Nectin4/PRR4, a New Afadin-associated Member of the Nectin Family That Trans-interacts with Nectin1/PRR1 through V Domain Interaction*

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Nectins are adhesion molecules that participate in the organization of epithelial and endothelial junctions and serve as receptors for herpes simplex virus entry. They belong to the immunoglobulin superfamily, are homologues of the poliovirus receptor (PVR/CD155), and were also named poliovirus receptor-related (PRR) proteins. We identify a new member of the nectin family named nectin4. Peptide sequences of human and murine nectin4 share 92% identity, and as for other members, the ectodomain is made of three immunoglobulin-like domains of V, C, C types. In contrast to other nectin molecules, detection of nectin4 transcripts is mainly restricted to placenta in human tissues. Expression is broader in mouse, and interestingly nectin4 is detected at days 11, 15, and 17 during murine embryogenesis. Nectin4 interacts with afadin, a F-actin-associated molecule, via its carboxyl-terminal cytoplasmic sequence. Both molecules co-localize at cadherin-based adhesions junctions in the MDCKII epithelial cell line. Nectins are homophilic adhesion molecules, and recently heterophilic interactions have been described between nectin3/nectin1 and nectin3/nectin2. We confirmed these trans-interactions and also described nectin5 as the PVR/CD155 ligand. By means of several approaches, we report on the identification of nectin4 as a new ligand for nectin1. First, a soluble chimeric recombinant nectin4 ectodomain (nectin4-Fc) trans-interacts with cells expressing nectin1 but not with cells expressing nectin2, nectin3, or PVR/CD155. Conversely, nectin1-Fc binds to cells expressing nectin4. Second, nectin1-Fc precipitates nectin4 expressed in COS cells. Third, reciprocal in vitro physical interactions were detected between nectin4-Fc and nectin1-Fc. The nectin4-Fc/nectin1-Fc interaction was detected suggesting that nectin4 exhibits both homophilic and heterophilic properties. Using the same approaches we demonstrate, for the first time, that the V domain of nectin1 acts as a major functional region involved in trans-heterointeraction with nectin4 and also nectin3.

Nectin/poliovirus receptor-related (PRR) molecules are adhesion receptors that belong to the immunoglobulin superfamily. They were originally described as molecules homologous to the poliovirus receptor (PVR/CD155) and called PRR. Four members have been described: PVR/CD155, nectin1/PRR1/CD111, nectin2/PRR2/CD112, and nectin3/PRR3 (1–4). Their ectodomain is composed of three immunoglobulin (Ig)-like domains of V, C, C types and shares between 30 and 55% amino acid identity. Expression of nectin/PRR molecules is generally broad in tissues, including hematopoietic, neuronal, endothelial, and epithelial cells, except for nectin3, which displays a more restricted expression (1–7).

Nectin1/PRR1 (also named herpes immunoglobulin receptor (HIgR) or herpesvirus entry (HveC)) serves as HSV entry receptor (6, 8). Nectin1 appears to be the major HSV receptor as it mediates entry of all the HSV-1 and HSV-2 strains tested as well as of animal alphaherpesviruses. Nectin2/PRR2 (HveB) and PVR/CD155 (HveD) serve as receptors for a limited range of alphaherpesviruses (9, 10). Nectin1 and nectin2 are involved in the cell to cell spreading of the virus (11).

Both soluble and transmembrane nectin isoforms have been described in humans and rodents (2, 4, 12–17). The transmembrane isoforms are different in length and can be classed into two groups according to their carboxyl-terminal sequences. One group carries a conserved carboxyl-terminal motif (X/YV) that interacts with the PSD-95/Dlg/ZO-1 (PDZ) domain of the filamentous-actin (F-actin)-associated molecule called afadin (7). The other group does not carry this motif of interaction. Two different isoforms of afadin have been described: the long isoform (l-afadin), ubiquitously expressed, is associated with F-actin via a carboxyl-terminal region absent from the short isoform (s-afadin) (18). In turn, afadin interacts with molecular components of the tight junctions like ZO-1 (zonula occludens) and JAM (junctional adhesion molecule) (19, 20). Afadin also interacts with specific components of adherens junctions (AdJs) like nectins and ponsin (7, 21). A recent report demonstrates that afadin associates with the actin cytoskeletal regulator profilin (22). Afadin has also been shown to interact with the activated Ras proteins involved in cell signal transduction and known to control cell proliferation (23, 24). Altogether afadin acts as a “linker” between nectins and the cortical

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) AF426163 for human nectin4.

