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Prominent Role of the Ig-like V Domain in *trans*-Interactions of Nectins

NECTIN3 AND NECTIN4 BIND TO THE PREDICTED C-C′-C″-D β-STRANDS OF THE NECTIN1 V DOMAIN*

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Nectins form a family of integral molecules that belong to the immunoglobulin superfamily. Their ectodomain is made of three Ig-like domains (V, C, C). This family comprises at least five members, namely nectin1, -2, -3, -4, and poliovirus receptor (PVR), that are involved in different physiological and pathological processes. (i) Nectins are adhesion molecules localized at adherens junctions in epithelial cells. (ii) Some nectins act as poliovirus or α-herpesvirus receptors (nectin1). (iii) Nectin1 mutations are involved in orofacial developmental abnormalities in humans. Adhesion properties of nectins are mediated by Ca^{2+}-independent homophilic and heterophilic processes through ectodomain *trans*-interactions. We have described a nectin *trans*-hetero-interaction network: nectin3 binds to nectin1, nectin2, and PVR; nectin1 also binds to nectin4. In the present study we compared the affinities of the different *trans*-interactions mediated by nectin1. We found that the K_D of nectin1/nectin3 and nectin1/nectin4 interactions is 1 and 100 nM, respectively, whereas the K_D of the nectin1-mediated homophilic interaction is 1 μM. We show that nectin1/nectin3 and nectin1/nectin4 *trans*-hetero-interactions were mediated through *trans* V to V domain interactions, whereas C domains contributed to increase the affinity of the interaction. Nectin3 and nectin4 share a common binding region in the nectin1 V domain: (i) nectin3 strongly competed with nectin4 binding, (ii) nectin3 and nectin4 binding to nectin1 was reduced by a number of monoclonal antibodies directed against the nectin1 V domain, and (iii) the glycoprotein D of herpes simplex virus-1 that binds to the V domain of nectin1 reduced nectin3 and nectin4 binding. Finally, using chimeric nectin1/PVR receptors where PVR V domain β-strands were substituted with the corresponding regions of nectin1, the nectin3 and nectin4 minimal binding region on nectin1 V domain was mapped to the C-C′-C″-D β-strands.

Adhesion molecules play a fundamental role in regulating a number of physiological processes that govern cellular functions and tissue organization. They act by mediating cell-cell or cell-matrix interactions via their extracellular domains. They are usually anchored to the plasma membrane through tight interactions with components of the cytoskeletal architecture and of cellular signaling. Recently a new family of adhesion molecules, which belongs to the immunoglobulin superfamily and is related to the poliovirus receptor (PVR),[1] has emerged and has been named nectin/PRR (poliovirus receptor-related). This family comprises at least five different members identified both in humans and rodents: nectin/PRR1, -2, -3, -4, and PVR (1–10).

Members of the nectin family carry an ectodomain made of three immunoglobulin-like domains of V, C, C types that share identities ranging from 30 to 55%. Alternative splicing originates different isoforms with long, short, or no cytoplasmic tail (for review, see Refs. 11 and 12). Nectins are expressed in several compartments such as hematopoietic, neuronal, endothelial, and epithelial tissues (13–17). Nectin1, -2, and PVR also serve as herpes simplex virus (HSV) and animal α-herpesvirus receptors. Nectin1 (also named herpes immunoglobulin-like receptor (β isoform) or herpesvirus entry mediator C (α isoform)) represents the major HSV receptor because it serves as receptor for all the HSV1 and -2 strains tested, and beyond its role for entry of free virions, it enables cell to cell spread of virus (14, 18–20).

Nectins are components of adherens junctions and co-localize with E-cadherin in polarized epithelial cells (17). The mechanisms by which nectins are stabilized at adherens junctions remain unclear. Nectins interact with the scaffold molecule afadin (4, 5, 21), which carries a PDZ domain that binds to the C-terminal cytoplasmic consensus motif (K/R)*XX*(Y/L)V of most of the nectin isoforms. In turn, afadin binds F-actin and thus links nectin to F-actin. However, this interaction does not seem to be the only prerequisite for nectin localization as some nectin isoforms (nectin1β and PVRα) that lack the C-terminal motif still localize at adherens junctions (22).[2] As exemplified by PVRα, the μ1B subunit of the adaptor complex AP-1B binds a tyrosine-based motif located in the cytosolic domain of the molecule and promotes the basolateral sorting of the molecule

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[1] The abbreviations used are: PVR, poliovirus receptor; PRR, poliovirus receptor-related; HSV, herpes simplex virus; mAb, monoclonal antibody; gD, glycoprotein D; MDCK, Madin-Darby canine kidney; FACS, fluorescence-activated cell sorting; MFI, fluorescence level intensity; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt.

[2] N. Reymond, unpublished results.

(22). Different reports demonstrate that nectins are Ca^{2+}-independent homophilic adhesion molecules and that this property contributes in part to their specific localization at cell to cell adherens junctions (5, 10, 13, 17, 23, 24). Altogether these data point to multiple mechanisms that contribute to nectin localization and hence to their functions. Recently a new feature in nectin *trans*-interactions has emerged: nectins display heterophilic adhesion properties and bind in *trans* with other members of the family. These *trans*-hetero-interactions have been described between nectin3 and nectin1 and also between nectin3 and nectin2 both in murine and in human cells (5, 10). The *trans*-hetero-interactions appear to also involve other members of the nectin family, described as a nectin *trans*-hetero-interaction network. Nectin1, nectin2, and PVR ectodomains interact with nectin3 ectodomain. Nectin4 ectodomain interacts with nectin1 ectodomain (5). Nectin1 *trans*-interactions are mediated by its V domain inasmuch as a soluble nectin1 V domain lacking the two C domains readily binds nectin4 and nectin3.

