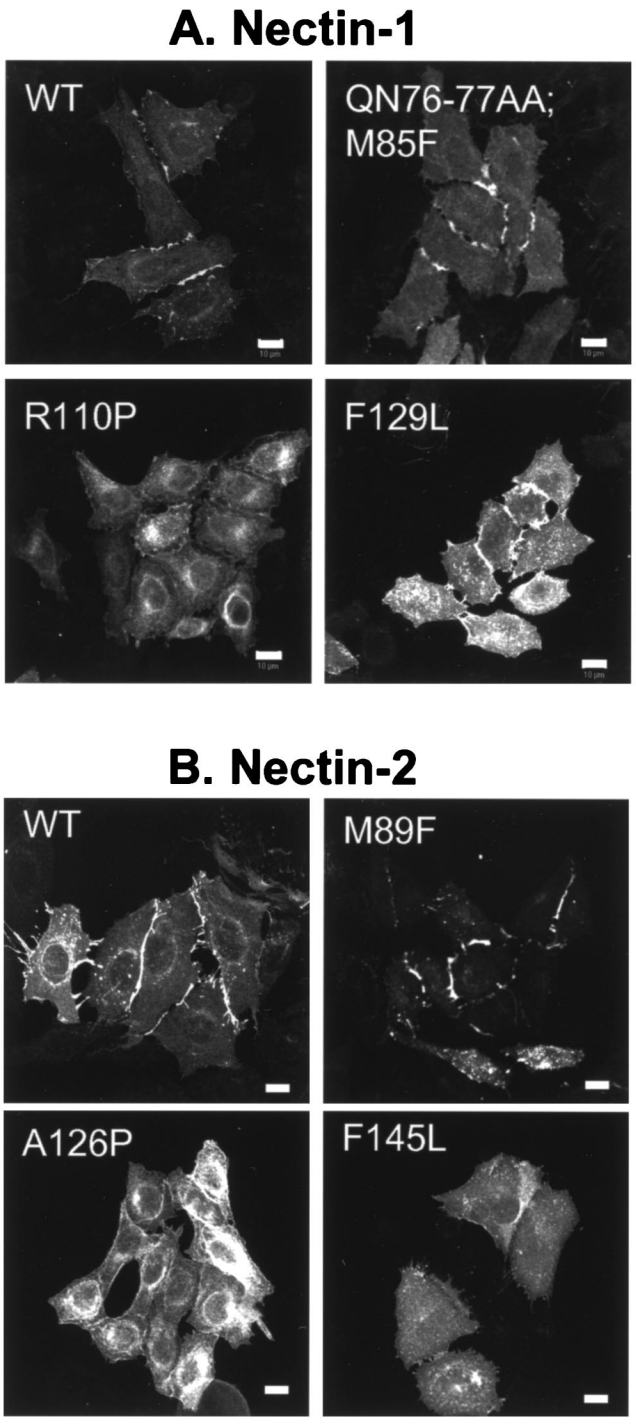

FIG. 3. Cellular localization of nectin-1 (A) and nectin-2 (B) in CHO cells transfected with plasmids expressing wild-type (WT) and mutant forms of each protein. The transfected cells were grown for a few generations in selective medium, as described in Materials and Methods, to obtain microcolonies of cells that did or did not express the relevant protein. Antibodies used were chicken anti-human nectin-1 (A) and rabbit anti-human nectin-2 polyclonal R146 serum (B), followed by Alexa 488 goat anti-chicken IgY or Alexa 488 goat anti-rabbit IgG (Molecular Devices). Bars, 10 μm.

CK31 binding, in comparison with the other mutants. The binding of nectin-3:Fc to CK31-positive cells was reduced significantly only for the R110P mutant (Fig. 4C and D).

Viral entry activities of the nectin-1 and nectin-2 mutants. Compared with the wild-type form of the receptor, region I mutants nectin-1 QN76-77AA;M85F and nectin-2 M89F have significantly reduced entry activity for HSV but retain PRV entry capacity at wild-type levels (24, 25). To assess the viral entry activities of the other nectin mutants, CHO cells, which normally lack receptors for alphaherpesvirus entry, were transfected with plasmids expressing the mutant and wild-type forms of nectin-1 or nectin-2 and then exposed to serial dilutions of the viruses indicated in Fig. 5. Because these viruses have inserts of the *lacZ* gene that can be expressed early after viral entry, the expression of β-galactosidase can be quantitated as a measure of viral entry (Fig. 5). Three viruses were tested with the nectin-2 mutants, in contrast to five with the nectin-1 mutants, because wild-type nectin-2 is not an entry receptor for wild-type HSV-1 or for BHV-1.

