Recruitment of Nectin-3 to Cell-Cell Junctions through trans-Heterophilic Interaction with CD155, a Vitronectin and Poliovirus Receptor That Localizes to αvβ3 Integrin-containing Membrane Microdomains*

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Nectins present a novel class of Ig superfamily adhesion molecules that, cooperatively with cadherins, establish and maintain cell-cell adherens junctions. CD155, the cognate receptor for poliovirus, undergoes cell-matrix contacts by binding to the extracellular matrix protein vitronectin. The significant homology of nectins with CD155 prompted us to investigate the possibility of their interaction. We determined that nectin-3 binds CD155 and its putative mouse homologue Tage4 in cell-based ligand binding assays. Coculture of nectin-3- and CD155-expressing HeLa cells led to CD155-dependent recruitment of nectin-3 to cell-cell contacts. In a heterologous coculture system with CD155 expressing mouse neuroblastoma cells, HeLa cell-expressed nectin-3 was recruited to contacts with CD155 bearing neurites. CD155 and nectin-3 colocalized to epithelial cell-cell junctions in renal proximal tubules and in amniotic membrane. Efficient interaction depended on CD155 dimerization, which appears to be aided by cell type-specific cofactors. We furthermore found CD155 to codistribute with αv integrin microdomains on the surface of transfected mouse fibroblasts and at amniotic epithelial cell junctions. Our findings demonstrate the possible trans-interaction between the bona fide cell-cell adherens type adhesion system (cadherin/nectin) and the cell-matrix adhesion system (integrin/CD155) by virtue of their nectin-3 and CD155 components, respectively.

The members of the nectin protein family have recently been recognized as a novel adhesion unit at cadherin-based cell-cell adherens junctions (1–3). Four family members, nectin-1, -2, -3, and -4, have been isolated (1, 3–6). Nectins form cis-homodimers on the cell surface that mediate trans-homophilic cell-cell adhesion (1–3, 7–10). Furthermore, trans-heterophilic interactions of either nectin-1 or -2 with nectin-3 and of nectin-1 with nectin-4 have been observed (1, 3). trans-Homophilic interactions appear to play a role in cell-cell adhesion at homotypic cadherin-based adherens junctions, such as those between epithelial or endothelial cells (1, 2, 9). trans-Heterophilic nectin adhesion, on the other hand, is thought to be involved in maintaining nonclassical, heterotypic adherens type junction, such as Sertoli cell-spermatid junctions in testis or puncta adherentia at synapses (11, 12). The nectin adhesion system has been proposed to act cooperatively with the classical cadherin system, and both adhesive units have been shown to interact through proteins associated with their cytoplasmic domains (14–16). Despite their relatively low homology (around 35% amino acid identity) nectins were grouped together functionally based on the interaction of their cytoplasmic domains with the F-actin-binding protein afadin (1–3). Although, not considered as nectins by this definition, the ectodomains of the poliovirus and vitronectin receptor CD155 (formerly PVR) (17) and that of its putative rodent homologue Tage4 (18, 19) share significant homology with nectins (54% amino acid identity between CD155 and nectin-2). In fact, the first members of the nectin gene family have been identified through their homology to CD155 (4–6). Together, CD155, Tage4, and nectins comprise a new group of Ig superfamily proteins. They are single span type I a transmembrane glycoproteins with three Ig-like domains (V-C2-C2) and a generally short cytoplasmic domain.

The CD155 gene is expressed in four isoforms. Two of the four alternatively spliced variants, CD155α and CD155β, are membrane-bound and serve as poliovirus receptor, whereas the significance of two secreted versions, CD155β′ and -γ, is not clear (20). CD155 mRNA is widely expressed, although in relatively low amounts, in human and CD155tg mouse tissues, including brain, spinal cord, heart, skeletal muscle, kidney, spleen, leukocyte, liver, lung, and placenta (20–23). In humans, CD155 is prominently expressed by enterocytes and cells of gastrointestinal lymphatic tissues (24). The ectodomain of CD155 establishes cell-matrix contacts by interaction with vitronectin (25). Although the overall structure of CD155 as an Ig superfamily protein and the similarity to nectins suggests a possible role in cell-cell adhesion, no binding partners that would support such a notion have been identified thus far. In fact, in contrast to nectins, there is no evidence for CD155-mediated trans-homophilic cell-cell adhesion or for CD155 cis-homodimerization (8–10). On the other hand, the short cytoplasmic domains of CD155α and CD155β interact with Tetex-1, a light chain subunit of the dynemin motor complex (26). This interaction is thought to mediate retrograde axonal transport of CD155 containing endocytic vesicles (26). In addition, the cytoplasmic domain of CD155α binds the mu1B subunit of the...
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clathrin adaptor complex, an interaction that is responsible for sorting of the CD155αc isotype protein to basolateral membranes in epithelial cells (27).