The distal V domain of nectin1 plays a central role not only in homophilic and heterophilic *trans*-interactions but also in the interaction with the envelope glycoprotein D (gD) of HSV, pseudorabies virus, and bovine herpes virus-1 (25, 26). Recent studies based on monoclonal antibody (mAb) competition or chimeric nectin1/PVR molecules identified the C-C′-C″ β-strands and intervening loops of the nectin1 V domain as the gD binding site. The A, B, D, E, F, and G β-strands do not appear to play any role in HSV entry (27, 28) In this study, we investigated the mechanisms by which nectin1 mediates heterophilic interactions with nectin3 and nectin4 and *trans*-homophilic interactions with nectin1. Using different approaches, we found the following. (i) The K_D of nectin3/nectin1 and nectin4/nectin1 interactions is 1 and 100 nM, respectively, whereas the K_D of the nectin1-mediated homophilic interaction is 1 μM. (ii) Nectin3 and nectin4 bound to a similar conformational region on nectin1 V domain, formed by C-C′-C″-D β-strands. (iii) In turn, nectin3 and nectin4 bound nectin1 through their V domains. (iv) The C domains of nectin3 and nectin4 contributed to increase the binding affinity of the *trans*-interaction with nectin1. (v) A soluble recombinant form of gD that binds to the C-C′-C″ β-strands interfered with the binding of nectin3 and nectin4 to nectin1.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Madin-Darby canine kidney II cells (MDCKII cells) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were cultivated in an air, 5% CO_2 atmosphere at constant humidity. Cells were purchased from ATCC (Manassas, VA).

Construction of hNectin1/PVR Chimeric Receptors—Chimeric primers overlapping the nectin1 and PVR sequences to be joined were synthesized both as sense and antisense. They were used separately with appropriate 5′ and 3′ primers to generate two fragments, one N-terminal and one C-terminal. The N-terminal and the C-terminal fragments were then mixed in equimolar amounts and joined through a polymerization reaction (20–25 cycles of denaturation-annealing-extension), which exploited the complementarity of the chimeric primer and the ability of the fragments to act both as primer and template for each other. The chimeras were cloned in pcDNA3.1 (Invitrogen) and sequenced for accuracy. Primer sequences were listed in a previous report (28) except for the N1-(77–102) chimera that was produced especially for this study. The N-terminal fragment was amplified with primer PVR5Nhe (GATT GCTA GCAT GGCC CGAG CCAT GGCC GCCG CGTG) and primer Mut10rev (CCTC AGCG AGGC ATTC CGCA GCTC GGGC CGCA GGAA TTCC ACAC GCTC). The C-terminal fragment was obtained with primer Mut10forw (antisense from Mut10rev) and primer PVR3Hind (ATTA AAGC TTCA CCTT GTGC CCTC TGTC TGTG G). The final product was obtained with primer PVR5Nhe and PVR3Hind.

DNA Transfection—MDCKII cells (2×10^5) were plated on 60-mm Petri dishes. After 12 h of incubation, cells were transfected with 2 μg of the appropriate cDNA expression plasmids by using FuGENE™ 6 reagent according to the manufacturer's recommendations (Roche Diagnostics). The cells were cultivated for 1 day, and the medium was replaced. Cells were selected in the presence of 1 mg/ml G418 to establish stable transfectants and sorted to get cells expressing an equal amount of receptor at the cell surface.

Construction, Production, and Purification of Soluble Forms of Nectins—The nectin1, -1V, -2, -3, -3V, -4, -4V, and PVR ectodomains were fused to the Fc fragment of the human IgG1. Most of these constructions and productions have already been described (5). The V domain of nectin3 (residues 1–167) was amplified with primer SB53.5 (CAAG AATT CATG GCGC GGAC CCTG CGGC CGTC CCCG) and primer SBR3V.3 (CTTG GTAC CTAA CACA GTTA CAGT TGTG AGGG). The V domain of nectin4 (residues 1–141) was amplified with primer R4S (GCGA ATTC ATGC CCCT GTCC CTGG GAGC CGAG ATG) and primer SBR4V.AS (GCGG TACC CACT CGGA GCCG CAGC CGCG CCTG G). Both PCR products were cloned in *Eco*RI/*Kpn*I sites of the COS Fc Link (CFL) vector (SmithKline Beecham) and transfected in COS cells with FuGENE™ 6. The proteins were purified from supernatants on Affi-Gel protein A. Purification was controlled by Coomassie Blue-stained SDS-PAGE (Fig. 1). CD28-Fc and TNF-R2-Fc (TR2-Fc) proteins were used as negative controls (SmithKline Beecham).

Antibodies—The mAbs R1.302, CK6, and CK8 directed to the V domain of hNectin1 were described previously (25, 27). The anti-PVR antibody PV.404 was described previously (28). The anti-nectin4 mAbs were obtained by immunization of mice with soluble nectin4-Fc. Two positive clones (N4.40 and N4.61) were isolated by screening hybridoma supernatants on nectin4-expressing MDCKII cells.

FACS and Cell Surface Binding Analyses—Cell surface expression was measured on different MDCKII transfectants using saturating concentration of mAbs R1.302, PV.404, N4.40, and N4.61 revealed by incubation with a phycoerythrin-labeled goat anti-mouse antibody (Immunotech). Analysis of soluble nectin-Fc binding (4×10^{-7} M) on MDCK cells was carried out at +4 °C for 60 min. After incubation, cells were washed twice with phosphate-buffered saline containing 5% fetal calf serum, and specific binding was revealed after incubation with a phycoerythrin-labeled goat anti-human IgG Fc fragment antibody (Immunotech). In blocking experiments with either mAbs or nectin-Fc, biotinylated nectin-Fc was used, and binding was revealed with phycoerythrin-labeled streptavidin (Immunotech). In Fig. 7, we calculated the binding of the nectin-Fc relative to the cell surface expression level of the target nectin: Binding factor = (MFI Nectin-Fc − MFI TR2-Fc)/(MFI mAb R1.302 − MFI IgG1), where MFI is mean fluorescence intensity.

Western Blot Analysis—Interaction between denatured forms of nectin1-Fc and mAbs or nectin3-Fc and nectin4-Fc was done as follows. 10 ng of nectin-Fc was heated in SDS sample buffer (60 mM Tris-Cl, pH 6.7, 3% SDS, 2% (v/v) 2-mercaptoethanol, and 5% glycerol), separated by 7.5% SDS-PAGE, semidry-transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Boston, MA), probed with a 1 μg/ml concentration of the indicated nectin-Fc or antibodies, and visualized by ECL (Amersham Biosciences).

ELISA—A sandwich enzyme-linked immunosorbent assay was used to define the region of nectin4 recognized by the anti-nectin4 mAbs N4.40 and N4.61. Ninety-six-well trays were coated with an antibody against the human Fc fragment (Sigma) at 10 μg/ml. After saturation of wells with phosphate-buffered saline containing 1% bovine serum albumin, 10^{-9} M nectin4-Fc or nectin4V-Fc was incubated with 2.5 μg/ml biotinylated mAbs followed by streptavidin-peroxidase and One Step ABTS (Pierce). Optical density was read at 405 nm.