The region II nectin-1 mutant R110P had significantly reduced entry activity for the HSV strains and PRV and no detectable entry activity for BHV-1 (Fig. 5A), and the region II nectin-2 mutant A126P completely lacked entry activity for all three of the viruses tested (Fig. 5B). Region III nectin-1 mutant F129L equaled wild-type nectin-1 in the ability to mediate entry of HSV-1, HSV-1/Rid1, and HSV-2 but showed reduced PRV entry activity and no BHV-1 entry activity. Region III nectin-2 mutant F145L showed a clear reduction in the capacity to mediate entry of HSV-2 and was apparently unable to serve as an entry receptor for HSV-1/Rid1 and PRV. Thus, mutations in each of the three regions of nectin-1 and nectin-2 resulted in reduced viral entry activity for at least one virus tested but the effects were different for the different viruses (summarized in Table 2).

Binding of nectin-1 mutants to soluble forms of viral gD. In previous studies, we found that soluble forms of alphaherpesvirus gD consisting of the gD ectodomain fused to the Fc portion of rabbit IgG (gD:Fc) could bind specifically to cells expressing appropriate alphaherpesvirus entry receptors (12, 13, 35), with one exception. We and others have been unable to detect the binding of soluble forms of gD to cells expressing nectin-2, perhaps because of the low affinity of this interaction (22, 25). The binding of gD:Fc to mutant or hybrid forms of nectin-1 does not always correlate with entry activity of the mutant receptors. For example, mutations in nectin-1 that caused only a slight or partial reduction in viral entry activity eliminated detectable binding of HSV-1 and HSV-2 gD:Fcs whereas binding of HSV-1/Rid1 gD:Fc occurred at intermediate levels roughly proportional to entry activity (24). Also, certain nectin-1/CD4 hybrid molecules could bind to HSV-1 gD:Fc and yet failed to mediate viral entry (12).

To determine the effects of the nectin-1 mutations on binding of gD:Fc, CHO cells were transfected with plasmids expressing the wild-type or mutant receptors and then tested for the ability to bind the HSV-1, HSV-1/Rid1, or PRV form of gD:Fc. Figure 6 shows that the binding of HSV-1/Rid1 and PRV gD:Fcs correlated roughly with the viral entry activities of the receptors, whereas binding of HSV-1 gD:Fc was barely above the background levels for any of the mutant receptors despite the near-normal entry activity of nectin-1 mutant