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The abbreviations used are: PRR, poliovirus receptor-related; PVR, poliovirus receptor; HSV, herpes simplex virus; Hve, herpesvirus entry; PDZ, PSD-95/Dlg/ZO-1; AdJ, adherens junction; MDCK, Madin-Darby canine kidney; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorter; kb, kilobase(s); HIgR, herpes immunoglobulin receptor; JAM, junctional adhesion molecule.

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actin and depicts a prototype of a scaffold molecule that exerts a central role in the organization of junctional complexes and in the recruitment of signaling molecules.

The function of nectin molecules is currently unknown. Recently mutations in the nectin1/PRR1 gene, leading to abnormal forms of the receptor, have been associated with cleft lip/palate-ectodermal dysplasia (25). Nectin2 mice display male sterility due to morphologically aberrant spermatozoa (26). A key property of nectins is their ability to form cis- and trans-dimers through their ectodomains and thus mediate cell-to-cell adherence (3, 7, 16). Indeed Ca\textsuperscript{2+}-independent homophilic trans-interactions have been documented for nectin1, -2, and -3, and heterophilic trans-interactions have been proposed between nectin3 and nectin1 and between nectin3 and nectin2 in mouse (16). Trans-interactions between nectins are dependent on cis-dimerization at the cell surface and may lead to the phosphorylation of cytoplasmic tyrosine residues (3). Thus the respective properties of nectins are probably not redundant. Nectins display different expression patterns, may interact or not with afadin, and most importantly may transheterointeract in a specific manner to regulate physiological processes through their respective cytoplasmic domains.

In this report we describe a new member of the nectin family called nectin4. We show that nectin4 (i) is structurally related to the nectin family members; (ii) is expressed mainly in placenta in human tissues, presents a broader distribution in mouse tissues, and is expressed in mouse embryo; (iii) is a 66-kDa protein that co-localizes and interacts with the PDZ domain of afadin; (iv) recruits afadin at cadherin-based adherence junctions; (v) is a Ca\textsuperscript{2+}-independent homophilic adhesion molecule; (vi) is a new ligand for nectin1 but not for nectin2, nectin3, and PVR/CD155; and (vii) binds nectin1 through the extracellular V domain interaction.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—Madin-Darby canine kidney II (MD-CKI) cells and COS cell lines 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\textsubscript{2} atmosphere at constant humidity. Cells were purchased from ATCC (Manassas, VA).

Cloning of Human and Murine Nectin4 cDNAs—Exons that composed human nectin4 cDNA were deduced from the analysis of the AL162592 genomic sequence (see first paragraph under “Results”). We designed primers encompassing the start codon and stop codon: R4S (GCGA ATTC ATGC CCCT GTCC CTGG GAGC CGAG ATG) and R4AS (CGTC TAGA TCAG ACCA GGTG TCCC CGCC CATT GATG). The full-length cDNA of nectin4 was then amplified from trachea mRNA (CLONTECH) by reverse transcription and polymerase chain reaction as described previously (4). Nectin4 cDNA was amplified without the leader sequence with primers PFLR4S (GCGA ATTC GGGT GAGC TGGA GACC TCAG ACGT GG) and R4AS and cloned in the pFLAG-CMV1 vector (Sigma) (pFLR4 vector). Blast analyses of the murine expressed sequence tag data base with the human cDNA sequence identified homologous murine expressed sequence tag clones. Full-length murine nectin4 cDNA was amplified from the mRNA of the myeloid cell line Da-1 with primers MR4S (CTGG GCAG GTCT ACCT TTCA GCC) and MR4AS (GTTT CCCC TAAG AAGA GCCG AAG) cloned in the pGEMTeasy vector.

Northern Blot Analysis—Human multiple-tissue Northern membranes (CLONTECH) were hybridized with the 999-base pair fragment (from 1 to 999). Murine multiple-tissue Northern membranes (CLONTECH) were hybridized with the full-length murine nectin4 cDNA probe. Both probes were labeled with \textsuperscript{32}P-dCTP as specified by the manufacturer (Amersham Pharmacia Biotech). The membranes were also probed with the \(\beta\)-actin probe to verify the loading conditions.

Yeast Two-hybrid System—Oligonucleotide adapters encoding the...
last 10 amino acids of the carboxyl-terminal sequences of the various human nectins and their mutants, as listed in Fig. 4, were cloned into the LexA DNA binding domain bait expression vectors pBTM116B and -C. cDNAs encoding the PDZ domain of two proteins were cloned into pACT2: pACPDZ-ADF6 (PDZ domain of afadin or AF-6) and pACScrubble (the four PDZ domains of Scribble) (a gift of Dr. J. P. Borg).