RESULTS

Characteristics of the Nectin3/Nectin1 and Nectin4/Nectin1 Interactions—Nectins were first described as homophilic adhesion molecules both in human and murine cells (5, 10, 13, 17, 23). Ectopic expression of nectin1, -2, -3, and -4 leads to a specific localization at cell to cell junctions and to cell aggregation *in vitro*. In a recent report we characterized the nectin *trans*-hetero-interaction network between all the known nectins (5). We described nectin3/nectin2, nectin3/PVR, nectin3/nectin1, and nectin4/nectin1 *trans*-hetero-interactions (5). Here we analyzed the affinity of *trans*-hetero-interactions between nectin3/nectin1 and nectin4/nectin1 as compared with the affinity of nectin1/nectin1 and gD/nectin1 interactions. For this purpose we used different forms of soluble nectins fused to

the Fc fragment of the human IgG1 and produced as dimers. Soluble dimeric nectins are thought to mimic *cis*-dimerization, which is a common characteristic in nectin physiology. The soluble receptors were purified by affinity chromatography to protein A, and their purity was checked by electrophoresis under reducing conditions (see "Experimental Procedures" and Fig. 1). Binding of serial dilutions of nectin3-Fc and nectin4-Fc to nectin1-expressing MDCKII (MDCK-N1) cells showed a marked difference (Fig. 2). Binding of nectin3-Fc was saturable at a concentration of 7 nM, whereas in the case of nectin4-Fc saturation was achieved at a higher concentration (250 nM). The apparent affinity, based on half-maximal binding, was higher for nectin3 binding to nectin1 (K_D = 1 nM) as compared with nectin4 binding to nectin1 (K_D = 100 nM). Under the same conditions, binding of gD(Δ290–299t) to nectin1 was saturable at 40 nM with K_D = 3 nM (Fig. 2). These results are in accordance with previously described gD/nectin1 affinity (saturation at 70 nM and K_D = 3 nM, Ref. 29) determined by ELISA. In addition we determined the apparent affinity for nectin1 homophilic interaction and found that K_D = 1 μM. These results highlight important differences between nectin *trans*-interaction affinities: we demonstrate here that homophilic interaction affinity is low. We observed that nectin3/nectin1 affinity was higher than that of nectin4/nectin1. This prompted us to carry out competitive experiments between nectin3-Fc and nectin4-Fc binding to MDCK-N1 cells. Whereas both nectins readily bound to MDCK-N1 cells (Fig. 3), preincubation of nectin3-Fc with MDCK-N1 cells inhibited by 79% the binding of nectin4-Fc (Fig. 3, *right*). Preincubation of nectin4-Fc did not inhibit nectin3-Fc binding to nectin1 (Fig. 3, *left*). Under these conditions, we controlled the level of cell surface-bound nectin4-Fc after nectin3-Fc binding. We observed a 70% reduction in nectin4-Fc level, suggesting that nectin3-Fc binding indeed displaced the cell-bound nectin4 (data not shown).

Nectin1 V Domain Regions Involved in Nectin3 and Nectin4 Interaction—The ability of nectin3 to compete with nectin4 binding to nectin1 suggests that nectin3 and nectin4 share, at least in part, a common binding site on nectin1. To further characterize nectin3 and nectin4 binding regions on nectin1, we carried out blocking experiments with a panel of anti-nectin1 mAbs (R1.302, CK6, and CK8) whose epitopes were previously mapped to the V domain (25, 27). First we further refined the nectin1 region recognized by these mAbs. For this purpose, we used chimeric nectin1/PVR receptors in which groups of β-strands and intervening loops of nectin1 V domain replace the corresponding regions in the PVRα V domain. The constructs were described in a previous study (28) (except N1-(77–102)) and are presented in Fig. 4*A* for convenience. They are named as N1 (nectin1) followed by numerals that define the nectin1 region present in the chimera. The different constructs were stably expressed in the MDCKII epithelial cell line. To ascertain that transfected MDCKII cells expressed comparable levels of each chimeric receptor, we performed FACS analysis using either the anti-nectin1 mAb R1.302 or the anti-PVR mAb PV.404 depending on the chimera tested (Fig. 5, *Expression column*). mAb R1.302 bound to the chimeras N1-(1–143), N1-(64–116), N1-(64–102), and N1-(77–102) but not to N1-(64–94), N1-(83–116), and N1-(77–94) (Fig. 4*A*). These results confirm previous data and define the C′-C″-D β-strands and the intervening loops (between residues 77 and 102) as the mAb R1.302 epitope. mAbs CK6 and CK8 recognized all the chimeras, thus making it possible to narrow the epitope to residues 83–94, which are predicted to encode the C″ β-strand (Fig. 4*A*), in accordance with the report of Krummenacher *et al.* (27). When preincubated with MDCK-N1 cells, all three mAbs inhibited nectin3 or nectin4 binding but at different levels (Fig. 4*B*). mAbs CK6 and CK8 gave similar results; they partially inhibited nectin3 and nectin4 binding (23 and 46% inhibition, respectively, with CK6), whereas mAb R1.302 was a powerful inhibitor of nectin3/nectin1 and of nectin4/nectin1 interactions (94 and 100% inhibition, respectively) (Fig. 4*B*). We also checked that mAbs were not displaced by nectin-Fc binding (data not shown). According to the results obtained with mAb R1.302, we infer that the C′-C″-D β-strands of the nectin1 V are part of the nectin3 and nectin4 binding site. The partial inhi-

FIG. 1. **SDS-PAGE analysis of the set of purified soluble chimeric nectins used in the present study.** Nectin-Fc was produced in COS cells, and proteins were purified from supernatants on an Affi-Gel protein A column. Two μg of protein were loaded, resolved under reducing conditions by 10% SDS-PAGE, and revealed by Coomassie Blue staining. *Lane 1*, nectin1-Fc; *lane 2*, nectin1V-Fc; *lane 3*, nectin3-Fc; *lane 4*, nectin3V-Fc; *lane 5*, nectin4-Fc; *lane 6*, nectin4V-Fc. Nectin1V-Fc and nectin1-Fc (*thick bands*) were already described and may correspond to variable levels of *N*-glycosylation (5).