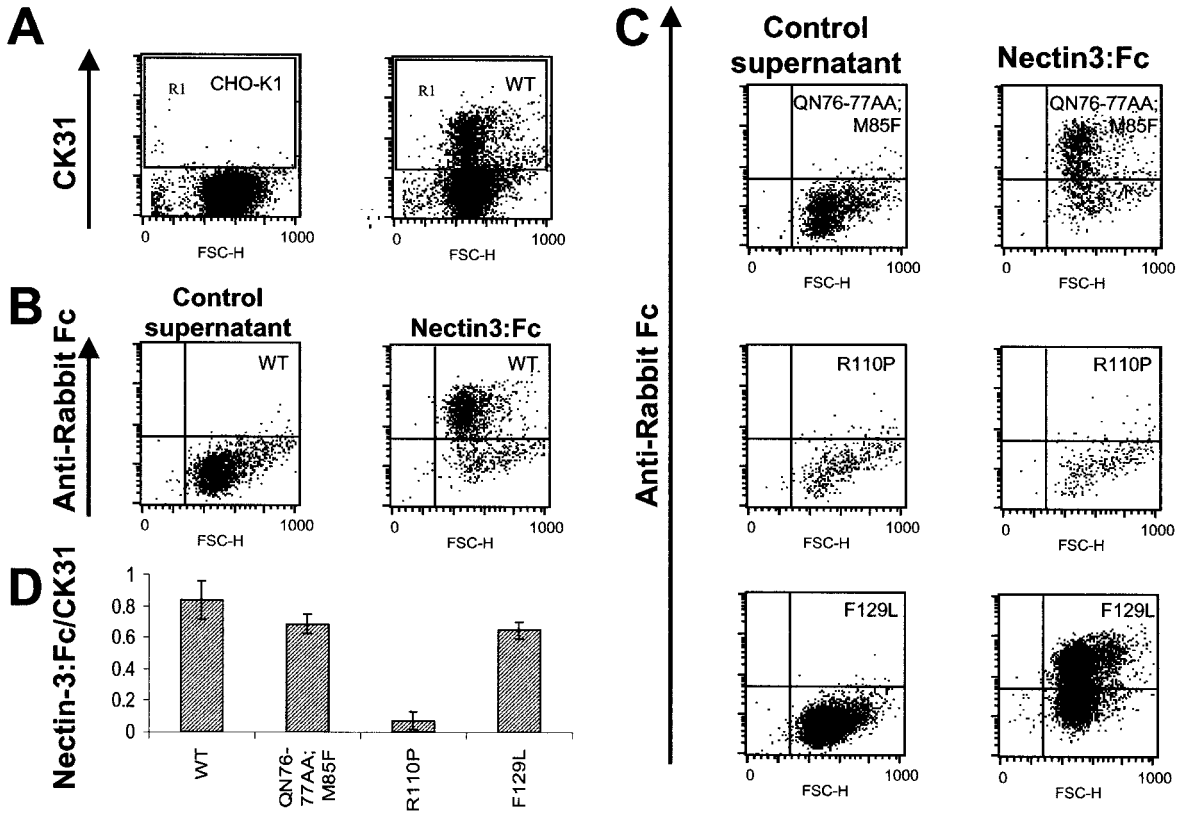

FIG. 4. Nectin-3:Fc binding to wild-type (WT) and mutant forms of nectin-1 expressed in CHO cells. CHO cells were transfected with plasmids expressing wild-type or mutant forms of nectin-1 and incubated in selective medium for several generations. The cells were then detached and incubated with a mixture of MAb CK31 (17), directed against the second C-like domain of nectin-1, and supernatant containing nectin-3:Fc (32) or control supernatant. Only cells that were positive for binding to CK31 (gate R1 in panel A) were included in the subsequent analysis of nectin-3:Fc binding. Panels B and C show nectin-3:Fc binding to cells expressing wild-type nectin-1 and mutants QN76-77AA;M85F, R110P, and F129L, respectively. Cells that failed to bind CK31 were excluded from panels B and C. Panel D shows the ratios of nectin-3:Fc-positive cells to CK31-positive cells (the number of events in the upper right quadrant divided by the number of events in the upper and lower right quadrants) for each transfected cell type. Results are representative of three independent experiments, and error bars indicate one standard deviation within the experiment shown.

F129L. We have no explanation for the anomalous behavior of HSV-1 gD:Fc but note that the single amino acid substitution responsible for the Rid mutation (Q27P) seems to eliminate it. Since the nectin-1 mutations have similar effects on the entry of HSV-1 and HSV-1/Rid1, the binding of HSV-1/Rid1 gD:Fc probably reflects more faithfully the interactions of virion-associated gD with the various receptor forms.

Coexpression of nectin-1 or nectin-2 mutants with gD. Previous studies have demonstrated that coexpression of an alphaherpesvirus gD with nectin-1 or nectin-2 in CHO cells can render the cells resistant to infection by the same or different alphaherpesviruses provided both the gD and virus could bind to the receptor (13). The expressed form of gD was shown to block virus access to the receptor without preventing receptor transport to the cell surface, as determined by CELISA. To explore the colocalization of gD and nectin-1 or nectin-2 and to determine whether gD expression has any effect on the surface localization of nectin-1 and nectin-2 and the mutant receptors, CHO cells were cotransfected with plasmids expressing the various forms of nectin-1 or nectin-2 and plasmids expressing HSV-1 gD or HSV-1/Rid1 gD. The cells were fixed with methanol and incubated with mixtures of anti-nectin-1 or anti-nectin-2 antibodies and anti-gD antibodies, followed by fluorescently labeled species-specific secondary antibodies. In separate experiments, it was shown that expression of HSV-1 or HSV-1/Rid1 gD alone resulted in diffuse distribution of gD across CHO cells with no localization at cell-cell contact sites (data not shown). Cells coexpressing wild-type nectin-1 and HSV-1 or HSV-1/Rid1 gD or wild-type nectin-2 and HSV-1/Rid1 gD showed clear colocalization of the nectin and viral gD at cell-cell contact sites but also over other parts of the cell surface (Fig. 7A and B WT columns), indicating that coexpression with gD resulted in some dispersal of wild-type nectin away from cell-cell contact sites (compare Fig. 3 and 7).