The integrity of all inserts was confirmed by DNA sequencing (Genome Express, Grenoble, France). Yeast assays were performed as described previously using a lithium acetate-based method. Plasmids were co-transformed into the Saccharomyces cerevisiae L40 strain harboring LacZ and HIS3 as reporter genes. Interaction between bait and prey was first monitored by a LacZ reporter assay: β-galactosidase activity was tested with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a substrate using the filter method. Yeast double transformants were transferred to nitrocellulose membrane (Protran BA-85, Bio-Rad). Immune complexes were washed three times with cold lysis buffer, heated in SDS sample buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0). Interactions were also evaluated by a HIS3 reporter assay by colony selection as double transformants were picked and dropped in quadruple minus HIS3 colonies. The mouse embryo Northern blot membrane (CLONTECH) was hybridized with murine nectin4 probe: a 3.7-kb transcript is mainly detected in placenta and in a lesser extent in trachea. B, mouse adult tissue mRNA blot membrane (CLONTECH) was hybridized with a murine nectin4 probe (see “Experimental Procedures”). A 3.7-kb transcript is detected in brain. Four different transcripts are detected in lung and testis. The mouse embryo Northern blot membrane was hybridized with murine nectin4 probe. A 3.7-kb transcript is present at embryonic days 15 and 17. A weak signal is seen at day 11 suggesting that nectin4 expression starts at day 11.d.p.c., days postcoitum; sh., skeletal; sp., spinal; ad., adrenal; PBL, peripheral blood leukocytes.

Cells in 100-mm dishes (5 × 106 cells) were washed three times with ice-cold PBS and then suspended in 750 ml of ice-cold lysis buffer containing 50 mM Hapes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, and 10% glycerol. A protease inhibitor mixture was added as recommended by the manufacturer (Roche Diagnostics). After a 30-min incubation on ice, solubilized material was clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Cell lysates were incubated for 12 h at 4°C with the appropriate antibody and then for 1 h at 4°C with 50 μl of protein A-Sepharose (Amersham Pharmacia Biotech). Immune complexes were washed three times with cold lysis buffer, 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% SDS-PAGE, semidy-transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Boston, MA), probed with the indicated antibodies, and visualized by ECL (Amersham Pharmacia Biotech).

Immunofluorescence—MDCKII cells were grown on 13-mm round glass coverslips as a confluent monolayer to reach optimal cell polarization. Cells were fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature. After blocking aldehydes with 50 mM NH4Cl for 10 min at room temperature, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, fixed cells were permeabilized with 0.2% gelatin (Sigma), 0.075% saponin (Sigma) in PBS. Fixed samples were first labeled with the appropriate primary antibodies for 60 min at room temperature and then were incubated with secondary antibodies for 60 min at room temperature. Finally samples were washed, mounted onto slides, embedded with 50% glycerol in PBS, and visualized using a confocal Leica microscope. Images were processed using Adobe Photoshop software.

Immunohistochemistry—Immunodetection of nectin4 was performed on frozen sections (5 μm) of human placenta using 10 μg/ml of the nectin4 C-terminal polyclonal antibody (pAb). Specimens were processed with the Universal DAKO ChemMate kit (Glostrup, Denmark) according to supplier recommendations, counterstained for 5 min in Harris hematoxylin, and mounted in DAKO glycergel mounting medium.

Construction, Production, and Purification of Soluble Forms of Nectin—Polymerase chain reaction amplification of the nectin4 ectodomain was performed by using primers R45 (GGCA ATTC ATGC CCTC GTCC CTGG GAGC CGAG ATG) and C1L45A (CCGG TACC AAGG ACAT CCAC AGTG ACCT GAGA ATGC). The nectin3 ectodomain was amplified with primers SBS3 (CAAG AATT CATG GGCA GCAG
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CCTG CGGC CGTC CCCG) and SBR3.3 (GTCA TCTA CATT TCAG ATCC TCCT GGTA CCA G). Nectin1-Fc, nectinIV-Fc, and nectin2-Fc constructions and productions have already been described (3, 10, 28).

Expression of Nectin4 in Human Tissues—Nectins display specific patterns of expression in human tissues. In contrast to PVR, nectin1, and nectin2 that are widely expressed in tissues, nectin3 expression is mainly detected in placenta and testis. Surprisingly these distributions may vary between human and mouse tissues as assessed by Northern blot experiments using commercial membranes. Indeed we and others found that murine nectin1 is expressed in brain and liver, whereas human nectin1 is widely expressed especially in brain and spinal cord but not in liver (6, 14). Also nectin3 is present in liver and kidney in mouse but not in human (4, 16). By means of a probe that encompasses the exons 3 to 6, we detected a major 3.7-kb transcript in placenta and, to a lesser extent, in trachea (Fig. 2A).