Studies of CD155 have been complicated by uncertainty over whether a functional rodent homologue of CD155 exists, a conundrum that has restricted and even misled earlier work. It is now clear that a mouse gene termed MPH, which was long held to be the mouse homologue of CD155, is, in fact, the homologue of human nectin-2 (formerly PR2 or PVR2)(4, 5). The rodent Tage4 gene, sharing only 46% amino acid identity with CD155, has recently been proposed as the possible CD155 homologue based on conserved gene structure and syngenic chromosomal localization (19). However, no functional relationship between these two molecules could be thus far established. Mice are not a natural host for poliovirus, and cultured mouse cells cannot be infected with poliovirus. Therefore, the development of transgenic mice expressing the human CD155 gene has greatly facilitated the study of CD155 function (21, 23).

CD155g mice are highly susceptible to poliovirus infection and develop disease symptoms very similar to poliomyelitis in humans, indicating that the human transgene functions similarly in mice as it does in humans.

Here we show that nectin-3 interacts with CD155, leading to unidirectional recruitment of nectin-3 to cell-cell contacts with neighboring CD155-expressing cells. This interaction was found to depend on dimerization/multimerization of CD155, which appears to be promoted by cell type-specific cofactors. Mouse Tage4 (referred to below as mCD155) showed similar affinity for nectin-3, providing the first evidence for a functional relationship between CD155 and its mouse counterpart. In vivo, nectin-3 and CD155 colocalized at lateral cell-cell junctions in epithelia of proximal kidney tubules and of the amniotic membrane. We determined that CD155 and α, integrin colocalize to membrane microdomains on the cell surface of mouse fibroblasts and to cell-cell and cell-matrix junctions in the amniotic membrane. We propose that CD155 and nectin-3 are mediators of trans-heterophilic cell-cell adhesion and may provide a link between the classical cell-cell adhesion system (cadherin/nectin) and the cell-matrix adhesion system (integrin/CD155).

MATERIALS AND METHODS

Cells and Plasmids—Human embryonal kidney cells (HEK 293), HeLa cells, African green monkey kidney cells (COS-1), mouse fibroblast cells (Ltk−), mouse neuroblastoma cells (Neuro2a), and rat pheochromocytoma cells (PC12) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium containing penicillin/v Streptomycin and 10% fetal bovine serum (Hyclone) or 15% fetal bovine serum medium containing penicillin/
streptomycin and 10% fetal bovine serum (Hyclone). Human embryonal kidney cells (HEK 293), HeLa, L(tk−, 1802, 11002), mouse neuroblastoma cells (Neuro2a), and rat pheochromocytoma cells (PC12) were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium containing penicillin/streptomycin and 10% fetal bovine serum (Hyclone) or 15% fetal bovine serum (PC12 cells).

Fusing the coding sequence of the extracellular domains of CD155, nectin-3, or nectin-3 upstream of the open reading frame of the secreted, heat-stable human placental alkaline phosphatase (SEAP)2 in vector pAPtag2 (28) (J. Flanagan, Harvard University) resulted in pCD155-AP, nectin-3-AP, and pNectin-1-AP, respectively. pAPtag4 (J. Flanagan) was used to express untagged SEAP protein. pCDNA-CD155(AO) (G. Bernhardt, University of Hannover, Germany) contains the entire CD155αc cDNA in pCDNA3. For eukaryotic cell transfection, eukaryotic cell transfection, the full-length open reading frames of mouse nectin-1α, mouse nectin-2α, and mouse nectin-3α were cloned into pCDNA3.1, resulting in pCDNA-nectin-1, pCDNA-nectin-2, pCDNA-nectin-3, respectively. Two I.M.A.G.E. consortium clones, 4188634 and 4953896, containing the full-length open reading frame of mouse Tage4 in vector pCMV Sport6 were obtained from ATCC. These plasmids afford eukaryotic expression and were used directly for DNA transfection. Plasmids encoding the human αc and β integrin subunits were kindly provided by Drs. Eric Brice and Mark Ginsberg.

Stably transfected HEK 293 cell lines secreting CD155-AP, nectin-3-AP, or nectin-1-AP fusion proteins or untagged SEAP alone were produced by cotransfection of empty pCDNA3.1 vector (carrying a neo selection marker) with either pCD155-AP, pNectin-3-AP, pNectin-1-AP, or pAPtag4, respectively. Cells were subjected to selection with 500 µg/ml G418 (Invitrogen), and cell clones were isolated and screened for AP activity in the supernatant. HeLa, HEK 293, Ltk−, and Neuro2a, and PC12 cells stably expressing full-length CD155αc, nectin-1α, nectin-2α, or nectin-3α were derived by DNA transfection with pCDNA-CD155(AO), pCDNA-nectin-1, pCDNA-nectin-2, or pCDNA-nectin-3, followed by selection with 500–750 µg/ml G418. The cell lines were screened in order to obtain clones with similar expression levels.