For all of the nectin-1 mutants, there was some colocalization of the mutant receptor and both HSV-1 (Fig. 7) and HSV-1/Rid1 (data not shown) gD at cell-cell contact sites. While colocalization cannot be considered a formal proof of protein-protein interaction, this finding suggests that some capacity for interaction of the mutant receptors with authentic membrane-bound gD persisted, despite the reduced entry activity of two of the mutants (QN75-76AA;M85F and R110P) for HSV-1 or HSV-1/Rid1. As with wild-type nectin-1, there was also dispersal of the nectin-1 mutants and gD over other

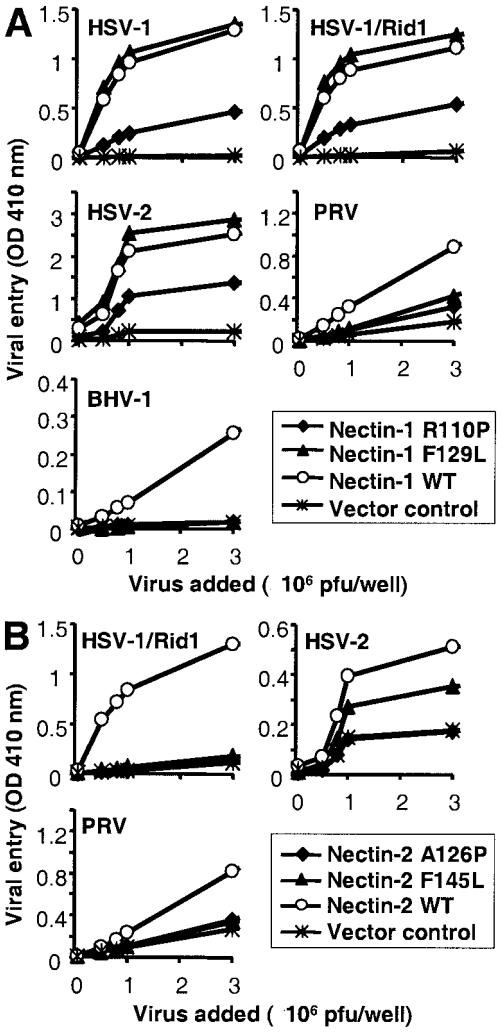

FIG. 5. Viral entry activities of wild-type (WT) and mutant forms of nectin-1 (A) and nectin-2 (B). CHO cells were transfected with plasmids expressing wild-type or mutant forms of nectin-1 or nectin-2 as indicated, exposed to β-galactosidase-expressing reporter viruses as indicated, and, after 6 h at 37°C, incubated with 3 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml and 0.5% NP-40 in PBS for quantitation of β-galactosidase activity. Viral entry is expressed as optical density (OD) at 410 nm. The values shown are means of triplicate determinations. Error bars indicate one standard deviation; error bars are not shown if the standard deviation was less than 5% of the mean.

TABLE 2. Summary of effects of nectin-1 and nectin-2 mutations

Protein	Viral entry					Nectin-nectin interactions	
	HSV-1	HSV-1 Rid1	HSV-2	PRV	BHV-1	Homotypic	Heterotypic with nectin-3
Nectin-1							
Wild type	+[a]	+	+	+	+	+	+
QN76-77AA; M85F	⇓	⇓	⇓	+	+	+	+
R110P	↓	↓	↓	⇓	⇓	⇓	⇓
F129L	+	+	+	↓	⇓	↓	+
Nectin-2							
Wild type	NA[b]	+	+	+	NA	+	ND[c]
M89F	NA	⇓	⇓	+	NA	+	ND
A126P	NA	⇓	⇓	⇓	NA	⇓	ND
F145L	NA	⇓	↓	⇓	NA	⇓	ND