We then analyzed the expression of murine nectin4 in adult tissues and found a broader expression in murine tissues: nectin4 is expressed in brain, lung, and testis. However, we found a marked difference between transcript sizes. Whereas a major 3.7-kb transcript was detected in brain, four different transcriptions were detected in lung, and at least six in testes.

RESULTS

Molecular Cloning of Human and Murine Nectin4—Blot analyses of the nonredundant and human expressed sequence tag database with sequences from nectin ectodomains identified new sequences related to nectins. Among them, an amino acid sequence (GenBankTM accession number AF160477) was identified sharing 30% identity with the nectin 3 ectodomain. Blast analyses of the high-throughput genome database with these sequences identified a clone of 149,475 base pairs named AL162592. This clone was subsequently analyzed with the Genescan program, and nine exons were identified: encoding putative leader sequence, V domain, first C domain, first half and second half of the second C domain, transmembrane, and intracytoplasmic region (three exons). According to the sequence homology with other nectin members, two primers were designed. One encompassed the leader sequence including the predicted ATG, and the second encompassed the cytoplasmic domain including the predicted stop codon. They were used to amplify this new member by reverse transcription and polymerase chain reaction from trachea mRNA (CLONTECH) (see “Experimental Procedures”).

The amplified fragment encoded for a protein of 510 amino acids long (55.5 kDa), which differed in one amino acid from AF160477 (Leu-146 (CTG) replaces Met-146 (ATG)) (Fig. 1). This CTG codon was also found in the murine nectin 4 sequence. A hydrophobic sequence (amino acids 352–372) corresponding to the transmembrane sequence is followed by a 139-amino acid long cytoplasmic sequence. This sequence shows a low degree of similarity with those of nectin 1, -2, and -3. The C-terminal sequence ends by a valine, but the nectin consensus (A/E)XTV sequence known to bind the afadin PDZ domain is not conserved. The murine nectin 4 sequence is 508 amino acids long and shares 92% identity with its human counterpart. The peptidic cytoplasmic regions 412–436 or 410–434 (in italics in Fig. 1) in human and murine sequences, respectively, were found to be absent in some cDNA clones. This sequence encoded by exon 8 of the nectin 4 gene is probably deleted by an alternative splicing process.

In Vitro Binding Studies—In vitro functional interaction studies between nectins were done as follows. Ultrasound 96-well trays (Nunc) were incubated overnight at 4 °C with 1 µg/ml goat anti-human Fn affinity-purified serum diluted in PBS (Sigma). After three washes with PBS containing 0.5% Tween 20, wells were incubated with PBS containing 1% bovine serum albumin. After three washes, 10–7 of different chimeric Fc proteins (nectin1-Fc, nectinIV-Fc, nectin2-Fc, nectin3-Fc, and nectin4-Fc) were incubated for 2 h at 37 °C. After the washes, free anti-human-Fc antibodies were blocked with PBS containing 100 µg/ml human immunoglobulin G (Novartis) for 1 h at 25 °C. Biotinylated nectin4-Fc or nectin4-IV-Fc (10–7 µg/ml) was then incubated for 2 h at 37 °C in the absence of Ca2+. After three washes, 2 µg/ml streptavidin peroxidase was incubated for 1 h at 37 °C. After five washes, binding was assessed by incubation with the One-Step ABTS (2,2’-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate (Pierce).

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scripts were detected in lung and in testis (1.4, 2.1, 2.8, and 4.2 kb) that probably correspond to alternative forms of nectin4 mRNA (Fig. 2B). Thus, the expression pattern of nectin4 mRNA may vary between human and mouse tissues as described for other nectins. Moreover, murine nectin4 is expressed during embryogenesis. We found a weak expression of the 3.7-kb mRNA in embryos at day 11 and an increased expression at days 15 and 17 (Fig. 2C). These results point to a major role of nectin4 during embryogenesis.

**Interaction of Nectin4 with Afadin**—To identify the nectin4 protein, we performed a Western blot analysis of COS cells transiently transfected with an epitope-flagged nectin4 cDNA. Immunoprecipitation was carried out either with M1 or M2 anti-FLAG mAbs, and nectin4-blotted proteins were revealed with the M1 mAb. No signal was detected in COS cells transfected with the mock plasmid, whereas a 66-kDa band was detected in transfected cells and corresponds to nectin4 (Fig. 3A). This band co-migrates with the band detected in the total cell lysate. The apparent molecular weight of nectin4 is higher than that predicted by the amino acid sequence possibly due to N-glycosylation.