Antibodies—The following primary antibodies were used in this study: mouse mAbs 18 (1:2 hybridoma), D171 (1:10 hybridoma supernatant), or 10 µg/ml for blocking experiments), P286, P403, P275 (all at 10 µg/ml), all against CD155 (24, 30, 31); rat mAbs 105-A1 (1:5 diluted hybridoma supernatant) specific for mouse nectin-3 (1); mouse mAb SB6 against human placental alkaline phosphatase PLAP-1 (1:500; Sigma); rabbit polyclonal antiserum raised against commercially available (Chemicon) purified α, integrin (1:2000; M. Roivainen, National Public Health Institute, Helsinki, Finland) and purified hexahistidine-tagged CD155 (NAEZ-5; 1:1000; produced in collaboration with M. Roivainen) (32). The secondary antibodies were anti-rabbit IgG (1:500), mouse IgG (1:1000, both Alexa488-conjugated; Molecular Probes, Inc., Eugene, OR), anti-mouse IgG (1:500), anti-rat IgG (1:1000), anti-rabbit IgG (1:500; all Cy3-conjugated; Jackson ImmunoResearch), anti-mouse IgG (1:200; AP-conjugated; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-rabbit IgG (1:1000, horseradish peroxidase-conjugated; Jackson ImmunoResearch).

Ligand Binding Assays—Receptor-AP in situ ligand binding was performed as previously described (33, 34), with some modifications. Briefly, HEK 293 cell supernatants containing AP fusion protein probes were diluted with growth medium to obtain a uniform AP activity of 2500 A 405 /µg/ml for all probes. COS-1 cells transiently transfected with expression vectors for individual target proteins were incubated with AP fusion protein probes for 1 h at 37 °C. The probes were then aspired, and cells were washed five times with warm Hanks’ balanced salt solution (HBSS), containing 0.5 mg/ml bovine serum albumin. The cells were then fixed with 4% formaldehyde and 80% acetone in PBS for 2 min and washed twice with HBSS/bovine serum albumin, followed by heating of the culture dishes at 65 °C for 4 h in order to inactivate cell endogenous, heat-labile AP. The monolayers were washed twice with double-distilled H2O, and bound AP fusion probes were detected by incubation with 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium colorimetric AP substrate (Sigma). Alternatively, stable HEK 293, HeLa, L(tk−), and PC12 cell lines expressing individual target proteins were produced and processed as described above. The effect of cross-linked CD155 binding to nectin-3, CD155-AP supernatant was preincubated with anti-AP mAb SB6 (1:500) for 1 h at room temperature before being added to the target cells. Alternatively, a fusion protein between the CD155 ectodomain and the Fc portion of mouse IgG2α (CD155-Fc) was used instead of CD155-AP (35). This protein is expressed as a dimer due to the biological activity of the Fc region (35). Binding of CD155-Fc was assessed by subsequent incubation with anti-mouse IgG secondary antibodies conjugated to AP. For antibody blocking of nectin-3-AP binding to CD155-expressing cells, the cells were preincubated with 10 µg/ml of an unrelated mouse mAb or mAb D171, P286, P403, or P275 for 30 min at 37 °C before the addition of CD155-3-AP.

In order to quantify cell-bound AP fusion proteins, confluent 6-well plates of HeLa, HeLa-CD155, Ltk−, and Ltk−/CD155 cells were incubated with AP fusion probes as above. However, instead of fixing the cells after the washing step, cells were lysed in 10 mM Tris, pH 7.4, 1% Triton, and the lysate was incubated at 65 °C for 45 min. The AP activity of the binding reaction was determined electrochemiluminescently at 405 nm using 9 mg/ml p-nitrophenolphosphate (in 1 M diethanolamine, pH 9.8, 0.5 mM MgCl2) as a colorimetric, water-soluble substrate. In parallel, 106 cell equivalents of total lysate were separated on 7.5% SDS-PAGE followed by Western blot analysis with CD155-specific antisera (NAEZ-8 (1:5000), anti-rabbit horseradish peroxidase-conjugated; Roche Molecular BioSciences). Cocluturer Assays, Tissue Sections, and Commumofluorescence Analysis—HeLa, HeLa-CD155, and HeLa-nectin-3 (or HeLa-nectin-2) cells

2 The abbreviations used are: SEAP, secreted, heat-stable human placental alkaline phosphatase; AP, alkaline phosphatase; mAb, monoclonal antibody; HBSS, Hanks’ balanced salt solution; PBS, phosphate-buffered saline; BS3, bis-(sulfosuccinimidyl)sulfate.

3 J. Zhan and E. Wimmer, unpublished results.
RESULTS

Interaction of CD155 and Nectin-3 in Cell-based Ligand Binding Assays—In order to test for interactions between nectin/CD155 family members, we employed a method developed by Flanagan and Leder (33, 34) that makes use of recombinant receptor-AP fusion proteins as affinity probes. Four affinity probes were expressed in HEK 293 cells by fusing the ectodomains of CD155, mouse nectin-1, mouse nectin-2, and mouse nectin-3 to SEAP (see Fig. 1A), a genetically modified derivative of the human PLAP-1 gene (36). The fusion proteins are targeted for secretion into the medium by virtue of the signal sequence endogenous to each of the receptor moieties. Between 2500 and 10,000 A405/ml·h of AP activity was detected in the various culture supernatants, compared with 1–2 A405/ml·h in supernatants of untransfected cells. Western blot analysis with anti-PLAP-1 mAb 8B6 was performed in order to ascertain the predicted size of the expressed proteins (Fig. 1B). All fusion proteins were detected as a single immune reactive band with no visible truncation products. Although the calculated molecular masses of the recombinant proteins were between 90 and 100 kDa, the apparent molecular masses were found to be between 105–140 kDa due to varying amounts of N-linked glycosylation (Fig. 1B).