[a] A plus sign indicates that activity is equivalent to that observed for wild-type nectin-1 or nectin-2. One or two downward arrows denote somewhat or drastically reduced levels of activity, respectively.
[b] NA, wild-type nectin-2 is not able to mediate entry of some of the viruses used.
[c] ND, not determined.

parts of the cell surface. The region I nectin-2 mutant M89F clearly localized primarily to cell-cell contact regions, both in cells transfected with the receptor only (Fig. 3) and in cells cotransfected with the receptor and HSV-1/Rid1 gD (Fig. 7). The gD was distributed diffusely across the cell and did not colocalize with nectin-2 M89F, indicating a lack of interaction between the two molecules. Cells cotransfected with nectin-2 mutant A126P (region II) and HSV-1/Rid1 gD showed intracellular and cell surface staining for receptor and gD, with no evident localization to cell contact sites. It is unclear from the images whether there is significant colocalization of the mutant receptor and gD. Region III nectin-2 mutant F145L exhibited more localization to cell contact sites when coexpressed with gD than when expressed alone (compare Fig. 3 and 7). This finding, coupled with colocalization of mutant F145L with gD at cell-cell contact sites, suggests a physical interaction between these two molecules (perhaps a *trans* interaction), despite the significantly reduced entry activity of the mutant receptor.

DISCUSSION

Wild-type forms of nectin-1 and nectin-2 differ in their interactions with other nectins and in their entry activities for alphaherpesviruses. Whereas nectin-1 can engage in homo-

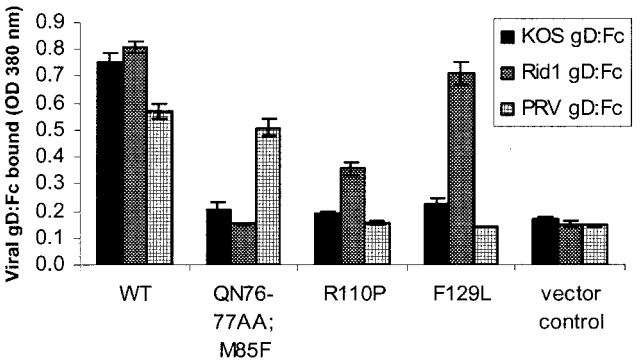

FIG. 6. Binding of viral gD:Fc to CHO cells expressing wild-type (WT) or mutant forms of nectin-1. CHO cells were transfected with plasmids expressing the various forms of nectin-1, replated in 96-well plates, and then incubated with culture supernatants containing the various forms of gD:Fc indicated. After washing away of unbound gD:Fc, the cells were fixed and incubated with an antibody specific for rabbit Fc and the horseradish peroxidase detection system described in Materials and Methods. Results are expressed as optical density (OD) at 380 nm and are means of triplicate measurements, with error bars indicating one standard deviation. The results shown were obtained with replicate cultures of the transfected cell populations analyzed for antibody binding as described in the legend to Fig. 2 and are representative of three independent experiments.