Previously we showed that nectin2α and -δ could cis-homo-(α/α and δ/δ dimers) or cis-heterodimerize (α/δ dimers) (3). A single cell suspension of COS cells expressing nectin4 was incubated in the absence or presence of the cell surface crosslinker BS3. Cell lysates were then analyzed by SDS-PAGE. A 130-kDa protein was detected with the nectin4 C-terminal pAb (arrowhead) providing evidence that nectin4 forms lateral cis-dimers (Fig. 3B). Higher molecular weight complexes were detected, but their composition is still unknown. Similar complexes were also described for nectin3 (16).

Most of nectin isoforms interact with the scaffold afadin molecule. To analyze the interaction of nectin4 with afadin, we analyzed the interaction of nectin4 with endogenous afadin in COS cells. Immunoprecipitation was carried out with antiafadin monoclonal antibody. The 66-kDa band specific for nectin4 was co-immunoprecipitated with endogenous afadin in COS cells (Fig. 3C), and conversely the 205-kDa band specific for endogenous l-afadin was co-immunoprecipitated with nectin4 (Fig. 3D). These results provide evidence that nectin4 interacts with afadin in a similar manner to nectin1α, nectin2α, and -δ, and nectin3α.

Interaction between nectins and afadin occurs between the carboxyl terminus of nectin and the PDZ domain of afadin. As shown in Fig. 4, some nectins carry the C-terminal consensus sequence (A/E)XYV (nectin1α, nectin2α and -δ, and nectin3α and -β). However, this sequence is absent from some nectin isoforms (nectin1β and nectin3γ) (16). Nectin4 carboxyl-terminal sequence does not fit with the nectin consensus sequence but ends by a valine as the consensus sequence does. It was of interest to test whether the C-terminal region of nectin4 interacts with the afadin PDZ domain as well as to compare both groups of nectins, i.e. those that carry the consensus C-terminal sequence and those that do not. To this end, nectin C-terminal peptides (Fig. 4) were fused to the LexA binding domain and challenged with the PDZ domain of afadin (1006–1124) or the PDZ domains of Scribble (used as a negative control) fused to the GAL4 activation domain in a yeast two-hybrid assay. The results in Fig. 4 show that afadin PDZ domain interacted with the C-terminal portion of nectin4 despite the absence of the (A/E)XYV consensus sequence and also interacted with nectin3α and -β as well as with nectin1α, nectin2α, and nectin3β in agreement with previous reports. As for other nectins, β-galactosidase activity for the nectin4/afadin interaction was detectable within the first 5 min. This suggests that the nectin interaction with afadin is similar to that of the other nectins. No interaction was detected between afadin and the C-terminal amino acids specific to nectin1β, nectin3γ, and PVRα. As expected, EphA7 and the papillomavirus E6 peptides interacted with afadin and huScribble PDZ domains, respec-

| Test          | C-ter peptides | Control pACT2 β-gal - HIS | afadin/PDZ β-gal - HIS | Scribble/PDZ β-gal - HIS |
|---------------|----------------|---------------------------|------------------------|-------------------------|
| Control pBTM116 | SFISKK3WYV     | -                         | -                      | -                       |
| Nectin1α      | RTTEPR3C6CP    | -                         | -                      | -                       |
| Nectin2α      | SLISRRAVYV     | -                         | +                      | -                       |
| Nectin2α      | GFVMSRAMYV     | -                         | +                      | -                       |
| Nectin3α      | SVISR5EWYV     | -                         | +                      | -                       |
| Nectin3αYV    | SVISR6REW      | -                         | -                      | -                       |
| Nectin3βα     | LYNPREHYV      | -                         | +                      | +                       |
| Nectin3βγ     | LGVRAL6DT      | -                         | -                      | -                       |
| Nectin4       | IYINGRGLHLV    | -                         | +                      | -                       |
| Nectin4βV     | IYINGRGLH     | -                         | -                      | -                       |
| Nectin4δV     | IYINGRGLH     | -                         | -                      | -                       |
| Nectin4δV     | SOQPST6GTR     | -                         | -                      | -                       |
| E6            | LHLHT6GIQV     | -                         | +                      | -                       |
| E6            | MSCTSSR6R6TQL  | -                         | -                      | +                       |
**Fig. 5. Nectin4 co-localization with afadin in confluent MDCKII epithelial cells.** The polarized MDCKII cell population partially expressing FLAG-tagged nectin4 was double-stained with anti-mouse afadin mAb and a goat anti-mouse rhodamine (Molecular Probes) and then with the fluorescein isothiocyanate-labeled M2 anti-FLAG mAb (Sigma). **A**, *left*, nectin4 expression by FACS analysis with the fluorescein isothiocyanate-labeled M2 anti-FLAG mAb. *Right*, Western blot analysis of nectin4 in MDCK cells. **B**, *left*: arrow, nectin4 localization at cell-cell junctions between two adjacent expressing nectin4 cells; arrowhead, absence of nectin4 expression at junctions between a cell expressing nectin4 and a cell that does not express nectin4. *Middle*, endogenous afadin expression. *Arrow*, strong expression of afadin at cell-cell junctions between two adjacent nectin4 expressing cells; arrowhead, afadin expression in the absence of nectin4. *Right*, merge analysis of nectin4 and afadin expression. **C**, confocal images in the XZ plane of MDCKII cells double-stained with anti-ZO-1 and nectin4, nectin1α, l-afadin, or E-cadherin antibodies. *IB*, immunoblot; *IP*, immunoprecipitation.
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Nectin4 Co-localizes with Afadin at AJs in Epithelial Cells—