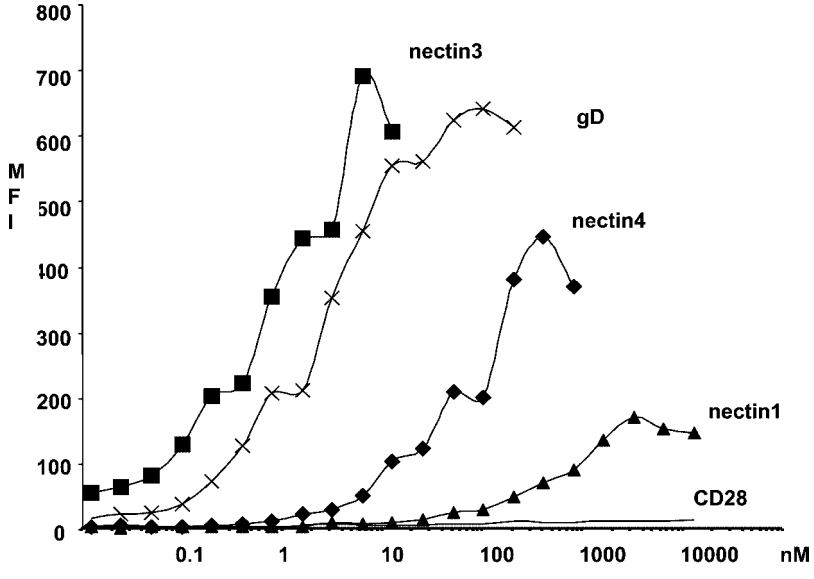

FIG. 2. **Comparison of nectin3, nectin4, nectin1, and gD binding to nectin1-expressing MDCKII cells.** MDCK-N1 cells were incubated with increasing concentrations of the different soluble proteins. Bound nectin-Fc was detected with a phycoerythrin-labeled goat anti-human IgG Fc antibody as described under "Experimental Procedures." Bound gD was detected with the mAb H170 followed by a phycoerythrin-labeled goat anti-mouse antibody. Samples were analyzed by FACS, and MFI was used to quantify cell surface binding. The results shown are representative of three independent experiments.

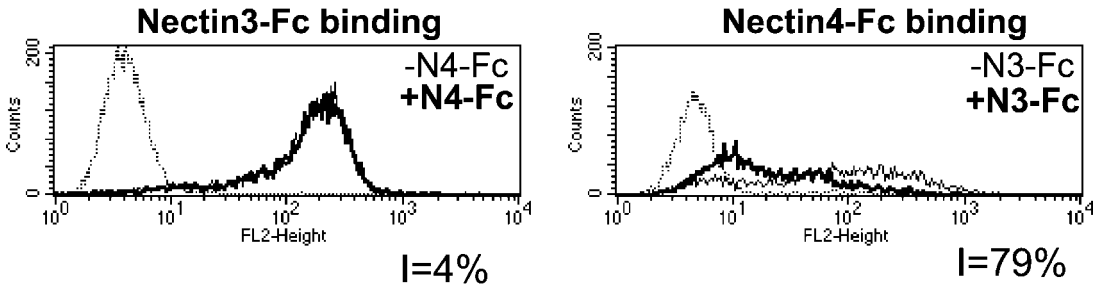

FIG. 3. **Reciprocal inhibition of nectin3-Fc and nectin4-Fc binding to MDCK-N1 cells.** Biotinylated nectin3-Fc (10 nM) (*left*) or nectin4-Fc (500 nM) (*right*) binding was performed by FACS in the presence (*black line*) or absence (*gray line*) of nectin4-Fc (*left*) or nectin3-Fc (*right*), respectively. Bound biotinylated nectin-Fc was measured using phycoerythrin-labeled streptavidin. Samples were analyzed by FACS, and MFI was used to quantify cell surface binding. *I*, inhibition.

bitions observed with mAbs CK6 and CK8 may indicate that the C″ β-strand of the nectin1 V domain does not carry full binding activity.

Glycoprotein D Interferes with Nectin3 or Nectin4 Binding to Nectin1—The V domain of nectin1 carries the HSV gD binding site (25, 26, 28). Thus, transmembrane nectin1 lacking its two C domains confers infectability to an HSV-resistant cell line, and soluble nectin1V-Fc protein blocks virus entry (25, 26). The gD binding site and the HSV entry site on nectin1 were subsequently located between residues 77–102 (predicted C-C′-C″). Binding of gD to nectin1 was inhibited by mAbs R1.302, CK6, and CK8. These data, associated with the data described in Fig. 4*A*, suggest that nectin3 and nectin4 share with gD at least part of their binding site on nectin1. To check this possibility, binding of either nectin3-Fc or nectin4-Fc on MDCK-N1 cells was performed in the presence of the soluble recombinant form of gD(Δ290–299t). We found that preincubation of gD with MDCK-N1 cells led to a 55 and 77% reduction of nectin3 and nectin4 binding levels, respectively (Fig. 4*C*, compare *white* and *black histogram bars*). The level of gD binding was reproducibly reduced after nectin3-Fc binding (20%) but not after nectin4-Fc binding suggesting that, in this case, nectin3-Fc, but not nectin4-Fc, partially displaced nectin1-bound gD (Fig. 4*C*, *gray histogram bars*). These results are in accordance with the different affinities described in Fig. 2 where the affinity of the nectin3/nectin1 interaction was higher than that of gD/nectin1, which in turn was higher than that of nectin4/nectin1.

C-C′-C″-D β-Strands of Nectin1 V Domain Carry the Nectin3 and Nectin4 Binding Activity—Results of blocking experiments by mAbs R1.302, CK6, and CK8 and by gD suggested that nectin3 and nectin4 share, at least in part, a common binding site on nectin1 (Fig. 4, *B* and *C*). Inasmuch as binding reductions may be due either to competition at the level of the binding site or to steric hindrance or conformational changes induced by the mAbs or gD, we measured the direct binding of nectin3-Fc and nectin4-Fc to MDCKII cells expressing the chimeras presented in Fig. 4*A*.