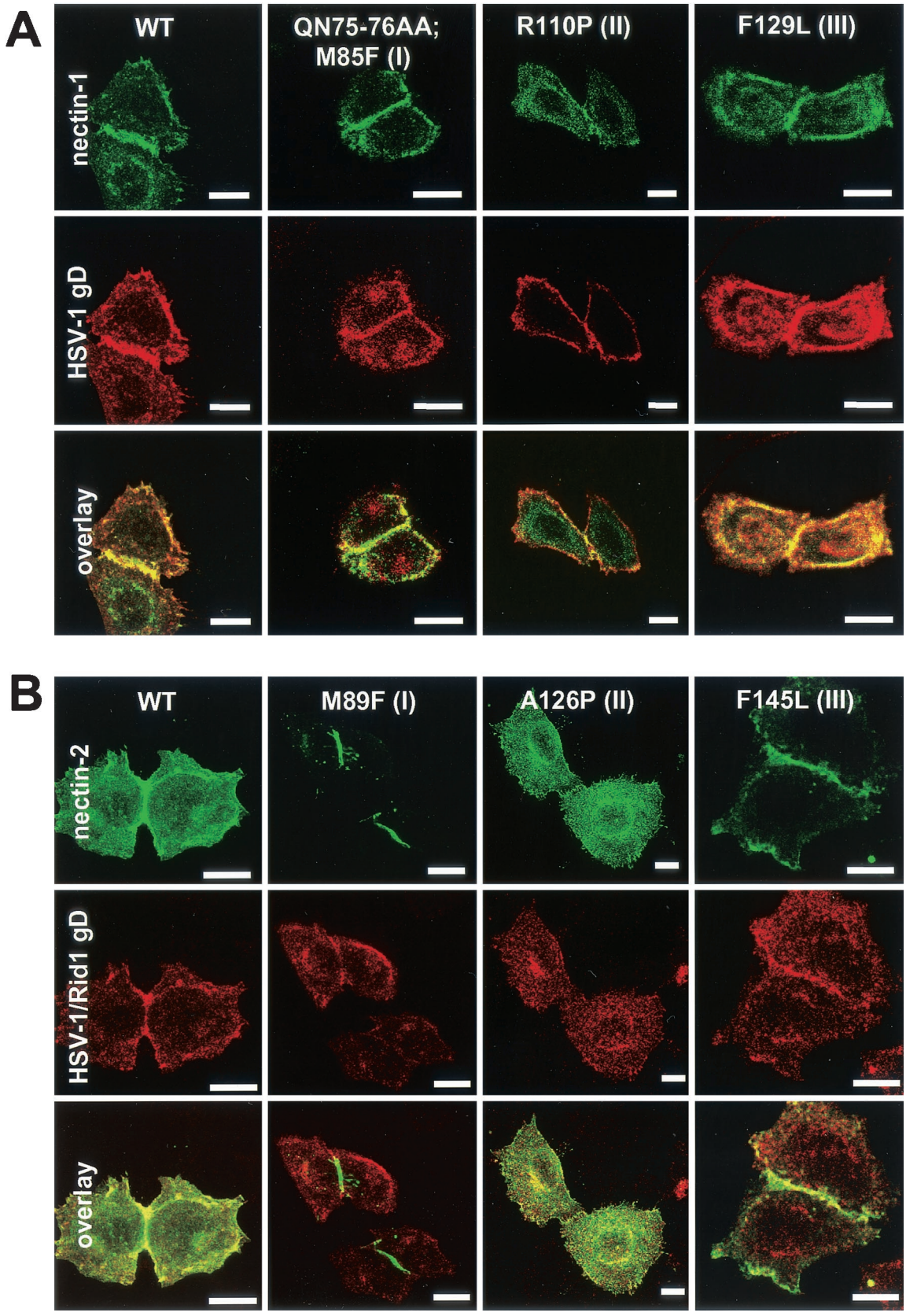

FIG. 7. Cellular localization of nectin and viral gD in CHO cells cotransfected with HSV-1 gD and various forms of nectin-1 (A) and HSV-1/Rid1 gD and various forms of nectin-2 (B). Antibodies used were chicken polyclonal IgY against human nectin-1 and rabbit polyclonal serum R7 against HSV-1 gD in panel A and rabbit polyclonal R146 serum against human nectin-2 and DL6 mouse MAb against HSV-1 gD in panel B. Overlays show colocalization of nectin-1 and HSV-1 gD and colocalization of nectin-2 and HSV-1/Rid1 gD, respectively. Bars, 10 μm. WT, wild type.

typic interactions and heterotypic *trans* interactions with both nectin-3 and nectin-4 and can mediate the entry of all of the alphaherpesviruses used in this study, nectin-2 engages in homotypic interactions and heterotypic *trans* interactions with nectin-3, but not nectin-4, and mediates the entry of HSV-1/Rid mutants, HSV-2, and PRV, but not wild-type HSV-1 or BHV-1 (see citations in the introduction). Despite these differences, we show here that the mutations introduced into three different regions of nectin-1 had, for the most part, effects similar to those of mutations introduced into the equivalent regions of nectin-2. The various activities of the nectins outlined above were affected differently by mutations in the three regions, however (Table 2).