Given the recent reports of heterophilic interactions of nectin-3 with both nectin-1 and nectin-2 (1, 11, 12), we asked whether CD155, the closest known relative of nectin-2, would be capable of a similar interaction with nectin-3. For this purpose, COS-1 cells, transiently transfected with individual nectin/CD155 family members, were incubated with AP fusion proteins as indicated in Fig. 2. First, we tested whether our
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Fig. 3. Inhibition of nectin-3-AP binding to PC12-CD155 cells by CD155-specific mAbs. Monolayers of parental rat PC12 cells (first bar) or PC12 cells stably expressing CD155 (second through sixth bars) were incubated with growth medium containing a 10 μg/ml concentration of an unrelated mouse mAb (first and second bars) or 10 μg/ml CD155-specific mAbs D171, P286, P403, or P275. After 30 min, antibodies were removed, and cells were incubated with nectin-3-AP for 1 h. Cells were washed extensively and then lysed, followed by heat inactivation of any endogenous AP activity. Heat-stable AP activity originating from cell-bound nectin-3-AP was quantitated spectrophotometrically using the water-soluble AP substrate para-nitrophenylphosphate. The amount of nectin-3-AP binding in the absence of CD155-specific mAbs was set to 100%. Three mAbs, D171, P286, and P403, were found to inhibit nectin-3-AP binding to CD155 by 54, 69, and 68%, respectively. mAb P275 had no inhibitory effect. Results are the average of three independent determinations.

Experimental system can reproduce the previously established interactions between nectin family members. Indeed, intense staining, indicating a strong interaction, was observed between cell surface-expressed nectin-3 and the nectin-1-AP probe (Fig. 2A) or the nectin-2-AP probe (Fig. 2B) as well as between nectin-1 and nectin-3-AP (Fig. 2D). Under the conditions of the assay, virtually no homophilic binding resulted from incubation of COS-nectin-3 cells with nectin-3-AP (Fig. 2C). These results support previous results of Satoh-Horikawa and colleagues, who have shown that nectin-3 and nectin-1 or nectin-3 and nectin-2 predominantly undergo trans-heterophilic rather than trans-homophilic interactions (1). When COS-1 cells were transfected with either human CD155 or mouse CD155/Tage4, significant binding of nectin-3-AP resulted from both proteins (Fig. 2, E and F). Judging from the intensity of the staining, the intraspecies interaction between mCD155/Tage4 and nectin-3-AP (containing the mouse nectin-3 ectodomain) appeared to be slightly more effective (Fig. 2, compare F and E). To rule out the possibility that the observed interactions might be mediated by the AP moiety of our fusion proteins, we incubated CD155-transfected cells with untagged AP protein. As expected, no binding was observed (Fig. 2G). Likewise, nectin-3-AP did not bind to COS-1 cells transfected with an empty expression vector (Fig. 2H), ruling out binding to COS-1 endogenous proteins. It is worth noting that in the reverse binding assay, in which CD155-AP was used to detect nectin-3 expression, we did not observe any significant interaction (Fig. 2I). This result suggests that, similarly to the interaction between other nectins (1–3), the binding site for nectin-3 lies within the N-terminal Ig-like domain of CD155.

The above findings present the first evidence for an interaction of CD155 with a bona fide cell-cell adhesion molecule, nectin-3, and for a functional homology between human CD155 and its mouse counterpart. We therefore propose that Tage4 be referred to as mCD155.

Interaction of Nectin-3 and CD155 Requires Dimerized CD155—To our surprise, we noticed that the reverse ligand binding assay, using soluble CD155-AP to detect nectin-3 expressed on the cell surface, did not result in significant binding (Figs. 2I and 4F) on any of the cell lines tested (HeLa, HEK 293, COS-1, L(tk−), PC12). During a parallel analysis of the interaction between nectin-2 and nectin-3, we made the curious observation that preincubation of HEK 293-nectin-2 cells with nectin-2 rat mAbs 17B10 and 6B3, followed by nectin-3-AP (A) or CD155-AP (C) or CD155-AP (D); nectin-1-AP (E); CD155-AP that was cross-linked prior to the addition of anti-AP mAb 275 (G); CD155-Fc plus AP-conjugated anti-mouse IgG antibody (H). Cell surface-expressed nectin-2 presents a poor substrate for nectin-3-AP (A) but becomes a good substrate upon antibody cross-linking of nectin-3 (C). No such effect is observed when CD155-AP (known not to interact with nectin-2) is used as probe (compare B and D). Whereas nectin-1-AP binds well to nectin-3-expressing cells (E), no binding is seen with CD155-AP (F). CD155-AP only binds to nectin-3 when cross-linked with an anti-AP antibody (G). Similarly, a dimeric CD155-Fc binds to nectin-3 on the cell surface (H). Bar, 100 μm.