Previous studies have demonstrated that nectins co-localize with afadin and E-cadherin at AJs in MDCKII epithelial cells (7). The localization of nectins in AJs is thought to be mediated in part by their interaction with l-afadin that connects nectins to F-actin. We therefore examined the subcellular localization of nectin4 in MDCKII cells used as a model of polarized epithelial cells. Confocal microscopy was performed on a mixed MDCKII cell population where 40% of the cells stably expressed nectin4 (Fig. 5A, left). Nectin4 was detected after immunoprecipitation and immunoblotting with the nectin4 C-terminal pAb and showed a 66-kDa band as seen in COS nectin4 cells (Fig. 5A, right). Nectin4 was found to localize with afadin at intercellular junctions (Fig. 5B). This localization at intercellular junctions was only seen between adjacent cells both of which expressed nectin4 (Fig. 5B, arrow) and not when only one cell expressed nectin4 (Fig. 5B, arrowhead). Endogenous afadin was detected at cell junctions in all MDCKII cells, possibly as the consequence of the association with other endogenous partners (Fig. 5B, arrowhead). Interestingly afadin was highly concentrated at sites where nectin4 was expressed suggesting that nectin4 contributes to the recruitment of afadin at cellular junctions, probably by means of the PDZ-mediated interaction (comparison between arrow and arrowhead).

We next compared the localization of nectin4 with that of nectin1α in MDCKII epithelial cells and found that both nectins were distributed along the entire lateral membrane (Fig. 5C). However, nectins did not co-localize with the tight junction component marker ZO-1, providing evidence that their specific localization is at AJs. This distribution is similar to that of E-cadherin, which is also present at AJs and absent from tight junctions. Afadin localization is more restricted than those of nectin, probably at the junctional structure called zonula adherens (Fig. 7C). No colocalization was detected between afadin and ZO-1 in MDCKII cells expressing nectin4 or nectin1α.

Endogenous Expression of Nectin4—Northern blot analyses showed that a major specific 3.7-kb nectin4 transcript was detected in human placenta, murine brain, and mouse embryo. Immunohistochemical analysis of human placenta with anti-nectin4 pAb reveals expression of nectin4 in endothelial cells from vessels located inside the placental villi (Fig. 6A, right). This pattern confirms Northern blot results and is similar to that described for nectin2 (Fig. 6A, middle) (3).

Endogenous expression of nectin4 was analyzed in mouse embryo, brain, and placenta. A 66-kDa band compatible with nectin4 expressed in COS cells was detected in murine brain (Fig. 6B, lane 1). Lower apparent molecular weight bands of 58 and 64 kDa were detected in mouse embryo and in placenta, respectively (Fig. 6B, lanes 3 and 4). These latter forms could result from the expression of a shorter alternative form of nectin4 or to post-translational modifications. No expression was detected in other adult tissues such as spleen, liver, skeletal muscle, and kidney in accordance with Northern blot results.