Nectin3-Fc or nectin4-Fc binding to MDCK-N1 cells is presented in Fig. 5 (*Binding column*). Interestingly nectin3-Fc, but not nectin4-Fc, bound to the MDCK-PVRα thus confirming that PVR is a ligand for nectin3 as reported previously (5) (Fig. 5). To determine the nectin1 minimal region sufficient for nectin3 or nectin4 interactions, first we ascertained that nectin3-Fc and nectin4-Fc did not interact with MDCK cells expressing the N1-(77–94) chimera (Fig. 5). This suggests that the substitution of the C′-C″ β-strands of PVRα with those of nectin1 is sufficient to abrogate nectin3-Fc binding to PVRα and also that the C′-C″ β-strands of nectin1 are not sufficient for nectin3 and nectin4 binding activity. In an attempt to recover binding and to define the minimal binding site, we analyzed chimeras containing larger portions of nectin1 V domain in place of those of PVRα. No binding was detected with the N1-(83–116),

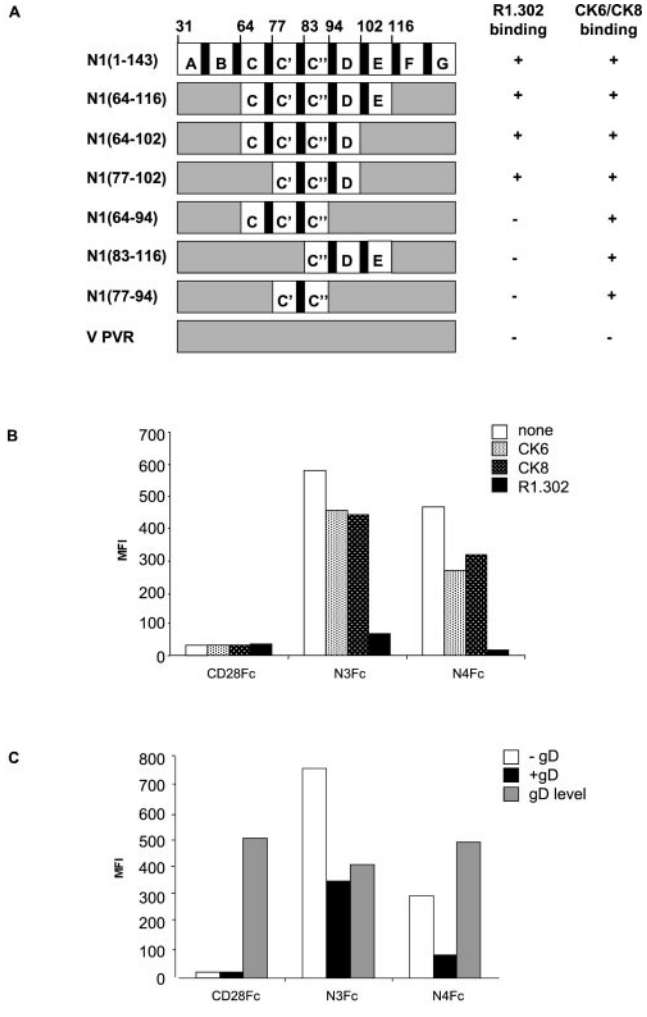

FIG. 4. **Schematic drawing of the nectin3 and nectin4 binding site on the V domain of nectin1.** *A*, schematic representation of the nectin1/PVR chimeras used in this study. Chimeric constructs obtained by PCR (see "Experimental Procedures") correspond to various homologous substitutions of β-strands and intervening loops in the V domain of PVRα (*gray*) by those of nectin1 (*black* and *white*). The rest of the molecule (not shown) (the two C domains and the transmembrane and intracytoplasmic regions) corresponds to PVRα. *Numerals*, amino acid positions in nectin1. The *R1.302* and *CK6/CK8 columns* correspond to the reactivity of the mAbs to the different chimeras: +, binding; −, no binding. *B*, inhibition of nectin3-Fc or nectin4-Fc binding to MDCK-N1 cells by mAbs CK6, CK8, and R1.302. Saturating concentrations of different mAbs were incubated with MDCK-N1 cells. Nectin3-Fc (10 nM) and nectin4-Fc (500 nM) binding analyses were done as described in Fig. 2. *C*, inhibition of nectin3-Fc or nectin4-Fc binding by gD(Δ290–299t) (100 nM). The *gD level* represents the level of gD remaining bound to MDCK-N1 cells following nectin3-Fc or nectin4-Fc incubations. The CD28-Fc recombinant was used as a control. The results shown are representative of three independent experiments.