In order to confirm the specificity of the interaction between CD155 and nectin-3, PC12-CD155 cells were preincubated with a panel of CD155-specific mAbs, followed by a nectin-3-AP binding assay (Fig. 3). Three mAbs, D171, P286, and P403, reduced binding of nectin-3-AP binding by 54, 69, and 68%, respectively, whereas mAb 275 had no effect. Interestingly, all three inhibitory antibodies are known to bind to the N-terminal Ig-like domain and also block binding of poliovirus to CD155, an interaction that is solely mediated by this domain (30, 31, 37). This result suggests that, similarly to the interaction between other nectins (1–3), the binding site for nectin-3 lies within the N-terminal Ig-like domain of CD155.

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on L(tk−)-nectin-2 cells (data not shown). This effect was specific for the interaction of nectin-2 with nectin-3-AP, since CD155-AP showed no binding to nectin-2 before or after antibody preincubation with 17B10/6B3 (Fig. 4, compare D and B). Furthermore, no cross-reactivity of mAbs 17B10 and 6B3 with nectin-3 was detected (data not shown). Since nectin-2 is already present in form of a dimer on the cell surface (9, 10), our data indicate that clustering of nectin-2 may be necessary for efficient trans-interaction with nectin-3.

The CD155-specific antibodies available to us did not cause a similar stimulation of nectin-3-AP binding to CD155-expressing cells; all of these antibodies were found to be inhibitory to this interaction or had no effect (Fig. 3). Since soluble CD155 does not form homodimers as nectins do, we speculated that the reason for the failure of CD155-AP binding to nectin-3 (Figs. 2I and 4F) is due to the monomeric nature of CD155. To address this issue, we cross-linked our CD155-AP probe by preincubation with AP-specific mAb 8B6 before adding the fusion protein to HEK 293-nectin-3 cells. We found that antibody-cross-linked CD155-AP now efficiently bound to nectin-3 (Fig. 4, compare F and G). To confirm this observation, we next tested the binding of a recombinant CD155 to which the Fc portion of mouse IgG2a was fused (CD155-Fc) (35). Due to the properties of the Fc region, this fusion protein spontaneously forms dimers. Binding of CD155-Fc to HEK 293-nectin-3 cells was detected with a secondary anti-mouse IgG antibody conjugated to AP. As can be seen in Fig. 4H, CD155-Fc bound well to nectin-3 expressed on the cell surface of HEK 293 cells, similar to the binding of the antibody-cross-linked CD155-AP (Fig. 4G). Our results indicate that CD155 dimerization/oligomerization is essential for efficient binding of nectin-3.

Cell Type-specific Cofactors Are Required for the Proper Display of CD155 on the Cell Surface and for the Interaction with Nectin-3—When the binding of nectin-3-AP to different CD155-expressing cell lines was compared, a considerable and reproducible difference was noticed. This was particularly striking in the case of CD155-expressing mouse L(tk−) versus human HeLa cells (Fig. 5). Although L(tk−)-CD155 and HeLa-CD155 cells both expressed high levels of CD155 (Fig. 5B, third and fourth lanes; compare with HeLa cell endogenous CD155 in first lane), very little nectin-3-AP binding above background (Fig. 5A, second bar) was observed on L(tk−)-CD155 cells (compare Fig. 5A, third and fourth bars). We do not believe this phenomenon to be a species-specific effect of mouse versus human cells, since CD155 expressed in other rodent cells, like mouse Neuro2a and rat PC12 cells, was efficiently recognized by nectin-3-AP (Fig. 3 and data not shown). We deemed it more likely that CD155 expressed on L(tk−) cells is not presented in a proper higher order structure (dimer, oligomer, or complex with other proteins). To address this point, we carried out surface cross-linking of L(tk−)-CD155 and HeLa-CD155 cells with the chemical cross-linker BS3 (Fig. 6). Four high molecular weight complexes containing CD155 were obtained from HeLa-CD155 cells (Fig. 6, third lane). The smallest, and most prominent, of these complexes was 155–160 kDa in size, which corresponds to the expected size of a CD155 dimer (Fig. 6, fourth lane). Three additional higher molecular mass CD155 containing complexes ranging from 250 to 400–450 kDa were observed (Fig. 6, third lane, arrowheads). The components of these complexes, besides CD155, are not known at present. In contrast, CD155 expressed on L(tk−) cells appeared to be exclusively present as the typical 75–80-kDa monomer (Fig. 6, fourth lane). We suggest that a correlation exists between the absence of these high molecular weight CD155 complexes on L(tk−) cells and the inability of nectin-3-AP to recognize CD155. This is most likely due to missing, as yet unidentified, cofactors on L(tk−) cells.
Nectin-3 Is Recruited to Cell-Cell Contacts through Interaction with CD155—The members of the nectin family have been shown to localize to homotypic cadherin-based cell-cell junctions both in cultured cells and in vivo (1, 2, 8, 9). Furthermore, we and others have shown that nectin-3 is also present at specialized heterotypic cell-cell junctions such as Sertoli cell-spermatid junctions in testis (11) or at synaptic puncta adherentia (12). Therefore, we asked whether CD155 and nectin-3 colocalize at sites of cell-cell contact. For this purpose, we devised an in vitro coculture system that allowed us to assay for trans-interactions between cells. When nectin-3 expressing HeLa cells (HeLa-nectin-3) were cocultured with parental HeLa cells until confluence (Fig. 7, A–D), nectin-3 distributed evenly to all junctions between neighboring cells (Fig. 7, B and D). There was no noticeable preference of nectin-3 localization to junctions between HeLa-nectin-3 cells over junctions between HeLa-nectin-3 and parental HeLa cells (Fig. 7, B and D). This distribution is similar to previously reported nectin-3 expression in L cells (1). We furthermore detected significant nectin-3 immunoreactivity on the cell membranes not involved in cell-cell contacts (Fig. 7B). To test for interaction of nectin-3 with CD155, HeLa-nectin-3 cells were cocultured together with HeLa-CD155 cells and parental HeLa cells (as an internal control) (Fig. 7, E–H). The cells were then stained simultaneously for nectin-3 and CD155 by indirect immunofluorescence with rat mAb 103-A1 and mouse mAb D171 and analyzed by fluorescence microscopy (see "Materials and Methods"). We found that wherever HeLa-nectin-3 cells came into contact with HeLa-CD155 cells, virtually all nectin-3 was unidirectionally attracted to cell contacts with the CD155-expressing neighboring cells (Fig. 7F and H). In contrast, when HeLa-nectin-2 cells were cocultured with HeLa-CD155, nectin-2 preferentially localized at junctions between HeLa-nectin-2 cells but not between HeLa-CD155 and HeLa-nectin-2 cells, suggesting a strong preference for trans-homophilic interaction of nectin-2 (J and L). 5, HeLa-CD155 cell; 3, HeLa-nectin-3 cell; 2, HeLa-nectin-2 cell. The white lines in G and K indicate the approximate location of cell junctions. Bars, 20 μm.
CD155) together with HeLa-nectin-3 and parental HeLa cells.