Nectin4 Is a New Ligand for Nectin1 through V Domain Interaction—Nectins are homophilic adhesion molecules, i.e. they are capable of self-interaction via their ectodomain on two adjacent cells. Recently it has been suggested that murine nectin3α could trans-heterointeract with nectin1α or nectin2α. Using a soluble chimeric form of the human nectin4 ectodomain fused to the Fc fragment of human IgG1 (nectin4-Fc) (Fig. 7A), we analyzed nectin4 trans-interactions with other nectins by three types of approaches: cell surface binding, precipitation, and enzyme-linked immunosorbent assay. First we analyzed the binding of nectin4-Fc on nectin-expressing COS cells (Fig. 7B). COS cells were transfected with nectin1α, nectin2α, nectin3α, nectin4, and PVRα. After 36 h, cell surface expression of nectins was assessed by FACS analysis using specific mAbs against the ectodomain (Fig. 7B, first column). Nectin4-Fc reacts only with cells expressing nectin1α but not with nectin2α, nectin3α, nectin4, and PVRα-expressing cells (Fig. 7B, second column). The percentage of cells expressing nectin1α and cells that bind nectin4-Fc is similar (31 versus 32%, respectively) and strongly suggests that nectin4-Fc binds to cells expressing transmembrane nectin1α. Conversely we performed the binding of nectin1-Fc and readily detected binding of cells expressing nectin4 (Fig. 7B, third column). Interestingly we found that nectin1-Fc binds nectin3α-expressing cells but not those expressing nectin1α, nectin2α, and PVRα-expressing cells, confirming that human nectin1 and nectin3 trans-heterointeract as recently suggested for the murine homologues (16). To further characterize these interactions, we analyzed the binding of the nectin1V-Fc (soluble nectin1 deleted from the two C domains) on different transfectants. Binding was similar to the nectin1-Fc suggesting that the V domain of nectin1 interacts with nectin4 and nectin3 (Fig. 7B, fourth column). In addition, we found that nectin4-Fc binding to nectin1 is blocked by the anti-nectin1 mAb R1.302 whose epitope has been localized to the V
**Fig. 7. Binding of nectin4-Fc, nectin1-Fc, and nectin1V-Fc on nectin-expressing cells.**

A, analysis of the different soluble receptors used in subsequent experiments. Two micrograms of purified soluble forms 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**, nectin2-Fc; **lane 4**, nectin3-Fc; **lane 5**, nectin4-Fc.

B, COS cells were transfected with nectin1/H9251, nectin2/H9254, nectin3/H9251, nectin4, or PVR/H9251 cDNAs. After 36 h cell surface expression was analyzed by a FACS using the R1.302, R2.477, anti-FLAG, or PV.404 mAbs, respectively. Differences observed in cell surface expression levels are yet unclear but were reproducibly found in four repeated experiments. Binding was performed with 40 μg/ml nectin4-Fc, nectin1-Fc, and nectin1V-Fc as described under "Experimental Procedures." **Horizontal bars** indicate fluorescence levels of negative controls and are used to calculate the percentage of cells that express nectins or that bind soluble nectin-Fc proteins.

C, preincubation of 10 μg/ml mAb R1.302 on nectin1-expressing COS cells blocks nectin4-Fc binding. **FSC-H**, forward scatter-height.
domain of nectin1 (Fig. 7C) (28). Second we carried out precipitation with nectin1V-Fc in COS cells expressing nectin4. As expected, nectin1V-Fc protein precipitates a 66-kDa band identified with the anti-nectin4 pAb. This band was not detected in COS cells transfected with the control cDNA (Fig. 8). Identical results were obtained with the nectin1-Fc protein (data not shown). These results demonstrate a direct interaction between nectin4 and nectin1 and confirm FACS observations. Third we tested interaction of biotinylated nectin4-Fc on different coated nectin-Fc molecules. Interaction was detected after incubation with streptavidin-peroxidase and ABTS substrate. We found that nectin-Fc molecules interact with afadin at cadherin-based AJs; (vi) nectin4 forms cis-dimers and this may contribute to increased trans-homophilic interaction affinities by enzyme-linked immunosorbent assay compared with cell surface binding.

**DISCUSSION**

Nectins are a growing family of adhesion molecules that belong to the IgSF, that are involved in cell to cell adhesion processes, and that serve as receptors for herpes simplex virus entry. Here we describe a new member of this family, namely nectin4. We show that (i) nectin4 is structurally and functionally related to other nectins; (ii) the ectodomain is formed of three immunoglobulin-like domains of V, C, C types; (iii) nectin4 is expressed mainly in placenta in human and in brain, lung, testis, and embryo in mouse; (iv) nectin4 interacts with afadin via its C-terminal cytoplasmic region; (v) nectin4 recruits and co-localizes with afadin at cadherin-based AJs; (vi) nectin4 forms cis-dimers at the cell surface and is a trans-homophilic adhesion molecule; (vii) nectin4 trans-heterointeracts with nectin1 and not with other nectins; and (viii) the region of nectin4/nectin1 and nectin4/nectin1 interactions resides in the V domain of nectin1. Nectin4 does not act as a receptor for alphaherpesviruses.2