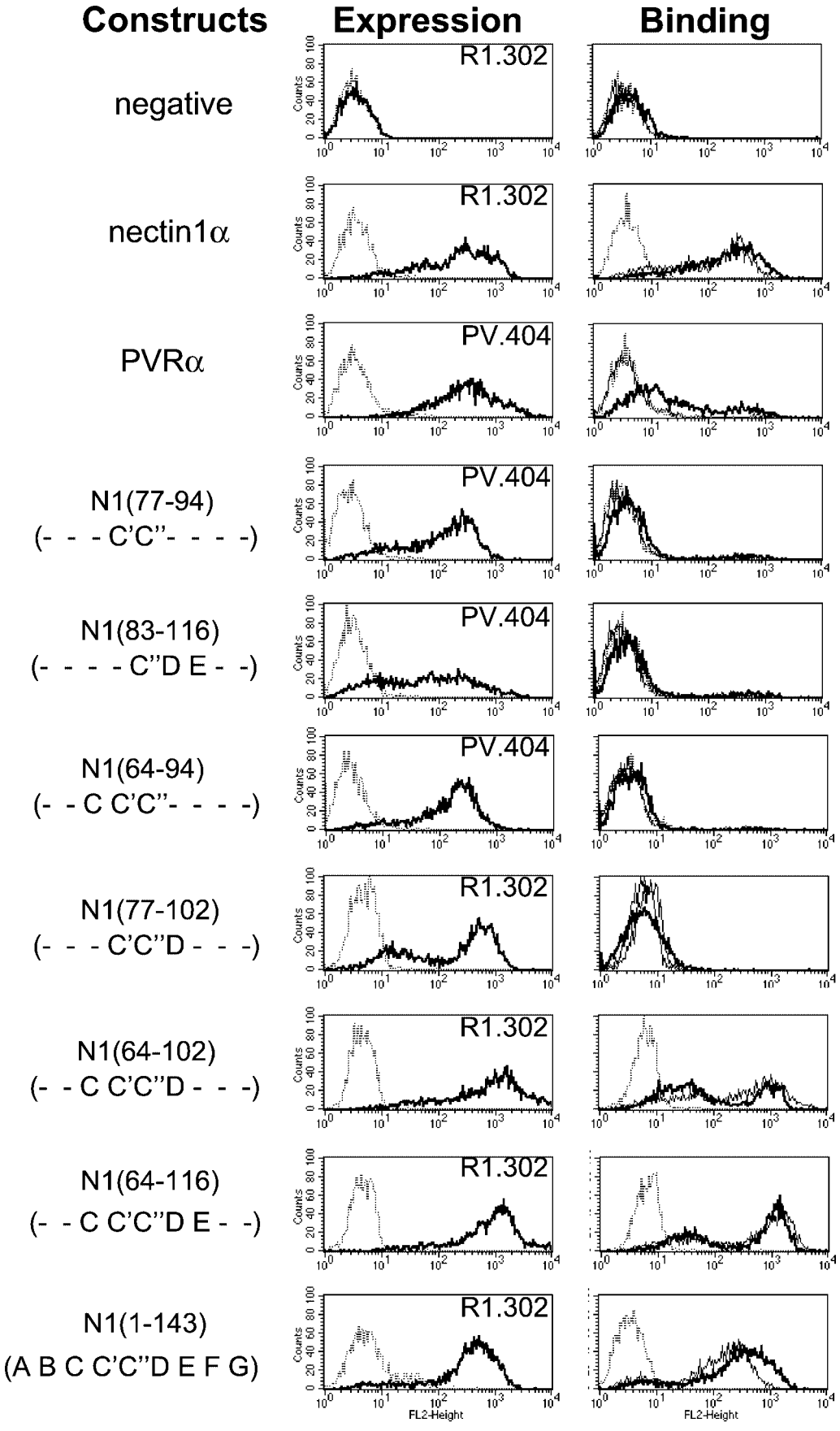

FIG. 5. **Nectin3 or nectin4 binding to MDCKII cells expressing nectin1/PVR chimeras.** Cell surface expression was monitored for each transfectant to ensure a similar expression level of the respective molecule at the cell surface (*Expression column*). Anti-nectin1 mAb R1.302 or anti-PVR mAb PV.404 (directed against PVR C domains) were used as specified in the *Expression column*. Mouse IgG1 was used as negative control (*dashed line*). The *Binding column* shows cell surface binding of nectin3-Fc (*black histogram*) or nectin4-Fc (*gray histogram*) to the MDCKII cells expressing the nectin1/PVR chimeras. CD28-Fc was used as a negative control (*dashed histogram*).

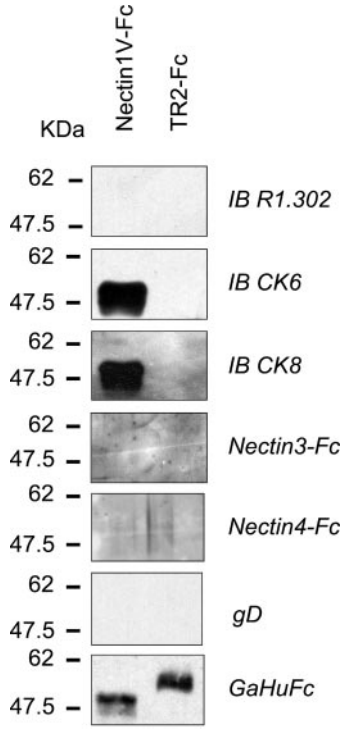

FIG. 6. **Interaction between the denatured form of nectin1V-Fc with biotinylated anti-nectin1 mAbs, nectin3-Fc, nectin4-Fc, and gD.** 10 ng of nectin1V-Fc or TR2-Fc (used as a negative control) proteins were analyzed on SDS-PAGE as described under "Experimental Procedures." 1 μg/ml for each of the respective biotinylated proteins was incubated with the membrane, and detection was achieved by using streptavidin-horseradish peroxidase. A blot was incubated with a horseradish peroxidase-labeled goat anti-human Fc antibody (*GaHuFc*) to control for the amount of recombinant protein loaded in each lane. *IB*, immunoblot.

N1-(64–94), and N1-(77–102) chimeras (Fig. 5). Nectin3-Fc and nectin4-Fc binding were readily detected with the N1-(64–102), N1-(64–116), and the N1-(1–143) chimeras. This demonstrates that (i) nectin3 and nectin4 bind to the same region in the nectin1 V domain, (ii) their binding site on nectin1 includes at least portions of C-C′-C″-D β-strands and intervening loops (residues 64–102), (iii) C and D β-strands are essential in both interactions, and (iv) the A, B, E, F, and G β-strands are not involved as nectin3 and nectin4 binding sites.

The above results suggest that the nectin3 and nectin4 binding site on nectin1 is conformational. This was confirmed by measuring binding to denatured nectin1. As expected, the denatured form of nectin1V-Fc was not recognized by the mAb R1.302 but readily recognized by both mAbs CK6 and CK8. The denatured form of nectin1V-Fc was neither recognized by gD nor by nectin3 and nectin4, strongly suggesting that the nectin1 binding region is also conformational (Fig. 6).