Together, these data indicate that nectin-3 preferentially undergoes trans-homophilic interactions with CD155 over trans-homophilic interactions, whereas nectin-2, under the experimental conditions of the assay, engages only in the trans-homophilic mode of interaction.

We extended our coculture system to the analysis of heterotypic cell-cell junctions by using a mouse neuroblastoma cell line, Neuro2a, stably transfected with CD155 (Neuro2a-CD155) together with HeLa-nectin-3 and parental HeLa cells. In contrast to the “HeLa-only” coculture described above, here HeLa-nectin-3 and HeLa cells were seeded first and allowed to grow for 2 days to a 70% cell confluence, during which time nectin-3 distributed evenly to all cell-cell junctions (similarly to Fig. 7B). Neuro2a-CD155 cells were then superseeded, followed by an additional 2 days of coculture. Cells were processed as above and analyzed by laser-scanning confocal microscopy (Fig. 8). Under the given culture conditions, a portion of Neuro2a-CD155 cells were found to extend long neurites. Like the rest of the cell surface, these neurites were richly decorated with CD155 protein (Fig. 8A, a1 and a2). Wherever CD155-positive neurites extended over a HeLa-nectin-3 cell, almost the entire nectin-3 pool of that cell was found to be associated with the crossing section of the CD155-bearing neurite (Fig. 8, B (b1 and b2) and D (d1 and d2)).

Fig. 8C (c1 and c2) depicts two CD155-expressing neurites that traverse the middle of several HeLa-nectin-3 cells and in some cases cross over the region of the underlying cell’s nucleus (arrowheads). As an interesting implication of this result, we conclude that the junction assembled by CD155 and nectin-3 can form anywhere on the cell surface. Our coculture data suggest to us that nectin-3 is being actively recruited away from HeLa-nectin-3 homotypic cell junction and incorporated into HeLa-nectin-3/Neuro2a-CD155 heterotypic cell junctions. Thus, nectin-3 protein at HeLa-HeLa cell junctions undergoes only weak and transient interactions, and upon encountering CD155 on Neuro2a-CD155 cells, it will be incorporated into a more stable adhesion complex. This leads to a concentration of nectin-3 along CD155-expressing surfaces until all nectin-3 has been has “mopped up” through interaction with CD155.