Recently, regions of nectin-1 that are critical for HSV entry and for the binding of nectin-3 and nectin-4 were mapped by analysis of nectin-1/CD155 hybrid molecules. The region found to confer HSV entry activity on hybrid molecules encompasses amino acids 64 to 94 (C-C′-C″ beta strands and adjacent loops) of nectin-1 (3). The region found to confer binding activity for nectin-3 and nectin-4 encompasses amino acids 64 to 102 (C-C′-C″-D beta strands and adjacent loops) of nectin-1 (11). Of the mutations described here, only the region I nectin-1 mutations fall within the regions mapped in these studies. Interestingly, the region I nectin-1 mutant (QN76-77AA;M85F) had significantly reduced HSV entry activity (24) but exhibited wild-type ability to bind nectin-3:Fc (Fig. 4), indicating that the residues critical to the binding the ligands (gD and nectin-3) must differ. The region II nectin-1 mutant R110P had a reduced ability to mediate HSV entry and was impaired for nectin-3:Fc binding. It is probably irrelevant that amino acid 110 falls outside the regions mapped by hybrid analysis for HSV entry and nectin-3 binding because it seems likely that the R110P mutation affected the overall conformation of the V domain, as discussed below. The region III nectin-1 mutant F129L, also located outside the regions mapped by hybrid analysis for HSV entry and nectin-3 binding, appeared not to be impaired for HSV entry or for nectin-3:Fc binding, although it was impaired for binding to HSV-1 gD:Fc, but not for binding to HSV-1/Rid1 gD:Fc. As discussed below, several nectin-1 mutations have been shown to eliminate the binding of wild-type forms of soluble HSV-1 gD:Fc without eliminating viral entry activity. With the caveats mentioned here and below, the results presented here are consistent with the mapping studies performed by hybrid analysis and provide more detailed information about specific residues of both nectin-1 and nectin-2 that are critical for HSV entry, PRV and BHV-1 entry, and homotypic *trans* interactions of nectin-1 and nectin-2.

Region I mutations (nectin-1 QN76-77AA;M85F and nectin-2 M89F) severely reduced HSV entry activity, but not PRV entry (or BHV-1 entry for nectin-1), and appeared to have no effect on *trans*-homophilic interactions or on binding of nectin-3:Fc to nectin-1 (Table 2). On the other hand, the region III mutation in nectin-2 (F145L) significantly reduced all activities of nectin-2 (with only a partial reduction in HSV-2 entry) whereas the equivalent mutation in nectin-1 (F129L) reduced PRV and BHV-1 entry and somewhat reduced homotypic *trans* interactions but appeared to have no effect on HSV entry activities and nectin-3:Fc binding. Thus, the amino acid substitutions in region I disrupt only HSV entry for both nectin-1 and nectin-2 whereas the amino acid substitutions in region III disrupt PRV (and BHV-1) entry and homotypic *trans* interactions but have less of an effect on HSV entry, particularly for nectin-1. The model of a nectin V-like domain in Fig. 8 shows the proposed positions of these regions, indicating that their probable locations are in loops that could be adjacent. This suggests that a domain critical to HSV entry is skewed toward the loop between the C′ and C″ beta strands but overlaps the domain skewed toward the loop between the F and G beta strands that is critical to PRV and BHV-1 entry and homotypic *trans* interactions.

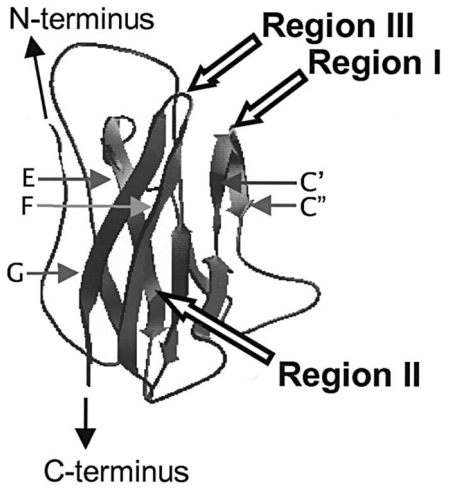

FIG. 8. Three-dimensional model of the V domain of nectin-2, modeled after the V domain of CD155 (Protein Data Bank accession number 1DGI) with SWISS-MODEL in first-approach mode and the Swiss-Pdb Viewer (29). Some of the predicted beta strands are labeled (C′, C″, E, F, and G).