trans-Hetero-interaction between Nectin4V, Nectin3V, and Nectin1V Domains—The results presented above identify a portion of the V domain of nectin1 as sufficient for nectin3 and nectin4 interaction. In a last series of experiments, we analyzed the nectin3 and nectin4 domains involved in this interaction. To address this issue, we developed monoclonal antibodies directed to the nectin4 ectodomain. We present here two mAbs (N4.40 and N4.61) isolated after immunization with the nectin4VCC-Fc recombinant protein (see "Experimental Procedures"). Their epitope was determined by ELISA using two recombinant forms of nectin4, one made of the entire ectodomain (VCC) and the other made only of the V domain (see Fig. 1). Both mAbs reacted with the nectin4VCC-Fc soluble protein, and only mAb N4.61 reacted with the nectin4V-Fc recombinant form (Fig. 7*A*). Thus the mAb N4.61 recognizes an epitope localized in the nectin4 V domain, whereas the mAb N4.40 recognizes an epitope present in one of the two C domains of nectin4. These mAbs were used in blocking experiments. First we controlled the binding of both mAbs to nectin4-expressing MDCKII cells (MDCK-N4). As shown in Fig. 7*B* both mAbs bound MDCK-N4. Preincubation of MDCK-N4 with the mAb N4.61 inhibited by 95% the nectin1-Fc/nectin4 interaction, whereas preincubation with mAb N4.40 did not (Fig. 7*C*, *bold black/thin black histograms*). Conversely, preincubation of nectin4-Fc with mAb N4.61 totally prevented interaction with MDCK-N1 cells. No inhibition was detected with mAb N4.40 (data not shown). The results provide evidence that the V domain of nectin4 is involved in nectin4/nectin1 interaction and highlight the importance of V domain in *trans*-interactions of nectins. To confirm a direct V-V *trans*-interaction, we analyzed the direct binding of nectin4V-Fc (soluble nectin4 lacking the C domains) to MDCK-N1V (transmembrane form of nectin1α lacking the two C domains, Ref. 25) as compared with the binding of nectin4V-Fc to MDCK-N1 and of nectin4-Fc to MDCK-N1. Serial dilutions of soluble nectins were incubated with transfected cells, and the cell surface binding factor was calculated relative to nectin1 expression (see "Experimental Procedures"). Nectin4V-Fc bound to nectin1V as well as nectin4-Fc bound to nectin1 at 4×10^{-7} M (Fig. 7*D*). However, deletion of the nectin1 or nectin4 C domains led to a 2-fold reduced binding factor when concentration decreased (Fig. 7*D*, compare *square* and *triangle* curves with *diamond* curve). These results demonstrate that (i) V domains of nectin4 and nectin1 are sufficient to mediate *trans*-interaction and (ii) C domains contribute to increase the affinity of this interaction. We obtained similar results between nectin3V and nectin1V (data not shown), thus strengthening and extending the major role of V domain in *trans*-hetero-interactions of nectins.

DISCUSSION

Nectins display common structural and functional features that allow their classification into a new family of cell-cell adhesion molecules. Their ectodomain is composed of three Ig-like domains of V, C, C types by which they engage in homophilic properties. Homophilic adhesion contributes in part to their specific localization at adherens junctions in epithelial and endothelial cells (13, 17). Nectin1 and nectin2 have also been described as HSV receptors, and nectin1 serves as receptor for all the HSV strains tested. The envelope gD of HSV plays an essential role during viral entry into cells and binds to the distal V domain of nectin1 (25, 29).

We and others recently described a new property of the nectins, *i.e.* that nectin ectodomains bind in *trans* to each other in a well defined fashion (5, 10). Four different *trans*-hetero-interactions were identified: nectin3/PVR, nectin3/nectin2, nectin1/nectin3, and nectin1/nectin4 (5). Thus, nectin1 ectodomain is able to share at least four different partners: in this case, gD, itself (homophily), and nectin3 and nectin4 (heterophily). These different combinations of *trans*-interactions probably play a central role in nectin physiology. We took advantage of the fact that the nectin1 site involved in gD binding has been well characterized to investigate the site on nectin1 involved in *trans*-hetero-interactions with nectin3 and nectin4. We show the following. (i) K_D for nectin3/nectin1 interaction is lower (1 nM) than that for nectin4/nectin1 interaction (100 nM). (ii) Nectin3 and nectin4 share at least in part a common binding site on nectin1 V domain. The binding site on nectin1 is conformational and includes the C-C′-C″-D β-strands of the V domain. (iii) Nectin3 and nectin4 V domains alone, in the absence of C domains, are sufficient to bind to the nectin1 V domain. (iv) Nectin3 and nectin4 C domains contribute to in-