**Nectin-3 and CD155 Colocalize at Lateral Cell-Cell Junctions of Kidney and Amniotic Epithelia**—In order to confirm the physiological significance of the interaction between nectin-3 and CD155, we searched for sites of colocalization of both proteins in mouse tissues. Cryosections of CD155tg mouse kidney and amniotic membranes were stained simultaneously with antibodies 103-A1 and NAEZ-8 and analyzed by laser-scanning confocal microscopy (Fig. 9). Basolateral expression of CD155 was seen in epithelial cells lining the proximal tubules of the kidney, decreasing in intensity toward the most apical portions of the lateral cell-cell junctions (Fig. 9A, a1). Nectin-3 was most concentrated in the anterior aspects of lateral cell-cell junctions (Fig. 9B, b1), consistent with its localization at cadherin-based adherens junctions that has been described in other epithelia (1). The expression level of nectin-3 decreased toward the basal aspect of the lateral membranes (Fig. 9B, b1). Significant colocalization of CD155 staining and nectin-3 staining could be detected in the medial region of the lateral cell.
contacts (Fig. 9C, c1). A very similar observation was made in the epithelium of the amniotic membrane, except that here CD155 was found mainly along the lateral cell-cell junctions and less at the basolateral side of the cell (Fig. 9, D–F, d1–f1, and d2–f2). We consider it possible that nectin-3 and CD155 may be involved in maintaining the distinct compartmentalization and polarity of the epithelial cell membrane in the transition zone between the apical and basolateral membrane region of epithelial cells.

cis-Colocalization of CD155 with αvβ3 Integrin—The basolateral distribution of CD155 in epithelia (24, 27, 38), reminiscent of an integrin expression pattern, and the fact that both CD155 and αv integrins serve as vitronectin receptor led us to speculate on a functional connection between CD155 and integrins. We therefore set out to analyze their subcellular expression and possible colocalization in cultured cells. A mouse mAb specific for the αvβ3 integrin heterodimer (clone LM609; Chemicon) and a rabbit polyclonal antibody against αvβ3 were found to intensely and specifically stain L(tk−/) cells cotransfected with human αv and β3 integrin expression vectors (data not shown). When the cells were cotransfected to express αv, β3, and CD155, the observed punctate surface staining for αvβ3 integrin (Fig. 10, A and D) coincided almost completely with that of CD155 (Fig. 10, B and E). Although we presently do not know whether they physically interact, the fact that both CD155 and αvβ3 are targeted to identical subcellular membrane microdomains suggests a functional relationship, possibly their presence in a common supermolecular adhesion complex. Moreover, no significant colocalization was observed between αvβ3 and nectin-1, nectin-2, or nectin-3, attesting to the specificity of the observed colocalization with CD155 (data not shown).

Finally, we determined that CD155 and αv integrin colocalize at the basolateral membranes in human amniotic epithelium (Fig. 10, G–I and insets). Unlike the colocalization with nectin-3 that only occurred at lateral cell-cell contacts (Fig. 9F), CD155 and αv colocalized in the entire basolateral region. Adequate staining in human amniotic epithelium was only obtained with a polyclonal antibody against αvβ3 and not with the αvβ3-specific mouse mAb LM609. Thus, it is not clear whether the observed staining reflects expression of αvβ3 or that of another αv-containing heterodimer cross-reacting with this polyclonal antibody. For instance, this polyclonal antibody very efficiently stained focal contacts in HeLa (data not shown), a cell line that reportedly does not express αvβ3 but does express αvβ6 (39, 40). Unfortunately, we were unable to test colocalization of nectin-3 and CD155 in human amnion due to the unavailability of suitable nectin-3 antibodies specific for the human protein.
CD155 and rabbit polyclonal antibody NAEZ8 against CD155 (as between the cadherin/nectin adhesive systems. Direct interactions such in mediating heterophilic adhesion between the integrin and bone fide extracellular matrix protein vitronectin, an interaction that may also occur through their associated proteins. Indication that an interaction between the cadherin and integrin system may also be explained by steric hindrance, due to the close association of CD155 and αβ3 integrin. However, it is presently unknown whether CD155 and αβ3 integrin interact physically. Interestingly, αβ3, αβ5, CD155, and their mutual ligand vitronectin are all up-regulated in glioma cells, and a positive correlation between metastatic/invasive properties of gliomas and αβ3 expression is well documented (49–53). In the future, it will be interesting to address the possible role of CD155 in such malignancies.

We have also begun to determine the mechanisms of the interaction that we have identified between nectin-3 and CD155. This heterophilic interaction is not a simple bimolecular reaction but rather requires the presence of CD155 in a higher order complex that facilitates dimer formation and proper display of CD155 on the cell surface. The correct presentation of CD155 needed for interaction with nectin-3 appears to be cell type-specific (Figs. 4–6). L(tk–) cells were found to be unable to assemble such a CD155 complex, possibly due to the lack of an as yet unidentified cellular cofactor(s). As a result, no functional CD155-mediated nectin-3 binding sites were formed on L(tk–)-CD155 cells (Figs. 4–6). Dimerization of binding partners alone, however, may not explain the complex interactions between nectin/CD155 family members. Although nectin-2 spontaneously forms homodimers in all cell lines that were tested (9, 10), cross-linking with anti-nectin-2 greatly facilitated binding of nectin-3-AP to HEK 293 nectin-3 cells (Fig. 4). Thus, cis-dimer formation may not be the sole requirement for efficient binding. Rather, the formation of higher order structures, such as nectin clusters or “lattices,” may be necessary. For example, the requirement of integrins to form supermolecular clusters at focal contacts has been well documented (see Refs. 44 and 54 and references therein). We suggest that this may also be the case for trans-interactions mediated by nectin/CD155 family members.