2 G. Campadelli-Fiume, unpublished results.
Based on sequence comparison in the nonredundant and expressed sequence tag databases we identified nectin4 and a genomic clone AL162592 that encompassed nectin4 exons. This genomic clone is localized in the 1q chromosomal region that may correspond to a region paralogous to the 3q (nectin3), 11q23 (nectin1), and 19q13 (nectin2 and PVR) chromosomal regions. Each of these regions includes paralogous genes belonging to the same family. These genes probably derive from a common ancestral region (32). We cloned the murine nectin4 cDNA that is 92% identical to the human sequence. Interestingly we identified a murine genomic clone AC087229 that encompassed murine nectin4 exons and localized in the murine chromosome 1. This region is syntenic to the human chromosomal region 1q (33) and strengthens the fact that these two genes are orthologous genes.

A key property of some nectins resides in their ability to interact with afadin via a consensus C-terminal amino acid sequence ((A/E/I)XYV). Nectin4 interacts with the PDZ domain of the F-actin-associated molecule called afadin despite the absence of this sequence. Indeed the C-terminal amino acid sequence of nectin4 (YINGRGHLV) does not fit with this sequence, but interaction with the PDZ domain of afadin is abolished by the terminal valine deletion, suggesting similar mechanisms of interaction as for other nectins. From Fig. 4, the new consensus sequence for nectins would be (K/R)XX(Y/L)V. Promiscuous interactions between PDZ domains and their target peptides have already been reported in other systems. The type II afadin PDZ domain is also very flexible and interacts with different carboxyl-terminal sequences present in some ephrin receptors, neurexin, Jagged, and JAM (20, 29, 30).

Confocal experiments performed on polarized MDCKII epithelial cells demonstrate that nectin4, as well as nectin1a, localizes at adherens junctions with E-cadherin and afadin and does not localize with ZO-1 at tight junctions. The precise subcellular localization of afadin in cell-cell junctional complexes was beyond the purpose of this study as we were interested in demonstrating nectin4/afadin interaction using MDCKII cells. In our experiments, afadin localization at cell-cell junctions is actually directed by nectin4 expression and confirms biochemical interaction studies. We noted that the localization at intercellular junctions was only seen between adjacent monolayers of which expressed nectin4 probably via a homophilic mechanism as demonstrated in Fig. 9. Interestingly we demonstrate that nectin4 binds to nectin1 through ectodomain trans-interaction (Figs. 7–9). The absence of nectin4 expression at intercellular junctions when only one cell expressed nectin4 is due to the undetectable expression of endogenous nectin1 in MDCKII cells (7). Moreover we failed to detect nectin4-Fc binding to MDCKII cells (data not shown). Trans-heterointeraction between nectin4 and nectin1 is specific as no interaction was detected between nectin4 and nectin2, nectin3, or PVR. Nectin4/nectin1 interaction is direct and does not involve other accessory molecules as nectin1 precipitates nectin4 and nectin4-Fc binds nectin1-Fc in an in vitro binding assay. In the course of identifying nectin4 binding partners, we demonstrated that human nectin1 trans-heterointeracts with human nectin3 as suggested by a previous report with the murine homologues (16). These data point to a fundamental role of nectin trans-heterointeractions as they are preserved in human and rodent. Preliminary results showed that PVR trans-interacts with nectin3 but not with nectin1, nectin2, and nectin4. Altogether our results depicts a complex “nectin network” that probably contributes to the regulation of epithelial and endothelial physiology (Fig. 10). Interestingly natural soluble isoforms of PVR and nectin1 have been described and may also participate to this regulation (13, 17).

We show here that nectin1 binds to both nectin4 and nectin3 through its V domain. The gD glycoprotein of HSV binds to the CC’C” region of the nectin1 V domain, and the epitope recognized by the mAb R1.302 was recently mapped to the C’C”D regions of the nectin1 V domain (27). This mAb blocks gD and nectin4 binding to nectin1 suggesting that both interactions share common binding regions in the nectin1 V domain. Fine mapping is in progress to define and compare the regions of nectin1 involved in nectin4 and nectin3 binding and conversely to identify critical interacting regions in the other nectin members.

Nectin4 expression is mainly restricted to endothelial cells in placenta and is expressed at embryonic days 11, 15, and 17 in mouse embryo. Previous reports have described the fundamental role of afadin in the organization of cell-cell junctions during mouse development (34, 35). Altogether these results point to a major role of nectin4 and possibly other nectins in embryogenesis.

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