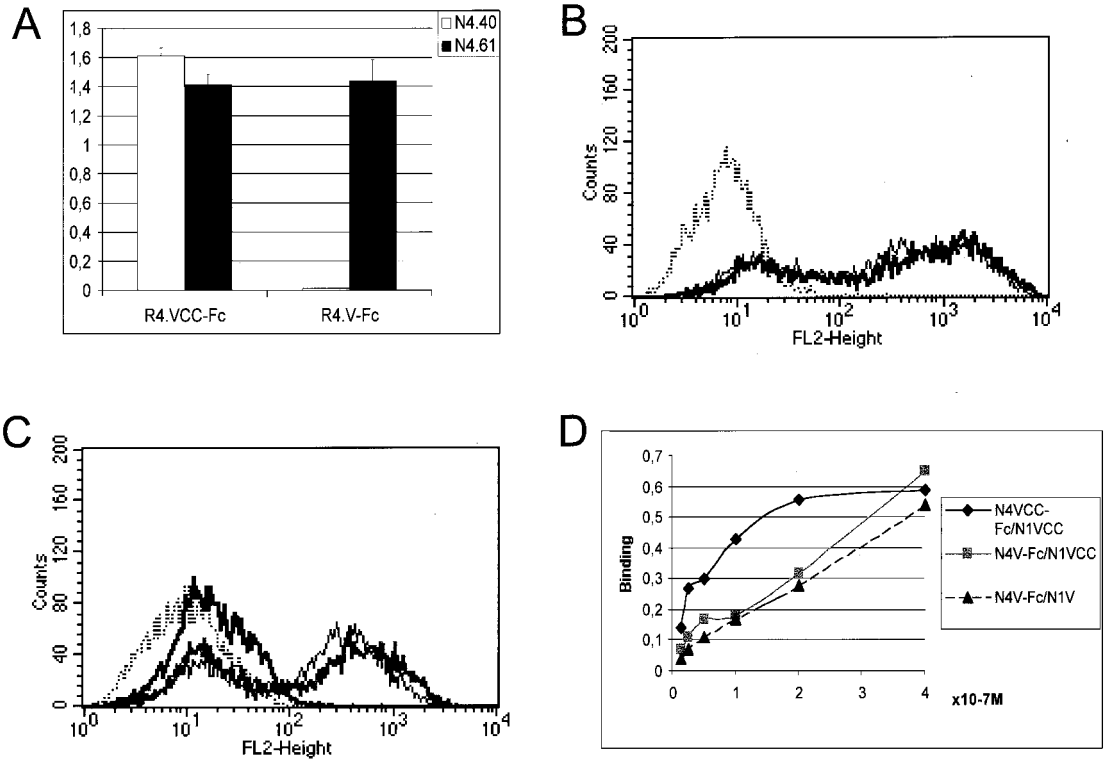

FIG. 7. **Mapping of nectin4 domain involved in nectin4/nectin1 *trans*-hetero-interaction.** *A*, localization of the epitope recognized by mAbs N4.40 and N4.61 by ELISA. ELISA was performed as described under "Experimental Procedures." N4.61 (*black bar*) reacts with both nectin4VCC-Fc and nectin4V-Fc. N4.40 (*white bar*) reacts only with nectin4VCC-Fc. *B*, FACS analysis of nectin4-expressing MDCKII cells with mAbs N4.40 (*gray histogram*) and N4.61 (*black histogram*). Mouse IgG1 was used as negative control (*dashed line*). *C*, nectin4-expressing MDCKII cells were preincubated with 10 μg/ml mouse IgG1 (*gray histogram*), mAb N4.40 (*thin black histogram*), and mAb N4.61 (*bold black histogram*). Nectin1-Fc was then incubated with pretreated cells, and binding was analyzed by FACS for each condition. TR2-Fc was used as negative control (*dashed line*). *D*, contribution of the nectin4 and nectin1 C domains to the binding affinity. Nectin4VCC-Fc binding on cells expressing nectin1α (*N1VCC*, curve with *diamonds*) was compared with the binding of nectin4V-Fc to the same cells (curve with *squares*). V to V domain interaction was analyzed between nectin4V-Fc and MDCKII cells expressing nectin1α lacking its two C domains (*N1V*, curve with *triangles*). Binding factor was defined as described under "Experimental Procedures."

crease the binding affinity. (v) A soluble recombinant form of gD interferes with the binding of nectin3 or nectin4 to nectin1.

Altogether our results provide evidence that *trans*-hetero-interactions between three different members of the nectin family are mainly mediated through direct V to V Ig-like domain interactions. Moreover, the V domain of PVR is essential for nectin3 binding as replacement of C′-C″ β-strands of PVR with those of nectin1 abrogated nectin3 binding (Fig. 5). Finally, we found that mAbs directed against the V domain of nectin2 and PVR blocked *trans*-interactions with nectin3 (data not shown). Thus, the V to V domain model of nectin *trans*-hetero-interactions could probably be extended beyond the presently described nectin3/nectin1 and nectin4/nectin1 interactions. This model has already been proposed for other receptors of the immunoglobulin superfamily and corresponds to an "end by end" model *versus* a "side by side" (or antiparallel) model. We demonstrate here that the deletion of the two C domains of nectins led to a reduction in binding affinity. C domains of nectins may contribute to position the V domain sufficiently distant from plasma membrane and maximize V to V interactions. Moreover, this end by end interaction is compatible with the size of the intercellular space at adherens junctions evaluated at 25 nm. The C domains may also contribute to lateral *cis*-dimerization essential for nectin *trans*-interaction although it has been shown that engineered soluble nectin1 V domain could form *cis*-dimer (26). Recently a novel structural motif, R(V/I/L)E, has been described in the V domain of the three known junctional adhesion molecule members (JAM1, -2, and -3) and shown to be essential for the *cis*-dimerization of the molecule (30). Interestingly this consensus motif is also present between the C″ and D β-strands in the V domain of nectin1 (positions 96–98), nectin4 (positions 120–122), and PVR (positions 114–116) and conserved in orthologous genes. Additional experiments will be necessary to assign this motif to the nectin *cis*-dimerization signal.

The V domain of nectin1 is also involved in homophilic interactions. Recently Krummenacher *et al.* (31) showed that nectin1-mediated homophilic aggregation was blocked by engineered soluble nectin1 V domain and by mAbs CK6 and CK8 that recognized the region (residues 83–94) on the nectin1 V domain (see Fig. 4*A*). However, a direct participation of the nectin C domains cannot be excluded as homophilic aggregation is also blocked by nectin anti-C domain mAbs (13, 23). Preliminary results suggest that soluble nectin1-Fc can bind cells expressing a chimeric nectin1α in which the V domain of nectin1 is substituted with the V domain of PVR. Although experiments are in progress, these data suggest that nectin *trans*-homophilic interactions may involve a *trans*-interaction between the nectin V and C domains.

At this point, we do not know whether nectin1 may be engaged in homophilic and heterophilic interaction at the same time to form with nectin3 or nectin4 (themselves homophilic) multimeric complexes at adherens junctions. In addition to these properties, nectin1 mediates HSV entry through interaction between the envelope gD and the C-C′-C″ β-strands of its V domain (28). Again this site overlaps with heterophilic and also homophilic binding regions on nectin1. Nectin1-mediated homophilic binding is blocked and reversed by gD(Δ290–299t) (31). We show here that the apparent affinity of the gD(Δ290–299t)/nectin1 interaction (3 nM) was more than 300-fold higher

than that of the homophilic interaction (1 μM) and probably accounts for the gD-induced reversion of nectin1-mediated homophily. gD also inhibited nectin3 and nectin4 binding to nectin1. Altogether these results highlight an overlapping region in the nectin1 V domain involved in homophilic, heterophilic, and gD interactions. X-ray structure determinations will lead to analysis of molecular events that govern each interaction. Interestingly the x-ray structure of the gD ectodomain has been resolved and showed a three-dimensional structure similar to a V-like immunoglobulin fold despite a primary amino acid sequence unrelated to any known protein (32). Thus gD/nectin1 interaction can be considered to be a V to V domain interaction. Recently Lange *et al.* (33) demonstrated that PVR binds to vitronectin through its V domain. Thus, we cannot rule out the existence of other ligands for the members of the nectin family. Mizoguchi *et al.* (34) reported an asymmetrical distribution of nectin1 and nectin3 at the pre- and postsynaptic sides of puncta adherentia junctions, thus identifying for the first time a natural site and a role for *trans*-hetero-interactions between nectin1 and nectin3. Mutations in the nectin1 gene have been associated with nonsyndromic cleft lip with or without cleft palate abnormalities (35, 36). Nectin1 gene is expressed in orofacial and skin epithelia in mouse embryo. Because nectin4 is expressed during mouse development, we hypothesize that *trans*-hetero engagement of nectin1 with nectin4, and probably nectin3, may regulate physiological processes during development.

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MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

Prominent Role of the Ig-like V Domain in *trans*-Interactions of Nectins: NECTIN3 AND NECTIN4 BIND TO THE PREDICTED C-C′-C"-D β-STRANDS OF THE NECTIN1 V DOMAIN

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