It is attractive to speculate that cofactors associated in cis with CD155 such as αβ3 integrin may aid in the formation of clusters of CD155 binding sites on the cell surface. Furthermore, the identification of CD155 as a vitronectin receptor (25) and its colocalization with αβ3 that we report here point to the possibility of a trimolecular vitronectin receptor (αβ3/CD155).

Our preliminary results indicate that nectin-3-AP binding to HeLa-CD155 cells does not block poliovirus binding or infection (data not shown). Poliovirus virions avidly bind monomeric CD155 in vitro (29), an observation suggesting that monomeric CD155 is an efficient cellular receptor for the virus. This is supported by the fact that L(tk–)-CD155 cells, which only express CD155 monomers (Fig. 6), present an excellent sub-

**DISCUSSION**

In this study, we have identified and characterized a novel interaction between nectin-3 and CD155, the latter being a receptor for poliovirus and for the extracellular matrix protein vitronectin. Our evidence suggests that CD155, which appears to be part of an αβ3 integrin-containing complex, may function as a bona fide cell-cell adhesion molecule by interacting with nectin-3, an adhesion receptor present at cadherin-based adherens junctions. We propose that this interaction may play a role in mediating heterophilic adhesion between the integrin and the cadherin/nectin adhesive systems. Direct interactions such as between the αβ3 or αβ5 integrins and E-cadherin have been described previously (41, 42). Our results provide a first indication that an interaction between the cadherin and integrin system may also occur through their associated proteins.

It has been shown recently that CD155 exhibits affinity for the extracellular matrix protein vitronectin, an interaction that may be involved in establishing cell-matrix contacts (25). The major receptors for vitronectin are members of the α integrin family, in particular αβ3 and αβ5. Integrins are considered the quintessential mediators of adhering epithelial cells to the underlying extracellular matrix (43). Their presence has been shown in at least two very distinct adhesion complexes. These are focal adhesions, in which they bridge the extracellular matrix and the actin cytoskeleton (44), and hemidesmosomes (45, 46) that tie into the intermediate filament network of the epithelial cell (see Ref. 47 and references therein). Our finding of cis colocalization of CD155 and αβ3 integrin on transfected mouse fibroblasts together with the fact that both proteins serve as vitronectin receptors seems to indicate their close association, possibly in a multiprotein adhesion complex. In addition, the CD155-specific antibody NAEZ-8 blocks the infection of rhabdomyosarcoma cells by Coxsackie virus A9, a virus that uses αβ3 as its cognate cellular receptor (48). Such a blocking effect could best be explained by steric hindrance, due to the lack of an as yet unidentified cellular cofactor(s). As a result, no functional CD155-mediated nectin-3 binding sites were formed on L(tk–)-CD155 cells (Figs. 4–6). Dimerization of binding partners alone, however, may not explain the complex interactions between nectin/CD155 family members. Although nectin-2 spontaneously forms homodimers in all cell lines that were tested (9, 10), cross-linking with anti-nectin-2 greatly facilitated binding of nectin-3-AP to HEK 293 nectin-3 cells (Fig. 4). Thus, cis-dimer formation may not be the sole requirement for efficient binding. Rather, the formation of higher order structures, such as nectin clusters or “lattices,” may be necessary. For example, the requirement of integrins to form supermolecular clusters at focal contacts has been well documented (see Refs. 44 and 54 and references therein). We suggest that this may also be the case for trans-interactions mediated by nectin/CD155 family members.

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*4. M. Roivainen, personal communication.*
strate for poliovirus binding and infection (17) while not supporting nectin-3-AP binding (Fig. 5). Our data indicate that several distinct CD155 populations exist on HeLa cells (Fig. 6). The majority of CD155 is present as monomers that may serve as poliovirus receptors, whereas CD155 pools that containimerized CD155, possibly in a complex with other proteins, may constitute the binding sites for nectin-3.

Finally, we have shown that mouse CD155/Tage4 interacts with nectin-3 much in the same manner as human CD155 (Fig. 2). This result provides the first functional evidence that mouse CD155/Tage4 is, indeed, the authentic rodent homologue of human CD155. In this context, it is interesting to note that both CD155 and Tage4 are overexpressed in colon carcinoma (13, 18).

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Note Added in Proof—Ikeda et al. (55) have shown heterogeneous trans-interaction of necl-5/Tage4 with nectin-3, consistent with our result that CD155 heterophilically trans-interacts with nectin-3. Ikeda et al. (55) furthermore showed that the heterotrans-interaction of necl-5/Tage4 with nectin-3 enhances cell motility. Reymond et al. (56) have previously suggested an interaction between CD155 and nectin-3 without presenting data.

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Recruitment of Nectin-3 to Cell-Cell Junctions through \textit{trans}\textsuperscript{-}Heterophilic Interaction with CD155, a Vitronectin and Poliovirus Receptor That Localizes to $\alpha_\nu \beta_3$ Integrin-containing Membrane Microdomains

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