There is reason to believe that the mutations in region II (nectin-1 R110P and nectin-2 A126P) caused a more severe alteration in the overall conformation of the V domain than did the mutations in regions I and III. First, the region II mutant molecules accumulated intracellularly to a greater extent than did the other mutant molecules. Although the region II mutants clearly were transported to the cell surface, as indicated by antibody binding to intact cells (Fig. 2) and residual viral entry activity (Fig. 5), results obtained with the anti-nectin-1 MAbs suggested an alteration of the conformational epitope recognized by CK41. Also, the mutations in region II had adverse effects on all of the functional tests used here that were less severe for nectin-1-mediated entry of the HSV strains but quite severe for all other activities. The effects of the region II mutations are perhaps not surprising, given that the positions altered are proposed to lie within the E beta strand (Fig. 8). The impetus for analyzing these amino acid substitutions came from finding the nectin-2 mutation in the dbSNP database. Possibly, the nucleotide substitution reported in this database resulted from sequencing error. Alternatively, the substitution exists in human populations even though we were unable to detect it in DNA samples from 200 European subjects.

Alphaherpesviruses that can enter cells via interactions with a particular cell surface receptor have, in general, been shown to express a form of gD capable of binding to this receptor. In

fact, gD is clearly the viral ligand for the entry receptors identified to date (4, 13, 19, 20, 35, 39). In these cited studies, various forms of gD were shown to bind to membrane-bound or truncated soluble receptor forms, with the exception of nectin-2. Mutant forms of nectin-1 have been identified, however, that retain at least partial viral entry activity for all HSV strains and exhibit partial binding of HSV-1/Rid1 gD:Fc and no detectable binding of HSV-1 or HSV-2 gD:Fc (24). There is no explanation for these findings, but they have been confirmed here, as shown in Fig. 6. Unfortunately, there is no good way of assessing the ability of authentic gD in virions to interact with the membrane-bound mutant receptors because binding of virus to cells is mediated by other glycoproteins interacting with heparan sulfate.

Confocal microscopy of cells expressing membrane-bound forms of both gD and wild-type or mutant forms of the nectins provided another approach by which to assess gD-receptor interactions. From the results presented in Fig. 7, we can conclude that there are interactions between wild-type nectin-2 and HSV-1/Rid1 gD whereas none were evident between the region I mutant and gD, consistent with the loss of HSV entry activity. Some interactions between the region III mutant and HSV-1/Rid1 gD were also evident, despite the loss of entry activity. The images could be misleading and, in any event, are in no way quantitative. It is also possible that weak interactions detectable by microscopy would not be sufficient for viral entry. In the case of nectin-1 and the nectin-1 mutants, all forms of receptor exhibited some colocalization with HSV-1 and HSV-1/Rid1 gD, even the region I mutant most impaired for HSV entry. In previous studies, we showed that wild-type nectin-1, when coexpressed with HSV-1 gD, remains detectable on the cell surface but loses the ability to bind to soluble HSV-1 gD:Fc (13). This indicates that cell-associated gD can block the gD-binding domain of nectin-1 by *trans* interactions between cells, *cis* interactions within the same cell, or both. A similar conclusion was drawn on the basis of results showing that coexpression of HSV-1 gD and nectin-1 reduced the cell surface binding of an anti-nectin-1 MAb specific for an epitope overlapping the gD-binding domain while enhancing the binding of another MAb specific for one of the C-like domains (18). All of these results, taken together, suggest that the colocalization of gD and nectin evident from confocal microscopy are indicative of gD-nectin interactions relevant to viral entry, at least for the wild-type receptors. Some of the mutations may impair entry without completely eliminating colocalization detectable by microscopy.

Our results indicate that structural requirements of the nectins for HSV entry, PRV and BHV-1 entry, and homotypic and heterotypic *trans* interactions are all different despite the evidence suggesting overlap in these various contact sites. Our results also define more precisely the specific regions of the nectins that are critical to entry of the different alphaherpesviruses and to homotypic *trans* interactions (Table 2). This information could, at least theoretically, be exploited in the design of antiviral agents that target the entry process while interfering as little as possible with the normal cellular functions of the nectins.

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